THE EFFECT OF GONADOTROPIN RELEASING HORMONE ON OPSIN GENE EXPRESSION AND SPECTRAL SENSITIVITY IN ZEBRA CICHLID FISH (*Metriaclima zebra*).

by

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Abstract

Sexual selection and the maintenance of species diversity in Lake Malawi cichlid fishes are greatly dependent on optical communication, which is influenced by environmental, physiological and endocrinological factors. The diversity in spectral sensitivity of cichlids has been partially attributed to differences in opsin gene expression, with each species preferentially expressing a subset of seven possible genes. Hormones such as gonadotropin releasing hormone (GnRH) can mediate changes in gene expression and the presence of GnRH immunoreactive fibers and GnRH receptors throughout the retinal layers make it an excellent candidate for mediating changes in visual processes. Effects of exogenous GnRH administration on the visual system of zebra cichlids (*Metriaclima zebra*) via prolonged release cholesterol implants and intubation was investigated using electroretinogram (ERG) recordings, quantitative real-time RT-PCR and *in situ* hybridization. Three week and ten week sampling periods were used in the intubation study. No obvious differences in spectral sensitivity were evident when looking at *a*-wave, *b*-wave and *d*-wave components of the ERG waveform in any of the treatment groups. A multiple mechanism model was used to describe the cone mechanisms mediating spectral sensitivity and this analysis showed that the activity of cones was shaped by opponent and non opponent cone interactions based on subsets of five opsin genes previously described in cichlids (*SWS1*, *SWS2b*, *RH2b*, *RH2aβ*, and *RH2aα*). Although differences in the spectral sensitivity between control and GnRH-treated fish were not evident on a functional level, there were changes in the gonadosomatic index in the intubation group. Quantitative real-time RT-PCR (qRT-PCR) and *in situ* hybridization demonstrated that treatment with a synthetic GnRH3 analogue using the oral intubation delivery system resulted in statistically significant changes in opsin gene expression in both three week and ten week treatment groups, specifically the upregulation of *RH2b* and the downregulation of *RH2a* opsin genes. Moreover, *in situ* hybridization analysis showed that the pattern of labeling for the *RH2a* and *RH2b* riboprobes corroborated the changes in opsin gene expression found in the qRT-PCR data. In contrast, GnRH treatment using the cholesterol implant delivery system did not result in significant changes in spectral sensitivity or opsin gene expression.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

A_1	vitamin A_1 based chromophore, 11-cis-retinal
A_2	vitamin A_2 based chromophore, 3, 4-didehydroretinal
anti-DIG/AP	anti-digoxigenin antibody conjugated to alkaline phosphatase
bp	base pair
Cq	critical cycle number
DIG	digoxigenin
ERG	electroretinogram
GnRH	gonadotropin releasing hormone
GnRH1	gonadotropin releasing hormone 1, hypothalamic releasing form of GnRH, also hypophysiotropic POA-GnRH
GnRH2	gonadotropin releasing hormone 2, mesencephalic form of GnRH, also chicken GnRH-II, [His^5, Trp^7,Tyr^8]-GnRH
GnRH3	gonadotropin releasing hormone 3, telencephalic form of GnRH, also sGnRH, TN-GnRH system
GnRH-ir	GnRH immunoreactive fibers
GnRH-R	GnRH receptor
GnRH-R1	GnRH receptor 1, also GnRH-Ra1
GnRH-R2	GnRH receptor 2, also GnRH-Rb2
GnRH-Ra1	GnRH receptor subfamily a1, found in cichlids

GnRH-Ra2	GnRH receptor subfamily a2
GnRH-Rb1	GnRH receptor subfamily b1
GnRH-Rb2	GnRH receptor subfamily b2, found in cichlids
GSI	gonadosomatic index
GTH-I	teleost gonadotropin hormone I
GTH-II	teleost gonadotropin hormone II
INL	inner nuclear layer
IPL	inner plexiform layer
k	weight coefficient of cone mechanisms
LH	luteinizing-hormone
LHRH	luteinizing-hormone releasing hormone, the former name for GnRH
LP	long pass filter
LWS	long-wavelength sensitive
MOBI	molecular biology grade
MS-222	tricaine methanesulphonate
MSP	microspectrophotometry
MWS	mid-wavelength sensitive
ND	neutral density
NoRT	non-reverse transcribed
NTC	non-template control
OPL	outer plexiform layer

PBS	phosphate buffered saline
PBST	PBS with 0.1% v/v Tween 20
PCR	polymerase chain reaction
POA-GnRH	hypophysiotropic preoptic area-GnRH system, also GnRH1
qPCR	quantitative PCR
qRT-PCR	quantitative reverse transcriptase PCR
RH1	rod opsin
RH2	rod opsin-like, mid-wavelength sensitive
RH2aα	most long-wavelength sensitive isoform of RH2
RH2aβ	isoform of RH2 with sensitivity in between that of RH2b and RH2aα
RH2b	most short-wavelength sensitive isoform of RH2
sGnRH	salmon GnRH, also GnRH3
SP	short pass filter
SSC	standard saline citrate
SSCT	SSC with 0.1% v/v Tween 20
SWS1	ultraviolet sensitive
SWS2	short-wavelength sensitive
SWS2a	a isoform of SWS2
SWS2b	b isoform of SWS2
T3	3,5,3'-triiodothyronine
T3IRD	T3 inner-ring deiodination

T4	thyroxine
T4IRD	T4 inner-ring deiodination
TBE	buffer containing Tris/Borate/EDTA
TN	terminal nerve
TN-GnRH	GnRH neurons originating from the terminal nerve
TSA	fluorescein tyramide amplification reaction system
UV	ultraviolet
UVS	ultraviolet-sensitive
VP	visual pigment
λ_{max}	wavelength of maximal absorption

Chapter 1

General Introduction and Literature Review

1.1 Overview

Vision is a valuable sensory system that facilitates and enhances an animal's ability to interact with its environment, as well as with other organisms in its habitat. In order for optical communication to occur, there must first be a system in place that allows for the perception and integration of what is conveyed by an optical signal. The vertebrate retina has a system of photoreceptors, composed of cones and rods, which enable the organism to see in light and dark conditions. The receptors contain photosensitive molecules, known as visual pigments (VP), made up of a light-absorbing chromophore, either 11-cis-retinal (A_1) or 3, 4-didehydroretinal (A_2), that is covalently bound via a Schiff's base to an opsin protein that captures photons (Wald, 1968; Yokoyama, 2000). The visual pigment molecules present in the outer segment of the photoreceptors are what confer sensitivity to specific wavelengths of light, with each VP generating a unique spectral range (Yokoyama, 2000).

Five classes of retinal opsin genes are found in vertebrates, one rod opsin (*RH1*) and four spectral classes of cone opsins, including very short-wavelength sensitive (*SWS1*), short-wavelength sensitive (*SWS2*), rhodopsin-like (*RH2*) and long-wavelength sensitive (*LWS*) (Okano et al., 1992; Yokoyama, 2000). The interaction between the chromophore and the opsin protein is what determines the wavelength of maximal absorption (λ_{max}) of the VP (Bowmaker, 1995). The absorption spectrum of the VP can be modified by amino acid substitution in the opsin protein, changes in chromophore usage, or changes in opsin gene expression (Carleton and Kocher, 2001). In teleosts, the mechanisms that cause these changes can occur during different life stages (Allison et al., 2003; Allison et al., 2006; Beaudet et al., 1997; Temple et al., 2008a; Temple et al., 2008b), and changes in retinal processing can be also affected by environmental, physiological and endocrinological factors.

Hormones are able to mediate effects on the visual system. There is a large amount of research on the effects of thyroid hormone (Alexander et al., 1998; Browman and Hawryshyn, 1992; Browman and Hawryshyn, 1994; Plate et al., 2002; Raine and Hawryshyn, 2009; Raine et al., 2010; Temple et al., 2008a; Temple et al., 2008b) and an increasing amount of interest in gonadotropin releasing hormone (GnRH), on visual systems. Studies have shown that GnRH, a hormone that can mediate reproductive effects on a physiological and behavioural level, plays a role in modifying sensory systems in fishes (Kawai et al., 2009; Maruska and Tricas, 2007; Stell et al., 1984; Stell et al., 1987; Behrens et al., 1993; Eisthen et al., 2000; Park and Eisthen, 2003; Umino and Dowling, 1991).

Cichlids are an immensely diverse group of fishes found in the African Great Lakes, and much of this diversity can be attributed to selection pressures influenced by female mate choice (Kocher, 2004). Sexual selection has led to reproductive isolation of species (Kocher, 2004; Seehausen and van Alphen, 1998) based primarily on body colour differences between heterospecific males. The mate choice of female cichlids can be dependent on their visual sensitivity, which in turn can be affected by differences in opsin gene expression (Kocher, 2004).

The significance of investigating the relationship between the visual systems of cichlids and the effects of a modulatory reproductive peptide is that these factors are ultimately tied to the maintenance of species diversity. The large amount of variation in body colouration among males of this family helps prevent hybridization of species. The visual sensitivity and mate-choice preferences of females can be compromised when there are changes in water clarity, as was seen in Lake Victoria (Seehausen et al., 1997; Sitoki et al., 2010). It is therefore necessary to understand the dynamics of vision and the reproductive hormones involved in mate choice as this can potentially impact biodiversity in African cichlids.

1.2 Cichlids Fishes of Lake Malawi

Cichlids of Lake Malawi have diverged recently in evolutionary history and approximately 600 mostly endemic species can be found living in close proximity (Fryer, 1996; Genner et al., 2004). Among the cichlids species, differences among feeding morphologies, habitat partitioning, and body colouration are evident. Tied in with these differences are behaviours and visual sensitivities that are species specific and help keep populations reproductively isolated. Sexual selection pressures are thought to be one of the governing factors that drive the explosive radiation of cichlids as it relates to speciation in terms of colour patterns (Kocher, 2004).

Cones mediate colour vision in teleosts, and it is the ability of the differing cone types to distinguish between wavelengths of light that permits color discrimination. Most teleost species, cichlids included, have a distinct morphological arrangement of cones, with both single and double cones present (Engstrom, 1963; Lyall, 1957). Cichlid cones are arranged in a square retinal mosaic, with a short wavelength visual pigment in the central single cone and two longer visual pigments in each of the double cones (Fernald, 1981; Levine and MacNichol, 1979).

Cichlids have an array of spectral sensitivities, which may be partially attributed to differences in opsin gene expression (Carleton and Kocher, 2001). As previously mentioned, the vertebrate opsin set includes one rod opsin and four cone opsins (Okano et al., 1992; Yokoyama, 2000). In Malawi cichlids, gene duplication events involving specific opsin genes has led to multiple gene isoforms of principally two classes of opsin genes: *RH2* - mid-wavelength-sensitive (*RH2a*α, *RH2a*β, *RH2b*) and *SWS2* – short wavelength sensitive (*SWS2a* and *SWS2b*). Malawi cichlids have seven classes of opsin genes, which include: *SWS1*, *SWS2a*, *SWS2b*, *RH2a*α, *RH2a*β, *RH2b* and *LWS* (Carleton et al., 2008). Each cichlid species differentially expresses a subset of the seven opsin genes, with many reportedly expressing only 3-4 cone opsin genes (Carleton et al., 2008) but new evidence shows that expression may be up to five opsin genes (Sabbah et al., 2010). The interspecific variation in gene expression provides each species with a unique range of spectral sensitivity (Carleton et al., 2008; Carleton, 2009; Parry et al., 2005; Spady et al., 2006).

Many teleosts possess ultraviolet-sensitive cones and have sensitivity in the UV spectrum (Bowmaker, 1995; Harosi and Hashimoto, 1983; Hawryshyn and Harosi, 1991; Hawryshyn and Harosi, 1994), although UV sensitivity is sometimes lost (Allison et al., 2006; Bowmaker and Kunz, 1987; Cheng and Flamarique, 2007) or regained (Allison et al., 2006; Beaudet et al., 1997) throughout their development. Several African cichlids have a UV opsin gene in their genome, but it is not always expressed by the fish as adults (Carleton et al., 2000). Each opsin protein is composed of a distinct amino acid sequence, which interacts with the chromophore to control the spectral absorbance characteristics of the visual pigments (Chang et al., 1995; Sakmar et al., 2002; Yokoyama and Radlwimmer, 1998). Although the amino-acid sequence of the opsin protein is what governs the absorbance of the pigment (Carleton et al., 2008), Carleton and Kocher (2001) suggested that the diversity in visual sensitivity of Malawi cichlids is not due to spectral tuning through opsin amino acid substitution, but due to changes in opsin gene expression. Other mechanisms of spectral tuning of cichlids have been attributed to the short-wavelength absorbing carotenoid pigments in the lenses of some cichlids (Thorpe et al., 1993) and variations in chromophore usage (Bowmaker, 1995; Temple et al., 2008b).

The diurnal activities of cichlids, including conspecific interactions, such as courtship and dominance displays, predator avoidance, (Couldridge and Alexander, 2002; Fernald, 1984; Fernald and Wright, 1985; Seehausen and van Alphen, 1998), and foraging (Lazzaro, 1987; Douglas & Hawryshyn, 1990), are greatly dependent on vision. When presented with the proper visual cues, females are able to correctly distinguish between conspecifics and heterospecifics (Seehausen and van Alphen, 1998), but do not discriminate when colour cues are masked (Seehausen et al., 1997; Seehausen and van Alphen, 1998), and preferentially select males with colour patterns most like that of their own species when only presented with closely related heterospecifics (Couldridge and Alexander, 2002).

Male cichlids often display bright nuptial colouration, and are able to mediate their colour patterns by aggregation or dispersion of granules in specialized pigment cells known as chromatophores (Fujii, 1993). The intensity of the body colour pattern displayed by the fish is related to the motivational state of the fish (Jordan et al., 2004; Muske and Fernald, 1987a; Muske and Fernald, 1987b), and hormonal (Fujii, 1993) or neuronal (Fujii, 1993) control mechanisms.

1.3 Gonadotropin Releasing Hormones

Originally thought to be only a single peptide, it has now been established that gonadotropin-releasing hormone, GnRH (also luteinizing-hormone releasing hormone or LHRH) actually encompasses three families of hormones, each having distinct molecular composition and expression sites in the brain (Fernald and White, 1999). Slightly different forms of this peptide appear in most vertebrates, including fish, and multiple forms can exist within a species. Initially, the nomenclature for GnRHs was based on the species in which it was first discovered, and the original title was maintained even if the same molecular form was subsequently discovered in another species. Based on phylogenetic and molecular analyses, current terminology designates GnRH1 as the hypothalamic releasing form (sometimes referred to as the hypophysiotropic preoptic area-GnRH system (hypophysiotropic POA-GnRH)), GnRH2 as the second, mesencephalic form, and GnRH3 as a third, telencephalic form (also TN-GnRH system) (Fernald and White, 1999; White et al., 1995; Yamamoto et al., 1995). To date, GnRH3 has only been found in teleosts (Chen and Fernald, 2006; Gothilf et al., 1996; Okubo et al., 2000; Parhar et al., 1998; White et al., 1995; Yamamoto et al., 1995). All GnRH forms are decapeptides with protein residues 1, 4, 9, and 10 conserved, and have modified amino and carboxyl termini (Chen and Fernald, 2008).

Since the first descriptions of a piscine GnRH found in salmon (Sherwood et al., 1983), it is now well established that teleosts possess at least two GnRH forms in their brains (Kinoshita et al., 2007). In most perciforms, three functionally and anatomically different forms of GnRH are expressed (Oka, 1997) now classified as GnRH1, GnRH2, and GnRH3 (Fernald and White, 1999; Gothilf et al., 1996; Okuzawa et al., 1997; Powell et al., 1995; Senthilkumaran et al., 1999; Weber et al., 1997). Each ligand is differentially distributed throughout the brain (Lethimonier et al., 2004), and GnRH2 ([His^5, Trp^7,Tyr^8]-GnRH) has been found to be the most ubiquitous and highly conserved form among vertebrate classes (Klausen et al., 2002; Millar et al., 1997). GnRH1 is known to control reproductive functions in vertebrates (Chen and Fernald, 2006). It acts on receptors found in the pituitary to trigger the release of gonadotropins (GTH-I and GTH-II in teleosts) from the pituitary (Baba et al., 1971; Sherwood, 1987; White et al., 1995). This peptide form has also been implicated in the control of growth hormone (Klausen et al., 2002; Carolsfeld et al., 2000; Li et al., 2002; Marchant et al., 1989; Melamed et al., 1995), prolactin (Weber et al., 1997) and somatolactin (Kakizawa et al., 1997).

Previous studies have postulated that GnRH1 is the most physiologically relevant form in perciformes as it relates to the induction of oocyte maturation, ovulation and maturation, as well as the main luteinizing hormone (LH) releaser (Zohar and Mylonas, 2001). GnRH2, originally isolated from the chicken brain (Miyamoto et al., 1984), but found to be almost ubiquitous in all vertebrates studied to date (Conlon et al., 1993; Millar, 2003; Mongiat et al., 2006; Sherwood et al., 1993), is produced in the midbrain tegmentum and neurons project widely throughout the brain, especially to the mid and hindbrain (Muske et al., 1994; Yamamoto et al., 1995). GnRH2 is sometimes found together with GnRH1 in the pituitary, but GnRH1 is thought to be the only GnRH peptide acting on gonadotropin release (Kim et al., 1995; Mongiat et al., 2006). GnRH2 may play a role in sexual behaviour and the neuroendocrine control of the gonads (Hofmann, 2006; Kauffman and Rissman, 2004a; Maney et al., 1997; Schiml and Rissman, 2000; Temple et al., 2003; Volkoff and Peter, 1999; White et al., 2002), and food intake and energy balance (Kauffman and Rissman, 2004a; Kauffman and Rissman, 2004b; Kauffman et al., 2005; Kauffman et al., 2006; Matsuda et al., 2008; Temple et al., 2003).

GnRH3 is localized in the terminal nerve ganglia (TN – located in the olfactory bulb) (White et al., 1995), which project to retinal and olfactory neuroepithelia (Grens et al., 2005; Kawai et al., 2009; Wirsig-Wiechmann and Wiechmann, 2002) and receive both glutamatergic and excitatory GABAergic synaptic inputs from brain regions, which are considered to be CNS centres for sensory integration (Nakane and Oka, 2010; Oka, 2010; Yamamoto and Ito, 2000). It has been suggested that GnRH3 originating from the TN has neuromodulatory capabilities acting on the forebrain, as well as influencing olfactory and retinal processing (Eisthen et al., 2000; Oka, 1992). TN-GnRH cell axons follow the optic nerve innervating the retina of teleosts, and synapse onto dopaminergic interplexiform cells, as well as falling adjacent to ganglion, bipolar, amacrine and horizontal cells (Grens et al., 2005; Munz et al., 1982; Stell et al., 1984; Zucker and Dowling, 1987). It has also been proposed that TN-GnRH neurons are responsible for controlling motivational state, including sexual behaviour (Abe & Oka, 2007).

The sexual behaviours reported being influenced by GnRH3 include nest-building (Ogawa et al., 2006; Yamamoto et al., 1997), aggression (Ogawa et al., 2006), and spawning behaviour (Volkoff and Peter, 1999). GnRH3 can also influence signal processing in sensory pathways (Behrens and Wagner, 2004; Behrens et al., 1993; Kinoshita et al., 2007; Maruska and Tricas, 2007), suggesting that relative to reproductive state, GnRH3 may modulate sensory input (Chen and Fernald, 2008; White et al., 1995).

GnRH acts via receptor binding, and as with GnRH ligands, multiple forms of GnRH receptors can be found in one species, and several forms of which have been cloned and identified in vertebrates (Bogerd et al., 2002; Chen and Fernald, 2008; Flanagan et al., 2007; Illing et al., 1999; Troskie et al., 2000; Wang et al., 2001).

1.3.1 GnRH Receptors

GnRH receptors (GnRH-R) belong to the superfamily of G-protein coupled receptors with seven hydrophobic alpha helix transmembrane domains linked by extra and intracellular loops (Chen and Fernald, 2008; Chen and Fernald, 2006; Millar, 2003). The extracellular transmembrane domains are normally responsible for binding, especially the third extracellular loop (Millar et al., 2004; Troskie et al., 1998). Classifications of GnRH receptors have been based on ligand-receptor relationships according to genes and C-terminal structure (Millar et al., 2004), C-terminal size (Guilgur et al., 2006), amino acid sequence of one extracellular loop and GnRH binding affinity (Troskie et al., 1998), and phylogenetic and structural analyses (Flanagan et al., 2007).

According to phylogenetic and structural groupings proposed by Flanagan and colleagues (2007), GnRH receptors in vertebrates can be subdivided into four subfamilies, grouped according to a set of three conserved amino acids in the third extracellular loop, as well as size ranges of the C-terminals. The subfamilies include GnRH-Ra1, GnRH-Ra2, GnRH-Rb1 and GnRH-Rb2 (Chen and Fernald, 2008). GnRH-Ra1 and b2 are found in teleosts, and each subfamily can bind with all three GnRH ligands. Each receptor has a significantly higher affinity for GnRH2 ligands (Chen and

Fernald, 2008), than for either GnRH1 or GnRH3 (Flanagan et al., 2007; Millar et al., 2004; Wang et al., 2001).

The three forms of GnRH ligands and two GnRH receptors have been identified in the cichlid *Astatotilapia* (*Haplochromis) burtoni* (Chen and Fernald, 2006). All three types of GnRH ligand can act on each of the two GnRH receptors as demonstrated biochemically (Bogerd et al., 2002; Illing et al., 1999; Lethimonier et al., 2004), and as implied from evidence from the retina, where both receptors are expressed but only GnRH3 is present (Chen and Fernald, 2006; Grens et al., 2005; Robison et al., 2001). The distribution of the two receptor types is distinctly different. GnRH-R1 is mainly found in the brain areas related to reproductive function and is highly expressed in the pituitary. These results suggest that GnRH-R1 may be important for regulating reproduction. Conversely, GnRH-R2 was detected widely throughout the brain, from the olfactory bulb to the medulla, as well as the pituitary. The expression of GnRH-R2 receptor mRNA levels have also been shown to vary with sex, social status and reproductive state at the level of the olfactory system in cichlids (Maruska and Fernald, 2010). Thus GnRH-R2 is located in a brain area which allows GnRH to modulate sensory, metabolic, and motor systems, further supporting the notion that GnRH may be responsible for changes in visual systems.

1.3.2 GnRH Delivery Systems

GnRH analogues can be subdivided into two types, agonists and antagonists. A GnRH agonist is an analogue which mimics effects of endogenous GnRH, such as stimulating the release of gonadotropin II (GTH-II) (Baba et al., 1971). GnRH antagonists block GnRH receptors and result in a drop in gonadotropin secretion (Dufour et al., 2010). The development of highly potent synthetic GnRH agonists opens new doors to hormonal manipulation therapies, and the hormones have been used successfully in controlling reproductive success in aquaculture (reviewed in Zohar & Mylonas, 2001).

GnRHs are structurally similar among fish species (Sherwood et al., 1994) and thus the use of GnRHs is generic and the same GnRH agonists have been successfully applied to a wide range of fish species (Zohar and Mylonas, 2001). Native GnRHs are rapidly degraded by endopeptidases upon administration by injection (Zohar and Mylonas, 2001). The decapeptide is cleaved between positions 5-6 and 9-10, inactivating the hormone. Due to amino acid and ethylamide substitutions, synthetic GnRH agonists are designed to be resistant to enzymatic degradation (Weil et al., 1991; Zohar et al., 1990) and are able to elicit stronger and more prolonged effects when compared to the native peptides. In some instances, the substitutions of the synthetic variants result in an increased binding affinity to GnRH-Rs (De Leeuw, 1988; Habibi et al., 1989; Pagelson and Zohar, 1992). Several methods of administration have been used to expose fish to GnRH, and investigations have compared the effectiveness of different delivery methods as well as vehicles. Methods studied include gill surface application (Sherwood and Harvey, 1986), dissolution into surrounding water (Sherwood and Harvey, 1986), dietary (Mikolajczyk et al., 2002; Solar et al., 1990; Thomas and Boyd, 1989), injection (including but not limited to intracranial, intraperitoneal, intramuscular, intracerebroventricular) (Lam et al., 1976; Matsuda et al., 2008; Plate et al., 2002; Plate et al., 2003; Weil and Crim, 1983) and sustained release or implantation (Carolsfeld et al., 1988; Crim et al., 1983; Crim and Glebe, 1984; Crim et al., 1988; Sherwood et al., 1988; Weil and Crim, 1983; Carolsfeld et al., 2000).

Drawbacks with injections are that the peptide is quickly degraded and excreted (Crim et al., 1988), and there can be increased stress to the fish, especially with repeated handling, which in some instances can lead to ovarian regression in fish that are more sensitive (Juario et al., 1984). Dietary administration provides a convenient and controlled method of treating fish with GnRH. Previously used oral delivery systems include feeding of GnRH injected shrimp (Thomas and Boyd, 1989) as well as oral intubation (Mikolajczyk et al., 2002; Solar et al., 1990).To maximize the efficiency of absorption, the hormones are also often administered in conjunction with a penetration enhancer as well as an antacid. Several methods have been devised to extend the release of GnRH analogues, the first systems being prepared using cholesterol (Weil and Crim, 1983). Variations using differing ratios of cholesterol and cellulose produced slow and fast releasing implants (Carolsfeld et al., 2000; Sherwood et al., 1988). Other developed means include injectable polyanhydride microspheres (Mylonas et al., 1995), pellets based on methacrylate embedding resin (Matsuyama et al., 1993; Scott et al., 1999), ethylene and vinyl acetate copolymer (EVAc) (Rhine et al., 1980), silicon (Weil and Crim, 1983), and coconut oil (Scott et al., 1999). The advantages of using dietary administration or a sustained release system is that they alleviate the need for numerous treatments, and thus reduce handling and stress of the fish.

1.4 Rationale for Study and Research Objectives

The presence of GnRH immunoreactive (GnRH-ir) fibers (generally originating from the terminal nerve, TN), and GnRH receptors in the teleost retina has been confirmed via immunocytochemistry (Grober et al., 1987; Maruska and Tricas, 2007; Munz et al., 1982) immunohistochemistry and *in situ* hybridization (Grens et al., 2005). Concentrated identification of the GnRH-ir fibers and GnRH receptors are found at the inner nuclear and inner plexiform layer (INL and IPL respectively). GnRH-ir fibers of the TN have been shown to synapse onto dopaminergic interplexiform cells, present in the amacrine cell layer, which project to the OPL (outer plexiform layer) and IPL (Dowling and Ehinger, 1978; Zucker and Dowling, 1987). Together with dopaminergic interplexiform cells, GnRH-ir fibers provide a neural connective pathway from the telencephalon, via the TN, to the photoreceptors (Behrens et al., 1993). On a morphological and physiological level, GnRH may act on the retina indirectly, via mediators such as dopamine and thyroid hormones. Dark adapted retinas incubated with GnRH3 are induced to increase light-adaptive spinule formation of the horizontal cells.

The horizontal cell processes extend into the OPL, which may be mediated by dopamine release from the dopaminergic interplexiform cells (Behrens et al., 1993). Horizontal cell gap junctions are uncoupled by dopamine (Baldridge et al., 1987), and provide negative feedback onto cone photoreceptors (Ramsden et al, 2008). This negative feedback governs opponent interactions between photoreceptors, which may be reflected in changes in spectral sensitivity. Application of GnRH to horizontal cells causes depolarization and changes in response amplitude to small-spot and full field stimuli, with cells most sensitive to application of GnRH3 when compared to mammalian GnRH (a GnRH1) or GnRH2 (Umino and Dowling, 1991). Thyroid hormones, which have been shown to play an important role in the cone distribution and opsin gene expression (Raine and Hawryshyn, 2009; Raine et al., 2010; Temple et al., 2008b), may to an extent be under the control of GnRH. For instance, retinal thyroid hormone regulatory processes (inactivation of thyroxine (T4) and 3,5,3'-triiodothyronine (T3) by inner-ring deiodination (T4IRD and T3IRD), were depressed in salmonids injected with GnRH, thus increasing T3 levels in the retina (Plate et al., 2002).

GnRH neurons are often found in conjunction with deep-brain photoreceptors responsible for regulation of circadian rhythms and integration of seasonal reproductive cues in non-mammalian vertebrates, creating a link between the photoperiod and reproductive axis (Saldanha et al., 2001). GnRH3 is only found in teleosts (Chen and Fernald, 2006; Gothilf et al., 1996; Okubo et al., 2000; Parhar et al., 1998; White et al., 1995; Yamamoto et al., 1995), and is the only GnRH isoform found in the retina, and is capable of binding to both types of GnRH receptors present in the retina (GnRH-R1 and GnRH-R2) (Chen and Fernald, 2006;Grens et al., 2005; Robison et al., 2001). The distribution of the GnRH receptors has been localized in teleosts. Specifically, as demonstrated in a Tanganyikan cichlid, GnRH-R1 was found sporadically distributed in the amacrine cell layer, whose neural networks are responsible for the lateral input to the transmission of visual information from the photoreceptors to the brain, while GnRH-R2 was found with high abundance in the ganglion cell layer (Maruska and Fernald, 2010). In Grens et al., (2005), *in situ* hybridization analysis showed GnRH-R expression in the retina, but the retinal sections illustrated did not show the photoreceptor layers very clearly. This line of evidence strongly supports my hypothesis that this reproductive peptide directly affects retinal processing via opsin gene expression and neuromodulation of photoreceptors, which may influence changes in the spectral sensitivity of cichlids as it relates to sexual selection and mate choice.

In this study, I incorporated electrophysiological, molecular and neuroendocrinological techniques to analyse spectral sensitivity, opsin gene expression, as well as using *in situ* hybridization to identify the spatial arrangement of cone photoreceptors in the retinal cone mosaic. The following research questions were aimed at exploring the effect of GnRH on the visual systems of cichlids:

(i) Does treatment with GnRH affect spectral sensitivity?

(ii) Does GnRH treatment affect opsin gene expression?

(iii) Does GnRH modify the spatial arrangement of cones in the retinal mosaic?

The results of this study will help elucidate the potential functional significance of GnRH at the level of the retina. Additionally, since vision is critical to the daily interactions of *M. zebra*, especially as it relates to mate choice and sexual selection, my research will provide valuable insight to the integrative role of vision and reproduction in the maintenance of cichlid biodiversity.

Chapter 2

Does treatment with GnRH affect spectral sensitivity in zebra cichlids (*Metriaclima zebra*)?

2.1 Introduction

Sexual selection in African cichlids involves highly dynamic optical sensory integration, with particular emphasis on body colour as a visual basis for mate choice. There is also strong sexual dimorphism between the sexes, with males exhibiting bold colouration and females being rather dull. Based on body colouration and displays by competing males, females select the individuals with whom to mate. Some of the explosive radiation exhibited by cichlids is attributed to the diversification of colour patterns (Kocher, 2004), and thus a large amount of the effort in vision research has focused on body colour and photoreceptor spectral absorption. Much of the information on spectral absorption data of individual photoreceptors has been obtained using microspectrophotometry (MSP) (Parry et al., 2005), and recent molecular studies have also looked into opsin gene expression of African cichlids (Carleton et al., 2008). However, MSP measurements unlike those obtained via electroretinogram recording (ERG), do not take into consideration opponent and non-opponent cone interactions representing the neural processing required for colour vision. These interactions shape the spectral sensitivities of cichlids, which are conferred by the expression of different opsin genes. The species investigated in my research, *Metriaclima zebra*, a rock-dwelling fish endemic to Lake Malawi, possesses five cone opsin genes (λ_{max}: *SWS1* - 368, *SWS2b* - 423, *RH2b* - 484, *RH2a*β - 519, *RH2a*α - 528 nm) (Parry et al., 2005). The spectral sensitivities conferred by the different opsins range from ultraviolet to long wavelength sensitive, forming the basis of colour vision in vertebrate systems.

Optical signaling in fishes is not solely reliant on the visual stimuli, but also highly dependent on the behavioural and social contexts in which they are presented. The dynamic factors of signaling must therefore be evaluated in addition to the static components of the visual appearance of cichlids. Optical signaling can be subject to rapid modification, which may be under the influence of neuronal or hormonal mechanisms. Hormone level fluctuations can play an important role in variations in the retinal cone complement (Allison et al., 2006; Raine and Hawryshyn, 2009; Raine et al., 2010), which may have dramatic effects on the visual performance and behaviour of an organism. Specifically, I have looked at the effects of GnRH on visual processes.

GnRH immunoreactive (GnRH-ir) fibers (generally originating from the terminal nerve, TN) are found in the retina or optic nerve of several vertebrates, (Fukuda et al., 1982; Wirsig-Wiechmann and Wiechmann, 2002; Wirsig-Wiechmann, 1993) and occur in high densities in the retinal tissue of fish (Oka and Ichikawa, 1991; Stell et al., 1984; Stell et al., 1987). In the teleost visual system, neuromodulatory effects of GnRH may be mediated via the TN-dopamine-interplexiform cell pathway (Behrens and Wagner, 2004; Grens et al., 2005; Maaswinkel and Li, 2003). The TN-GnRH neurons may have neuromodulatory functions that are influenced by physiological and environmental parameters experienced by a fish (Abe and Oka, 2002; Haneda and Oka, 2004; Oka, 1992; Oka and Matsushima, 1993). GnRH receptors (GnRH-R) have been found in the retina of several fish species, with multiple types in one organism, such as the Tanganyikan cichlid, *Astatotilapia burtoni* (Chen and Fernald, 2008). In cichlids, GnRH-R1 is distributed in the inner plexiform and inner nuclear layers, an area where lateral integration of optical information occurs (Grens et al., 2005).

In this study, I evaluated the effect of GnRH3 treatment on spectral sensitivity of cichlids using electroretinogram (ERG) recordings, by measuring changes in the *a*- *b*- and *d*-waves, which reflect the electrical activity of the photoreceptors and bipolar cells. My prediction was that GnRH treatment would modify spectral sensitivity, either by a direct neuromodulatory effect on the retina or indirectly through changes in opsin gene expression. In general, my analysis did not reveal changes in spectral sensitivity as a result of GnRH treatment.

2.2 Methods

2.2.1 Experimental Animals

Our original brood stock of *Metriaclima zebra* were obtained from the Nankumba Peninsula in Lake Malawi, Malawi through Old World Exotic Fish Incorporated (Miami, Florida, USA). The fish used in this study were F1 and F2 progeny of the original brood stock. Fish were kept visually isolated (by means of opaque barriers) in 5 L aquaria at 26-

$29^{o}C$, fed daily with TetraCichlid mini granules (Tetra Holding, Blacksburg, VA, USA) and Spirulina aquarium flake food (Ocean Star International, Burlingame, CA, USA). Fish were maintained under a 12 h:12 h L:D cycle, with lighting conditions provided by full-spectrum fluorescent lamps with BlueMax™ colour technology (Full Spectrum Solutions, Inc., Jackson, MI, USA). All experimental procedures and animal care protocols were in accordance with regulations set by Queen's University Animal Care Committee under the auspices of the Canadian Council for Animal Care.

2.2.2 GnRH Treatments

Experimental fish were treated with GnRH3 (salmon GnRH) by means of either a cholesterol implant or a GnRH solution via intubation. To manufacture the cholesterol pellets, I used salmon GnRH (pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH2; LHRH salmon) from Sigma-Aldrich. Synthetic salmon GnRH (pGlu-His-Trp-Ser-Tyr-D-Arg-Trp-Leu-Pro-NHC_2H_5; Ova-RH, Syndel Laboratories, Vancouver, BC) was used for the intubation solutions. GnRH3 was used as it is the only type found in the retina (Chen and Fernald, 2006; Grens et al., 2005), and it elicits physiological and behavioural responses in treated fish (Ogawa et al., 2006; Volkoff and Peter, 1999; Yamamoto et al., 1997).

2.2.2.1 Cholesterol Pellet Implants

Fish were divided into two experimental groups, control (receiving no implant) and GnRH treated (implanted with a pellet containing GnRH) (Figure 2.1). Pellets were fabricated based on the methodology outlined in Lee et al., 1986 with the exception that coconut oil was used as a binder instead of cocoa butter. In brief, GnRH was dissolved in 50 percent ethanol and added to powdered cholesterol. This mixture was dried, molten coconut oil was added, and the product was pelleted in a customized mold. The cholesterol pellets contained 15-20 percent coconut oil and 1.5 μg GnRH (for details on implant preparation and implantation see Appendix A).

Fish were anesthetized with tricaine methanesulphonate, (MS-222; 200 mg L^{-1} for 5 minutes) and a small ventral incision (0.5 cm) was made 0.5 cm posterior to the pelvic fins in order to insert the pellet into the peritoneal cavity of the fish with the aid of an implanter. Fish were left for two weeks prior to being tested via electroretinogram (ERG) recording (Figure 2.1).

2.2.2.2 GnRH Intubation

Fish were divided into three treatment groups, control, GnRH treated, and 10 week GnRH treated (Figure 2.1). Each fish received 10 μl of intubation solution per gram body mass, containing 0.01 mg ml^{-1} L-lysophosphatidylcholine (Sigma, St-Louis MO, USA), 1 mg ml^{-1} sodium bicarbonate, and 0.1 mg ml^{-1} GnRH (Ova-RH, Syndel Laboratories, Vancouver B.C.) in 0.65 percent saline, ensuring that each fish in the

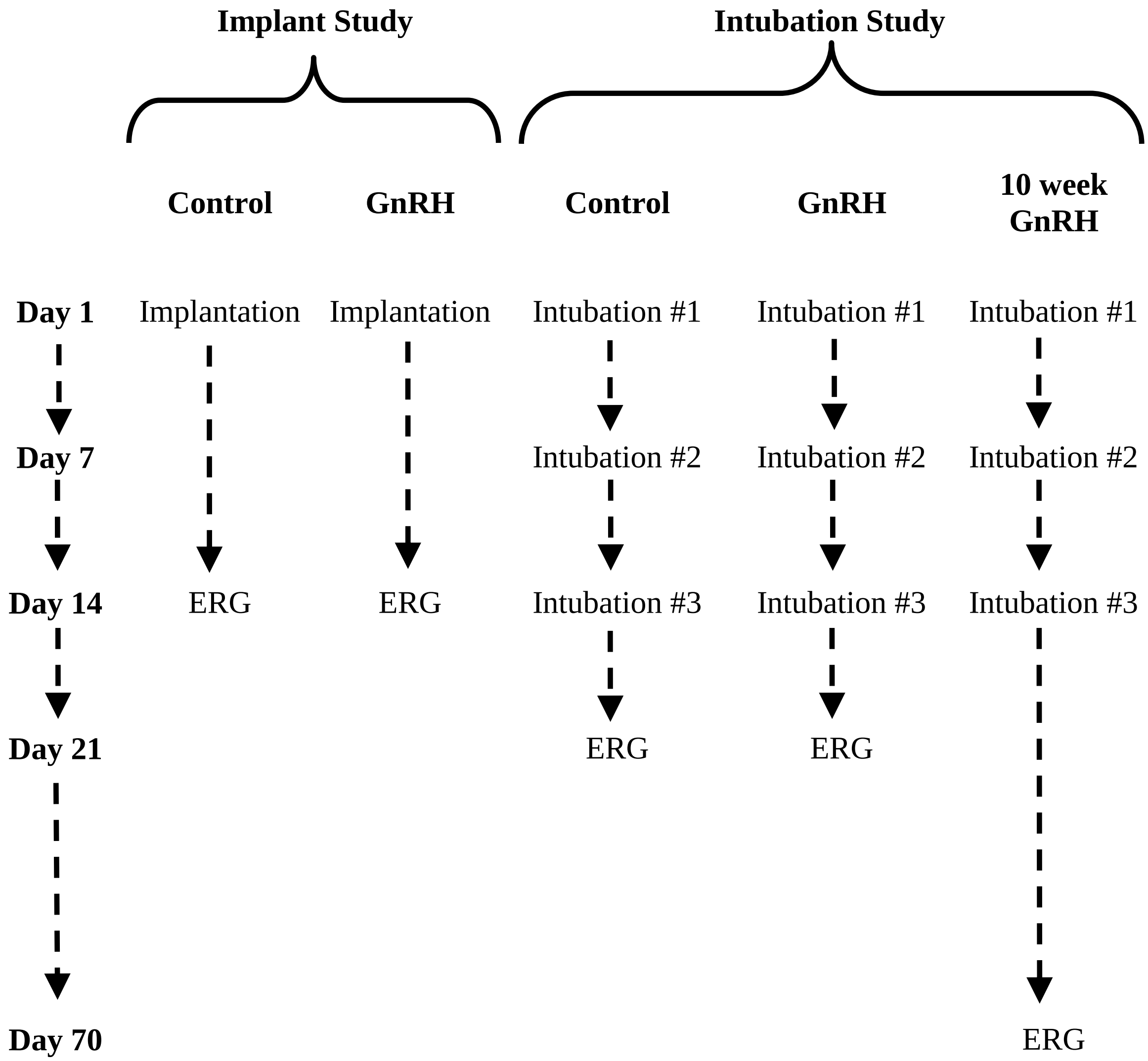

Figure 2.1. Flow chart demonstrating the time course of treatment and electroretinogram recording (ERG) for both the implant and intubation delivery methods. Fish in the implant study were treated on day 1 and sampled via ERG on day 14. Fish in the intubation study were treated on day 1, day 7 and day 14. In the intubation study, the control fish and the GnRH treated fish were sampled via ERG on day 21. The 10 week GnRH fish in the intubation study were sampled on day 70.

treatment group received 1 μl of GnRH per gram body mass. The same solution without GnRH was administered to the control group (see Appendix B for details on solution preparation). The volume to be administered was measured and preloaded into the tubing. The fish were lightly anesthetized with MS-222 (200 mg L^{-1} for 2-3 minutes) and subsequently intubated via the oral cavity by placing the tubing between the pharyngeal plates and sliding it into the stomach. This procedure was repeated twice more, 7 and 14 days after the first intubation. The fish were used for ERG recording either 21 (control and GnRH groups) or 70 (10 week GnRH group) days after the initial treatment (Figure 2.1). The later sampling period was instituted to examine fish that had been exposed for a significantly longer period in the event that GnRH did not have an effect on spectral sensitivity at 21 days after initial treatment. Modifications in opsin gene expression exert changes in visual pigment with cone outer segment renewal which requires new membrane to be produced and turned over in the outer segment (Bassi & Powers, 1990; Jonnal et al., 2010), a process that may require a time frame that extends beyond the 21 day sampling period.

2.2.3 Preparation of Fish

Prior to being used for ERG recording, fish were immersed in MS-222 (200 mg L^{-1}) buffered with sodium bicarbonate until respiratory collapse (stage IV anesthesia (Jolly et al., 1972)). Standard length (cm), total length (cm) and body mass (g) of the fish were recorded. The fish was further anesthetized by means of injection with methomidate hydrochloride (Maranil, 0.075 mg g^{-1} body mass) and immobilized by means of injection of pancuronium bromide (0.05 mg g^{-1} body mass) into several sites of the epaxial musculature. The fish was then positioned in a restraining cradle in a Faraday cage, with a tight-fitting mouthpiece inserted into the buccal cavity to irrigate the gills with aerated water (flow rate approximately 1.5 ml s^{-1}). The fish was covered with wet cheesecloth and the body drip-irrigated between trials. At the end of the experiment, the fish was sacrificed and both retinas were removed, under deep red illumination, for further opsin gene expression analysis.

2.2.4 ERG Recording and Analysis

Electroretinograms (ERG) were recorded from the cornea of the fish to determine the effects of GnRH on spectral sensitivity in *M. zebra* (see Appendix C for details on experimental set-up). Each fish was stimulated with wavelengths of differing light intensities to determine spectral sensitivity of the test fish. Test wavelengths were presented in a staggered sequence to prevent selective adaptation of any given cone mechanism, with the wavelength ranging from 300 to 700 nm in 10 nm intervals. For each wavelength, amplitude (μV) of the *b*-wave (Figure 2.2) response was plotted against the intensity of the stimulus (log photon irradiance ($\log_{10}$ photon cm^{-2} s^{-1}). The resultant response versus intensity curves were fitted individually by a third order polynomial function using a least squares method (Figure 2.3). The threshold intensity values were determined by making an interpolation using the criterion response level. The criterion

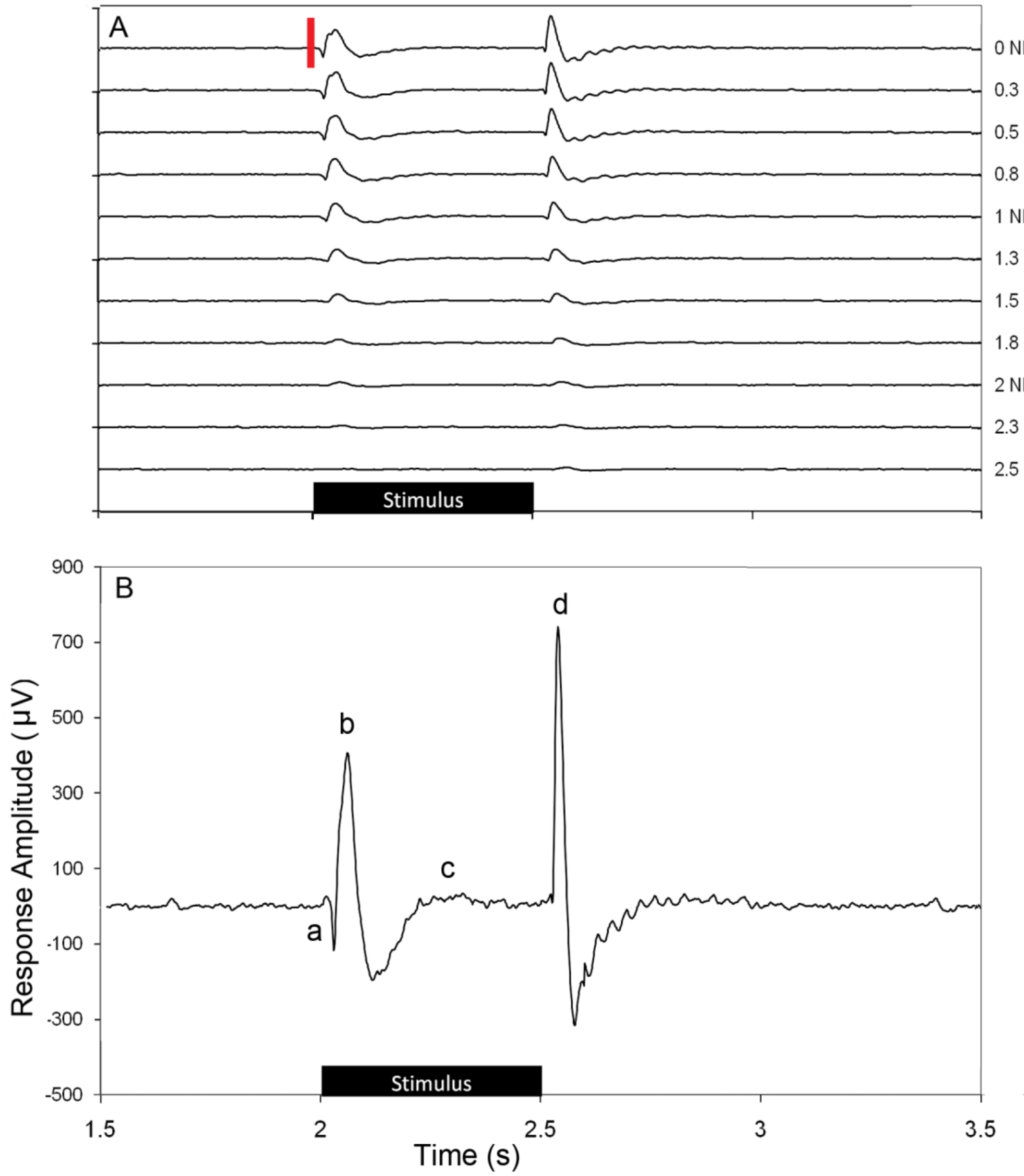

Figure 2.2. Electroretinogram (ERG) waveforms from one fish. (A) ERG traces at increasing intensities at 530 nm. Note that these waveforms were adjusted vertically for purposes of clarity. 0.0 ND (neutral density) corresponds to 16.241 log photon irradiance. The vertical scale bar (red) represents 1000 µV. (B) ERG waveform components showing the *a*-wave (photoreceptor response), *b*-wave (ON bipolar cell response), *c*-wave (pigment epithelium) and *d*-wave (OFF bipolar cell response) at a test wavelength of 530 nm at 0 ND 16.241 log photon irradiance.

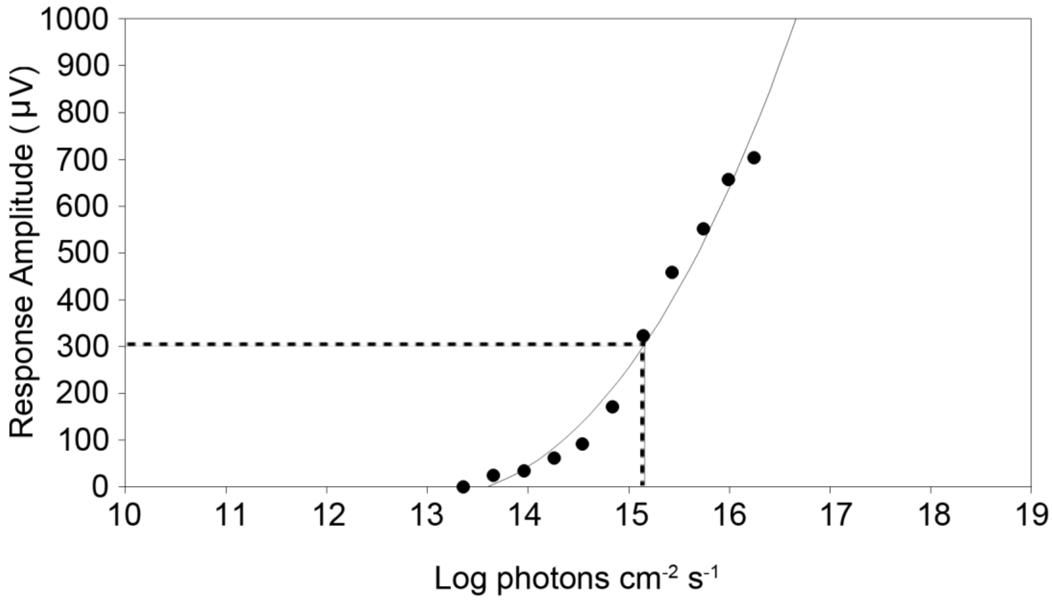

Figure 2.3. Response versus intensity curve for one fish at a test wavelength of 530 nm. The solid line represents a third order polynomial function fit with the least squares method. The dashed line illustrates the interpolation of threshold intensity for a criterion response value of 300 µV.

response was taken as the half maximum response level of noise-corrected response amplitude for the wavelength that yielded the highest response amplitude for a trial, which was then applied to all test wavelengths. The criterion response level consistently fell on the linear portion of the response versus intensity curve for all wavelengths for that trial. Log relative sensitivity was determined as the reciprocal of threshold intensity for a given test wavelength. Log relative sensitivity values were normalized between 0 and 1 to the highest values to generate normalized sensitivity versus wavelength curves to remove differences in absolute sensitivity between individuals. The data from all fish were subsequently pooled and averaged for each wavelength to obtain one mean normalized spectral sensitivity curve for each treatment group and adaptation condition.

For each fish, spectral sensitivity was measured under two background conditions: white, spectrally broad, background designed to adjust the sensitivity of the cone mechanisms to roughly the same level (see Appendix D for details on filter set used); and a yellow, long wavelength dominated background designed to partially light adapt the mid and long wavelength sensitive cone mechanisms (see Appendix D for details on filter set used).

2.2.5 Analysis of Spectral Sensitivity Curves

To correlate spectral sensitivity with cone visual pigments, the mean normalized spectral sensitivity curves were initially fit by visual pigment templates based on Govardovski, et al. (2000) using a least squares fit. It was assumed that the curves received input from five cone mechanisms previously identified by microspectrophotometry (MSP) and gene expression studies in *M. zebra*, *SWS1*, *SWS2b*, *RH2b, RH2aα,* and *RH2aβ*. Cichlids have a paired-pigment visual pigment system, consisting of mixtures of vitamin A derived chromophores, A_1 (11-cis retinal) and A_2 (3,4-didehydroretinal) (Carleton et al., 2008). Switching from an A_1 to an A_2 visual pigment for the same opsin has the effect of shifting the λ_{max} of the visual pigment to a longer wavelength, and the magnitude of this bathochromic shift was wavelength dependent such that visual pigments with a longer λ_{max} exhibit larger bathochromic shifts (Harosi and Hashimoto, 1983). To produce visual pigment templates, the absorption spectra of the A_1 - A_2 mixture (previously described in Govardovski et al., (2000) by equations 1,2,4,5a,5b for A_1 and equations 1,4,6a,6b,8a,8b for A_2) was based on the A_1/A_2 ratio, which was determined using a curve fitting process for each treatment group and adaptation condition. The proportion of the A_2 state was presented using a fraction parameter, *a* ($0 \leq a \geq 1$), and therefore the absorption spectra of a given cone type exhibiting an A_2 proportion of *a* was calculated as

$$A_2(a) = A_1 \cdot (1 - a) + A_2 \cdot a \qquad \text{Equation 1}$$

The long-wavelength portion of the spectral sensitivity curve was governed by *RH2a*, which typically manifests a peak in the range of 560-600 nm. The long-wavelength limb of this peak was used to find the A_2 percent. A least squares fit was utilized to allow Microsoft Excel to find a visual pigment template that best describes the long-wavelength limb of the spectral sensitivity curve by leaving the A_2 proportion and the weight coefficient of cone mechanism (*k*) unrestricted. This approach to determining the A_1:A_2 ratio was most accurate since shifts in λ_{max} occurring from substituting A_1 for A_2 are largest here, and the long-wavelength limb of the spectral sensitivity curve was governed by one cone class and should therefore be free from opponent interactions. The A_2 value was then kept constant for fitting the remainder of the peaks, while the *k* parameter was left unrestricted. For each peak, the visual pigment template was limited to a specific spectral range. Each visual pigment template was corrected for the effects of the lens spectral transmission (obtained from measurements made in our lab as per Lisney et al., 2010). The proportion of variance accounted for by the visual pigment templates (R^2) was also quantified.

In addition, a multiple mechanism model was used to determine the relative input weights of each of the cone mechanisms, by assigning positive (excitatory) or negative (inhibitory) weights to each mechanism (Hughes et al., 1998; Kalloniatis and Harwerth, 1990; Sperling and Harwerth, 1971). The multiple mechanism model takes the general form:

$$S_{\lambda 1,\lambda 2}(\lambda) = \sum k_i \cdot A_i(\lambda) \qquad \text{Equation 2}$$

where $S_{\lambda 1, \lambda 2}(\lambda)$ represents the regions of the spectral sensitivity curve enclosed by $\lambda 1$ and $\lambda 2$ to reflect the peaks and depressions present in the curve (Hughes et al., 1998), while taking cone interactions into consideration; $A_i(\lambda)$ denotes the absorption of the visual pigment template, corresponding to cone *i* (*i* = SWS1, SWS2b, RH2b, RH2bβ or RH2aα) at a wavelength λ (nm); and k_i represents the weight coefficient of cone mechanism i. The relative weights of the cone mechanisms (k) also vary for each of the interactions for all of the treatments and conditions.

The multiple mechanism model was fit to the mean normalized spectral sensitivity curves for each treatment group and adaptation condition using a least-squares approach. The portion of the spectral sensitivity curve analysed was within the bounds designated by $\lambda 1$ and $\lambda 2$ (Hughes et al., 1998; Kalloniatis and Harwerth, 1990; Sperling and Harwerth, 1971). To apply the model and ensure the best fit, the fewest possible cone interactions were selected to describe the curve (two or three), with the same set of cone mechanisms used to describe both adaptation conditions (white or yellow) for a particular treatment group (control and GnRH in the cholesterol implant study, and control, GnRH and 10 week GnRH in the GnRH intubation study). The k values (weight coefficients of each cone mechanism) and R^2 values were also determined.

2.2.5.1 Analysis of Spectral Sensitivity Using other ERG Waveform Components

In the previous section, spectral sensitivity was based on the ERG *b*-wave amplitude as the response, which reflects the activity of the ON bipolar cells. This represents the most common methodology for measuring spectral sensitivity. However, it was possible that GnRH may have acted on the retina at a different locus, which may have resulted in changes in other waveform components of the ERG response waveform. GnRH is released by efferent terminal nerve fibers onto interplexiform cells that span the outer plexiform and inner plexiform layers (Grens et al., 2005) and this may have changed the waveform in ways not represented in the *b*-wave. For instance, a signal suppression resulting from blocking glutamate uptake of the photoreceptors or a reduction in horizontal cell feedback on cones, as is caused by the application of cobalt chloride, causes a decrease in amplitude and temporal broadening of the *a* and *d*-wave (Ramsden et al., 2008). Increases in thyroid hormone (T4) serum levels under treatment with levothyroxine is accompanied by a shortening of peak times of *a* and *b*-waves in hypothyroid dogs, suggesting an effect at the level of the retina (Durieux et al., 2008). GnRH and GnRH receptors are present in several areas of the retina (Grens et al., 2005), which may permit it to act on the neural network such that the effect can be seen in other components of the ERG. To determine whether other salient features of retinal response were affected by GnRH treatment, the *a*-wave and *d*-wave components of the waveform were measured and spectral sensitivity determined and analyzed. The amplitude of the *a*-wave was measured from the baseline to the *a*-wave trough, and that of the *d*-wave from the breaking point of the *c*-wave to the peak of the *d*-wave. This analysis was restricted to the fish that were used in the intubation experiments.

The values obtained from GnRH treated fish were compared to the spectral sensitivity of control (non-treated) fish to determine whether GnRH causes changes that may have been missed by looking at the *b*-wave in the spectral sensitivity of cichlids. Spectral sensitivity was examined under spectrally neutral background conditions (white light adaptation) to ensure that all available cone mechanisms were expressed in spectral sensitivity curves. Chromatic adaptation with a yellow background was used to lower the sensitivity of the test fish to the long wavelength portion of the spectrum (for details adaptation conditions and quantum catches (number of photons (quanta) absorbed by the photoreceptor) see Appendix D). Each fish was allowed to adapt to the background conditions for 45 minutes prior to recording.

2.2.6 Evaluation of Differences in Gonadosomatic Indices

Testes and ovaries were removed and weighed to calculate the gonadosomatic index (GSI) (GSI = (gonad mass/body mass)×100) upon completion of the ERG. The GSI of female fish only were compared as very few males were available for comparison. The data were analysed statistically by means of a Kruskal-Wallis test (KW), as the assumptions of a parametric test were not met. Statistical analysis was completed in JMP 8.0.1 (SAS Institute, Cary, NC, USA).

2.3 Results

ERG responses to 500 ms flashes of light of increasing intensity for wavelengths ranging from 300 to 700 nm were recorded and compared for *M. zebra* under two different hormone administration techniques; the cholesterol implant and intubation delivery method. Analysis of the spectral sensitivity data showed evidence of cone mechanism activity including those that were mediated by five (SWS1, SWS2b, RH2b, RH2aβ and RH2aα) of the possible seven cone visual pigments that can be expressed by *M. zebra*.

2.3.1 Cholesterol Implant Study

Figure 2.4 shows that the spectral sensitivity curves for GnRH implanted and control fish were very similar in both background conditions, although the overall sensitivity of the GnRH treated fish was somewhat lower than that of the control fish. Under the yellow chromatic adaptation condition, curves for the control and treatment fish show a depression in the long-wavelength end of the spectrum, most evident at 550-640 nm (Figure 2.4B), when compared to the white chromatic adaptation condition curve. *M. zebra* have visual sensitivity that ranges from approximately 340-640 nm. Five separate peak sensitivities (a peak being any portion of the curve corresponding to a point or series of points which are above an adjacent point or set of points) are found at 370-380, 450, 480-500, 520-530, 530-550 nm. Troughs in the curves, which may indicate cone mechanism interactions, were also evident at approximately 390-410 and 460-470 nm. The maximum sensitivity in both conditions for both treatments was located at 500 nm.

Visual pigment templates corresponding to SWS1, SWS2b, RH2b, RH2aβ and RH2aα cone visual pigments were fit to the spectral sensitivity curves in Figure 2.5. The visual pigment templates did not provide very good fits to the curves for the SWS1, SWS2b, RH2b, RH2aβ cone pigments for all background conditions, with the exception of an adequate fit for SWS2b in the GnRH yellow background condition ($R^2 = 0.79$) (Table 2.1). This was to be expected since spectral sensitivity curves are typically shaped by opponent and non-opponent cone interactions. Good fits were evident for the Rh2aα

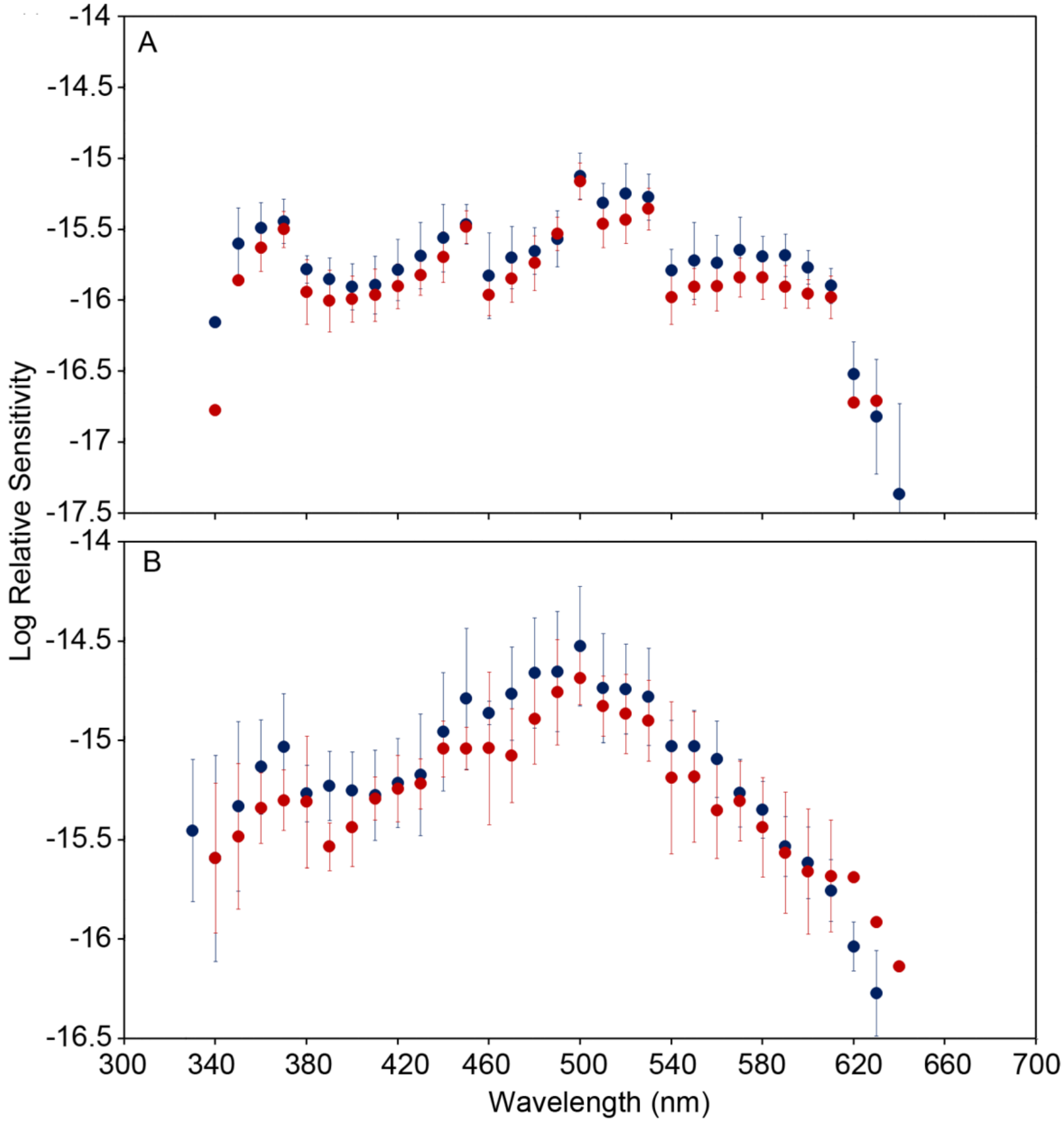

Figure 2.4. Mean spectral sensitivity curves for two different background conditions for the cholesterol implant study. (A) Log relative sensitivity (1/log photons $cm^{-2} s^{-1}$) using a white background condition. Blue filled circles represent the control fish (n = 6) and the red filled circles represent the GnRH treated fish (n = 7). (B) Log relative sensitivity using a yellow background condition. Blue filled circles represent the control fish (n = 6) and the red filled circles represent the GnRH treated fish (n = 5). Error bars represent ± one standard error of the mean.

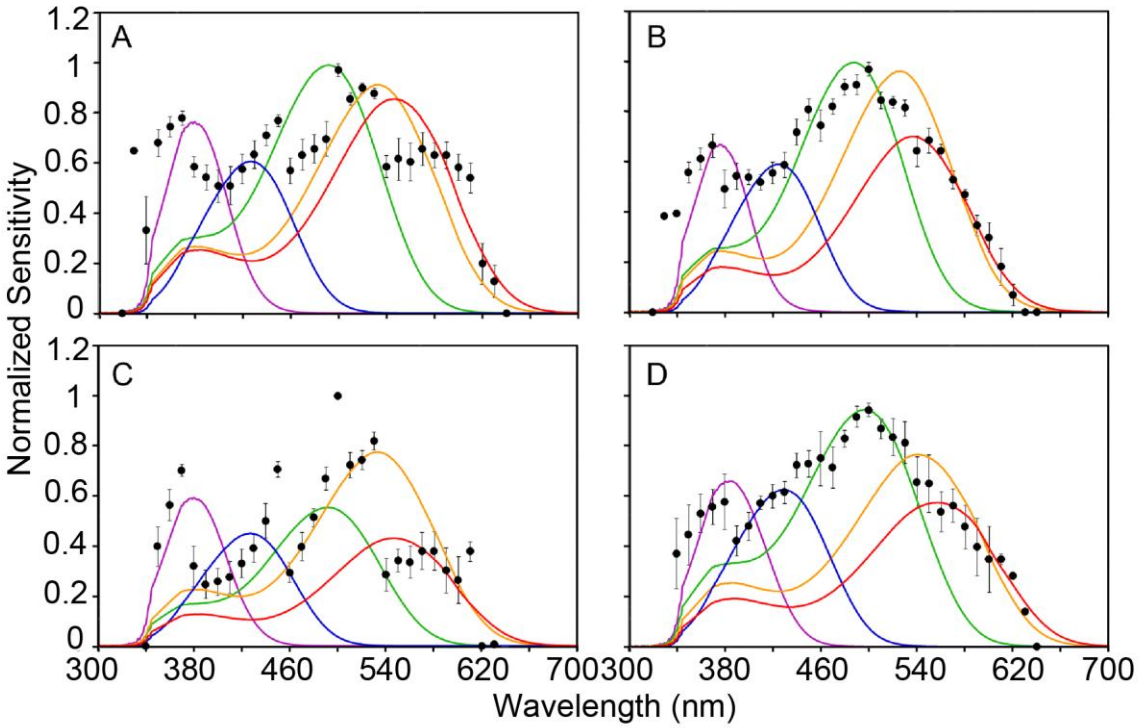

Figure 2.5. Visual template fits for mean spectral sensitivity curves for the cholesterol implant study. Mean normalized sensitivity for (A) control fish in the white background condition (n=6), (B) GnRH fish in the white background condition (n=7), (C) control fish in the yellow background condition (n=6), (D) GnRH fish in the yellow background condition (n=5). Visual pigment templates - purple = SWS1, blue = SWS2b, green = RH2b, orange = RH2aβ, red = RH2aα. Error bars represent ± one standard error of the mean.

Table 2.1. Results of least squares fitting of visual pigment templates (Govardovskii et al., 2000) to spectral sensitivity curves for two treatment groups (control and GnRH fish) determined using two background adapting conditions (white and yellow background adaptation conditions) for the cholesterol implant study.

Treatment Group	Background Condition	Visual Pigment Template					A_2 Percent
		SWS1	**SWS2b**	**RH2b**	**RH2aβ**	**RH2aα**	
Control	White	380[1]	428	492	531	547	49.4[4]
		(-3.80)[2,3]	(0.67)	(-3.21)	(-0.37)	(0.82)	
GnRH		380	428	492	531	547	49.4
		(0.54)	(-0.02)	(-0.46)	(0.20)	(0.60)	
Control	Yellow	372	426	489	526	537	24.7
		(-3.59)	(-1.17)	(-0.41)	(-2.56)	(0.98)	
GnRH		384	428	497	541	557	74.1
		(-2.39)	(0.79)	(-0.71)	(-0.50)	(0.89)	

[1] λ_{max} values (nm) for the visual pigment template fit
[2] R^2 values for the visual pigment template fit
[3] Negative R^2 values are due to the model being non-linear
[4] Percent A_2 values based on Equation 1

cone pigment for all background conditions apart from GnRH white background condition (R^2 = 0.60) (Table 2.1). Based on fitting the long-wavelength limb of the spectral sensitivity curve (see Methods), the percent A_2 for the control white background condition was 49.4 percent, and less for the control yellow condition (24.7 percent), whereby the λ_{max} of the control yellow condition was short-wavelength shifted in comparison to the control white condition. The percent A_2 of the GnRH white background condition was also 49.4 percent, which was less than that of the GnRH yellow background condition (74.1 percent), resulting in the λ_{max} values for the GnRH yellow background condition being long-wavelength shifted with respect to the GnRH white background λ_{max} values (Table 2.1).

Clearly, the visual pigment templates alone do not adequately describe the spectral sensitivity curves presented in Figure 2.5. However, when I use a multiple mechanism model (see Methods for fitting procedure), that takes into consideration both opponent and non-opponent cone interactions, the fit of the cone mechanism contributions to the spectral sensitivity points improves dramatically in comparison to the visual pigment template fits (Figure 2.6). The multiple mechanism fits depend on weighted negative opponent (inhibitory) and weighted positive non-opponent (excitatory) contributions to cone mechanisms represented by coloured line segments (each colour represents a different cone mechanism – the colour key was presented in the Figure 2.5 legend). The results of this modeling, including the weights contributing to the best fits are shown in Table 2.2. The R^2 values for cone mechanism fits to the spectral sensitivity

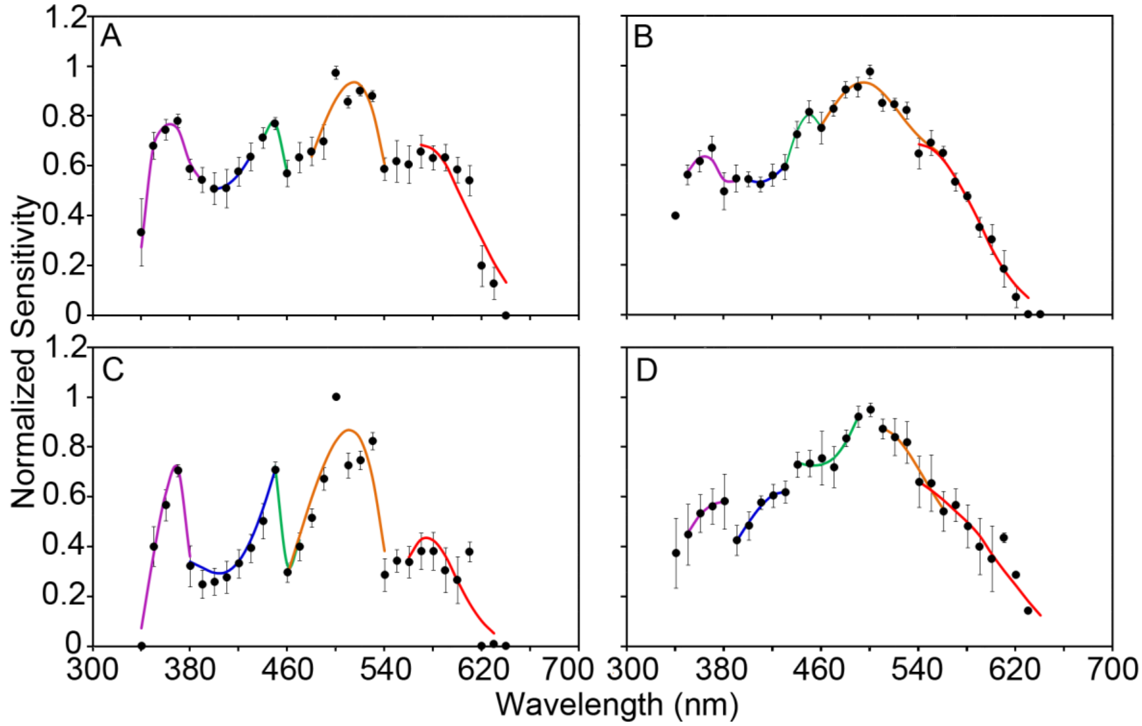

Figure 2.6. Spectral sensitivity curves fit with a multiple mechanism model for the cholesterol implant study. The multicoloured line represents the model fit for a particular cone mechanism that best describes the spectral sensitivity points (see Table 2.2). Mean normalized sensitivity for (A) control fish in white background conditions (n=6), (B) GnRH fish in white background conditions (n = 7), (C) control fish in yellow background conditions (n=6), (D) GnRH fish in yellow background conditions (n=5). Colour key for cone mechanisms - purple = SWS1, blue = SWS2b, green = RH2b, orange = RH2aβ, red = RH2aα. Error bars show ± one standard error of the mean.

Table 2.2. Results of multiple mechanism modeling showing the cone contributions (weight coefficients) to spectral sensitivity curves obtained using ERG recording for control and GnRH implanted fish for white and yellow background conditions for the cholesterol implant study.

Treatment Group	Background Condition	Cone Mechanism	Modeled Spectral Range (nm)	Weight Coefficients of Cone Mechanisms[1]					R^2
				K_{SWS1}	K_{SWS2b}	K_{RH2b}	$K_{RH2a\beta}$	$K_{RH2a\alpha}$	
Control	White	SWS1	350-390	-0.769	-1.106	+6.364	-	-	0.870
		SWS2b	400-430	+0.116	+0.077	+1.095	-	-	
		RH2b	440-460	-	+0.382	+6.337	-11.452	-	
		RH2aβ	480-540	-	-	-0.803	+7.569	-6.509	
		RH2aα	570-640	-	-	-	-0.998	+1.568	
GnRH		SWS1	340-380	+4.598	-3.795	-7.554	-	-	0.865
		SWS2b	380-450	+0.062	-0.190	+1.214	-	-	
		RH2b	450-470	-	+3.000	-13.028	+21.304	-	
		RH2aβ	460-540	-	-	-1.140	+10.122	-9.108	
		RH2aα	560-630	-	-	-2.189	+1.323	-0.248	
Control	Yellow	SWS1	350-390	-0.482	-0.711	+5.324	-	-	0.948
		SWS2b	400-430	+0.247	+0.225	+0.705	-	-	
		RH2b	430-460	-	+0.109	+3.181	-4.391	-	
		RH2aβ	460-560	-	-	+1.018	-1.873	+2.073	
		RH2aα	540-630	-	-	-	-0.159	+0.834	
GnRH		SWS1	350-380	-1.769	0.594	+5.832	-	-	0.897
		SWS2b	390-430	+0.020	+0.613	-	-	-	
		RH2b	440-490	-	+0.403	-0.404	+1.894	-	
		RH2aβ	510-560	-	-	+0.796	-0.584	+0.830	
		RH2aα	540-640	-	-	+0.417	-0.292	+0.720	

[1]A positive value for the weighting coefficient represents a non-opponent additive cone contribution while a negative value represents an opponent subtractive cone contribution.

points fall between 0.865 and 0.948. Figure 2.6 shows the spectral sensitivity curves for both treatments and background adaptation conditions fit with the multiple mechanism model described by Equation 2. For GnRH and control spectral sensitivity curves determined using the white background and the control spectral sensitivity curve determined using the yellow background condition, cone interactions were necessary to reproduce the SWS1 (UV-sensitive) cone mechanism (purple line segment) and SWS2 (short-wavelength sensitive) cone mechanism (blue line segment) regions, which were represented by cone weighing factors for SWS1, SWS2b and RH2b listed in Table 2.2.

The UV (purple line segment) and short-wavelength (blue line segment) sensitive region of the GnRH yellow background curve followed a similar pattern, with the difference being that only two cone interactions were required to produce a good fit for the SWS2b (blue line segment) sensitive region. The mid-wavelength portion of the spectral sensitivity curve (green line segment) required the input of SWS2b, RH2b and RH2aβ cone mechanisms. The long- (orange and red line segments) wavelength portion of the spectral sensitivity curve was best described by the interaction of either two or all three RH2 cones. The specific nature of the interactions (either excitatory or inhibitory) varied between background and treatment conditions, as did the weights of each cone class (Table 2.2).

The results from the cholesterol implant study indicate that GnRH did not have a significant effect on spectral sensitivity in either background condition. Indeed the spectral sensitivity curves were very similar in terms of the level of sensitivity and shape of curve and there was no qualitative difference in terms of the pattern of cone interactions underlying spectral sensitivity.

2.3.2 GnRH Intubation Study

The spectral sensitivity curves obtained for control, GnRH, and 10 week GnRH fish did not differ between treatment groups for either of the background adaptation conditions (Figure 2.7). Consistent with the cholesterol implant study, the visual sensitivity of *M. zebra* in the intubation study ranged from approximately 340-640 nm. For the white background condition, spectral sensitivity curves for all treatment groups had peak sensitivities evident at approximately 360-380 nm, 490-530 nm, and 560-580 nm, and troughs present at 400-430 nm and 540-550 nm. A similar pattern was present in the yellow background condition spectral sensitivity curves, where all treatment groups showed peak sensitivities at approximately 360-380 nm, 430-470 nm, 490-530 nm and 560-580 nm, and troughs approximately 390-420 nm, 480 nm and 540-550 nm. Maximum sensitivity for all curves was approximately 510 nm (Figure 2.7). All spectral sensitivity curves were fit by the same five visual pigment templates (SWS1, SWS2b, RH2b, RH2aα, and RH2aβ) used for the cholesterol implant spectral sensitivity curves (Figure 2.8). As was evident in the cholesterol implant data, the visual pigment templates failed to adequately describe the spectral sensitivity curves for the SWS1, SWS2b, RH2b, RH2aβ cone pigments for all background conditions, with the exception of a good fit for RH2b in the 10 week GnRH yellow background condition ($R^2 = 0.90$) (Table 2.3). On

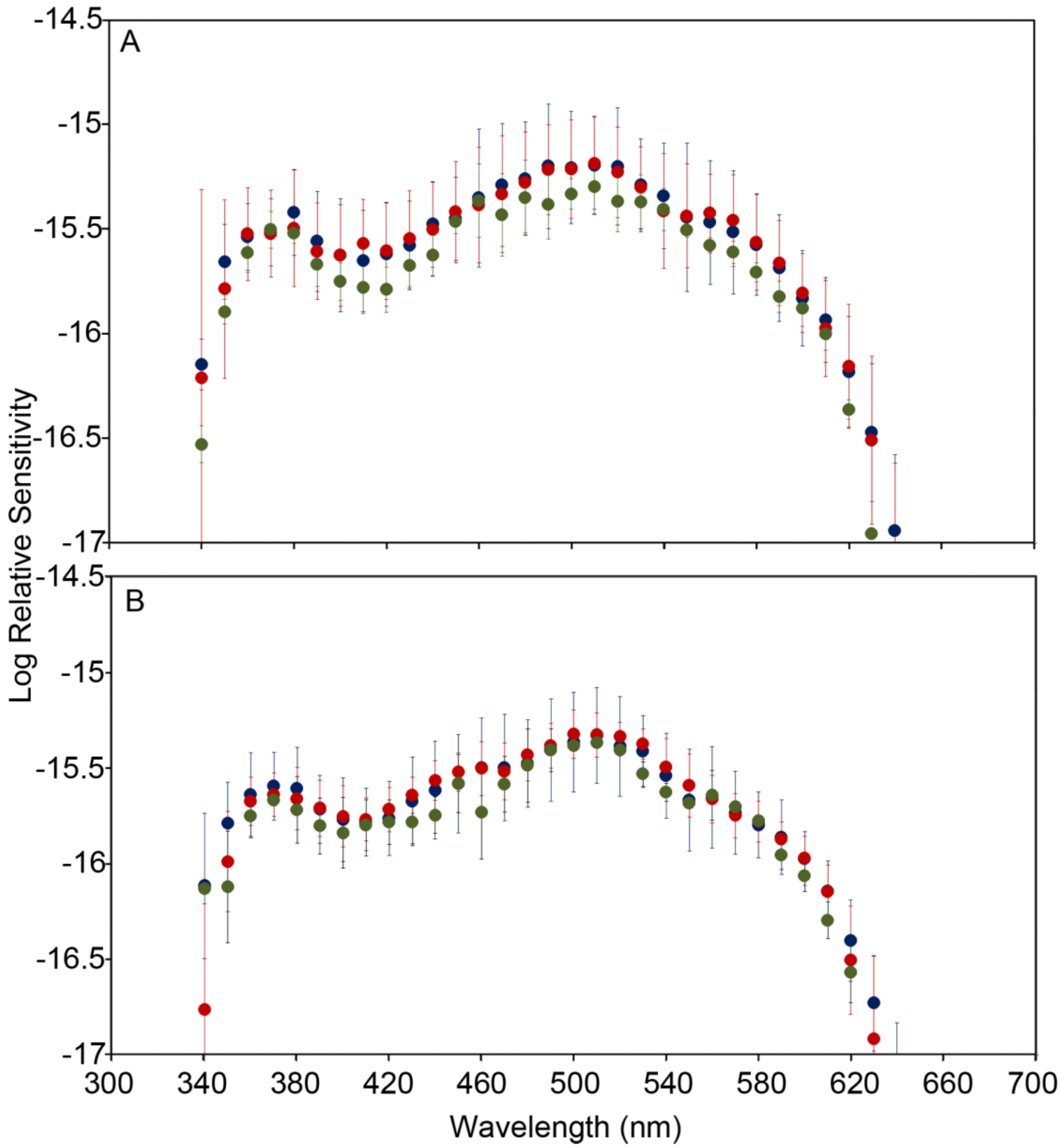

Figure 2.7. Mean spectral sensitivity curves for two different background conditions for the intubation study. (A) Log relative sensitivity (1/log photons cm^{-2} s^{-1}) using a white background condition. Blue filled circles represent the control fish (n=7), the red filled circles represent the GnRH treated fish (n=12), and the green filled circles represent 10 week GnRH fish (n=3). (B) Log relative sensitivity using a yellow background condition. Blue filled circles represent the control fish (n=7), the red filled circles represent the GnRH treated fish (n=11), and the green filled circles represent 10 week GnRH fish (n=3). Error bars represent ± one standard error of the mean.

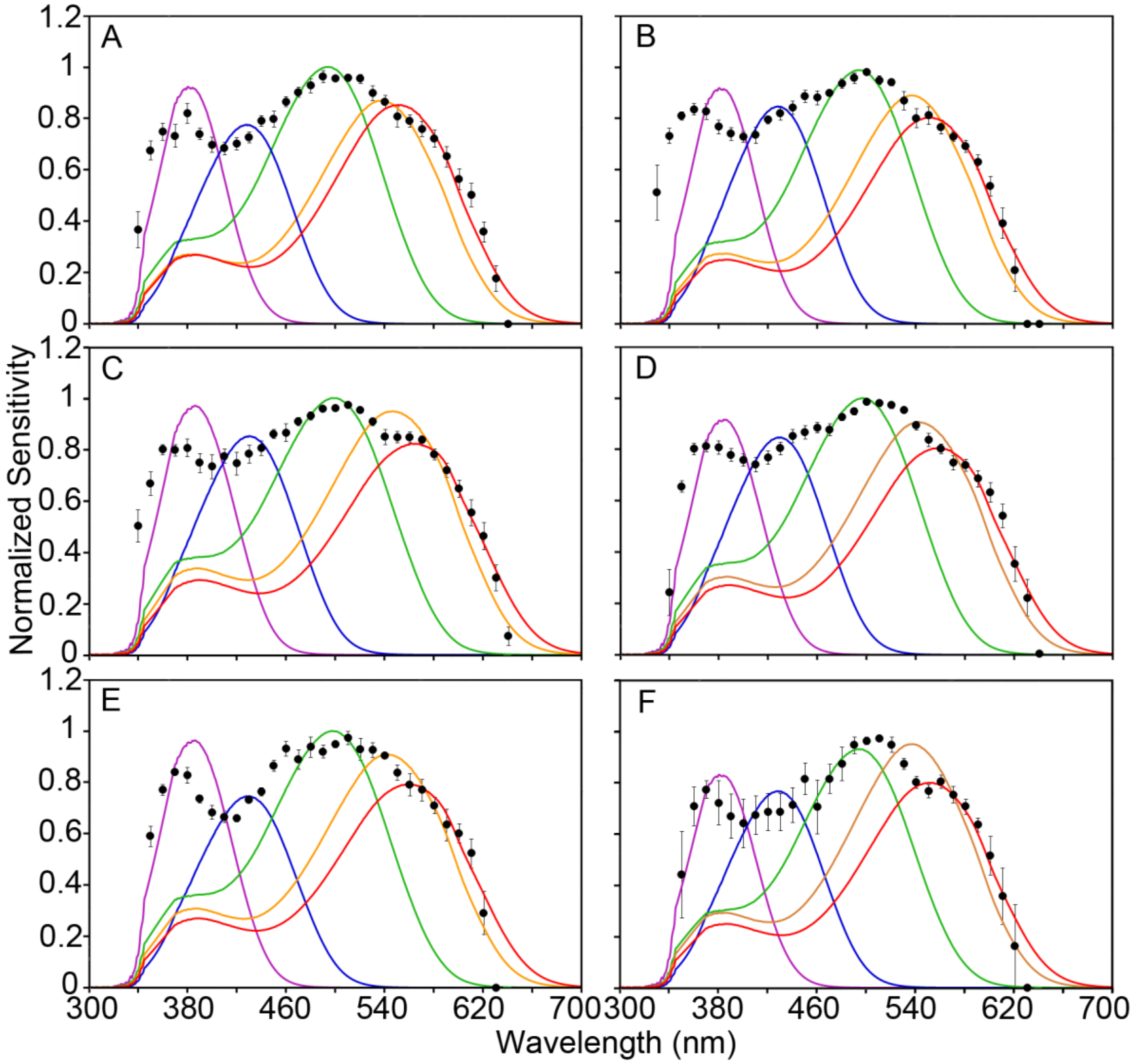

Figure 2.8. Visual template fits for mean spectral sensitivity curves for the intubation study. Mean normalized sensitivity for (A) control fish in the white background condition (n=7), (B) control fish in the yellow background condition (n=7), (C) GnRH fish in the white background condition (n=12), (D) GnRH fish in the yellow background condition (n=11), (E) 10 week GnRH fish in the white background condition (n=3), (F) 10 week GnRH fish in the yellow background condition (n= 3). Visual pigment templates (purple = SWS1, blue = SWS2b, green = RH2b, orange = RH2aβ, red = RH2aα). Error bars represent ± one standard error of the mean.

Table 2.3. Results of least squares fitting of visual pigment templates (Govardovskii et al., 2000) to spectral sensitivity curves for two treatment groups (control and GnRH fish) determined using two background adapting conditions (white and yellow background adaptation conditions) for the intubation study.

Treatment Group	**Background Condition**	**Visual Pigment Template**					**A_2 Percent**
		SWS1	**SWS2b**	**RH2b**	**RH2aβ**	**RH2aα**	
Control	White	380[1]	428	493	537	553	61.8[4]
		(-0.20)[2,3]	(-1.30)	(-1.44)	(0.37)	(0.93)	
GnRH		386	430	497	547	562	88.9
		(-6.94)	(-1.82)	(-1.07)	(-2.75)	(0.96)	
10 Week GnRH		386	430	497	543	561	79.0
		(-0.39)	(-3.57)	(-1.42)	(-0.33)	(0.93)	
Control	Yellow	380	428	493	537	553	59.3
		(-6.88)	(0.22)	(0.45)	(-0.81)	(0.97)	
GnRH		384	430	497	543	561	76.6
		(0.50)	(-1.08)	(-0.58)	(-0.28)	(0.93)	
10 Week GnRH		380	428	493	537	553	59.3
		(0.55)	(-1.36)	(0.90)	(-2.14)	(0.95)	

[1] λ_{max} values (nm) for the visual pigment template fit
[2] R^2 values for the visual pigment template fit
[3] Negative R^2 values are due to the model being non-linear
[4] Percent A_2 values based on Equation 1

the other hand, R^2 values ranged from 0.93 to 0.97 for RH2aα for all treatments and conditions (Table 2.3). The A_2 proportions of the control and GnRH groups were comparable for both adaptation conditions, with values falling between 59.3 and 61.8 percent and 76.6 and 88.9 percent respectively. The higher A_2 proportions in the GnRH group results in the λ_{max} values being long-wavelength shifted when compared to the control group. Conversely, the A_2 percent range for the 10 week GnRH was somewhat broader, falling between 59.3 and 79.0 percent, with the λ_{max} values for the yellow adaptation condition being slightly short-wavelength shifted when compared to the white adaptation condition.

A multiple mechanism model was used to describe the spectral sensitivity curves by incorporating the opponent and non-opponent interactions of the different cone mechanisms. The results of fitting the model to the data are presented in Table 2.4. The amount of variance accounted for by the model, as determined by the R^2 values ranged from 0.939 to 0.967. Figure 2.9 shows the results of fitting the multiple mechanism model to the spectral sensitivity curves for each of the treatments and conditions described by Equation 2.

For all control and GnRH spectral sensitivity curves, cone interactions were necessary to reproduce the SWS1 (UV-sensitive) cone mechanism (purple line segment) and SWS2b (short-wavelength sensitive) cone mechanism (blue line segment) regions, which were represented by cone weighing factors for SWS1, SWS2b and RH2b listed in Table 2.4. For the UV-sensitive (purple line segment) region of the spectral sensitivity

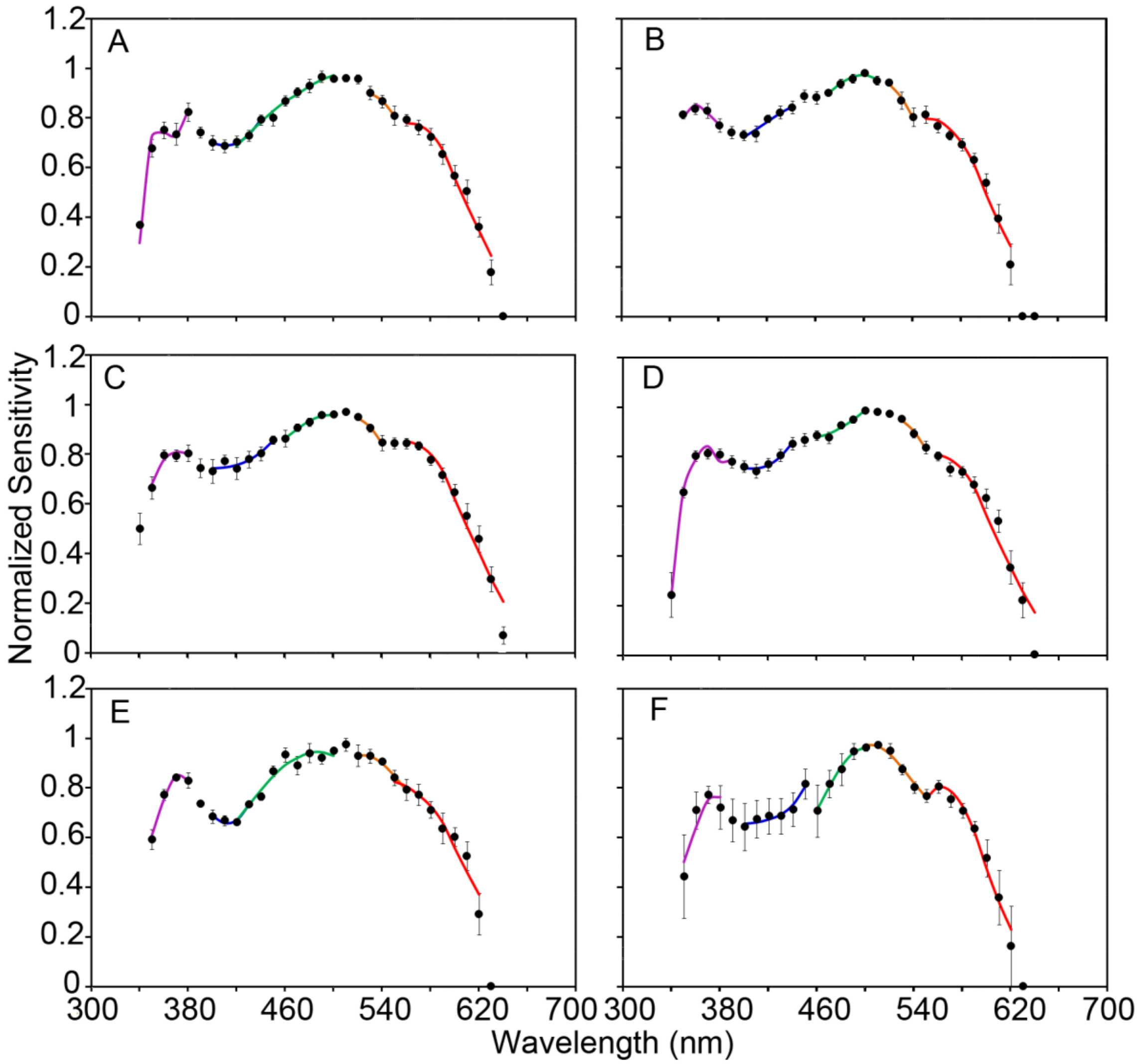

Figure 2.9. Spectral sensitivity curves fit with a multiple mechanism model for the intubation study. The multicoloured line represents the model fit for a particular cone mechanism that best describes the spectral sensitivity points (see Table 2.4). Mean normalized sensitivity for (A) control fish in white background conditions (n=6), (B) GnRH fish in white background conditions (n=7), (C) control fish in yellow background conditions (n=6), (D) GnRH fish in yellow background conditions (n=5), (E) 10 week GnRH fish in white background conditions (n = 3), (F) 10 week GnRH fish in yellow background conditions (n=3). Colour key for cone mechanisms - purple = SWS1, blue = SWS2b, green = RH2b, orange = RH2aβ, red = RH2aα. Error bars show ± one standard error of the mean.

Table 2.4 Results of multiple mechanism modeling showing the cone contributions (weight coefficients) to spectral sensitivity curves obtained using ERG recording for control and GnRH implanted fish for white and yellow background conditions for the intubation study.

Treatment Group	Background Condition	Cone Mechanism	Modeled Spectral Range (nm)	Weight Coefficients of Cone Mechanisms					R^2
				K_{SWS1}	K_{SWS2b}	K_{RH2b}	$K_{RH2a\beta}$	$K_{RH2a\alpha}$	
Control	White	SWS1	340-380	-4.555	+1.900	+13.437	-	-	0.965
		SWS2b	400-440	+0.268	+0.156	+0.156	-	-	
		RH2b	420-500	-	+0.322	+0.537	+0.526	-	
		RH2aβ	530-550	-	-	+0.254	+0.726	-	
		RH2aα	560-630	-	-	-	-0.564	+1.303	
GnRH		SWS1	350-380	-3.809	+1.311	+10.327	-	-	0.955
		SWS2b	400-440	+0.236	+0.215	+0.954	-	-	
		RH2b	460-500	-	+0.260	+0.738	+0.263	-	
		RH2aβ	520-540	-	-	+0.616	+0.438	-	
		RH2aα	560-640	-	-	-	+0.251	+0.614	
10 Week GnRH		SWS1	350-380	-2.634	-	-	+10.222	-	0.939
		SWS2b	400-430	-0.764	-0.580	-	-	+5.500	
		RH2b	420-500	-	+0.292	+0.843	+0.068	-	
		RH2aβ	520-560	-	-	+0.487	+0.004	+0.611	
		RH2aα	550-620	-	-	+0.289	-0.228	+0.919	

Treatment Group	Background Condition	Cone Mechanism	Modeled Spectral Range (nm)	Weight Coefficients of Cone Mechanisms					R^2
				K_{SWS1}	K_{SWS2b}	K_{RH2b}	$K_{RH2a\beta}$	$K_{RH2a\alpha}$	
Control	Yellow	SWS1	350-380	-3.074	+0.291	+11.433	-	-	0.967
		SWS2b	400-440	+0.178	+0.427	+0.713	-	-	
		RH2b	470-510	-	+0.246	+0.680	+0.352	-	
		RH2aβ	520-540	-	-	+0.579	+0.475	-	
		RH2aα	550-620	-	-	-	-0.056	+0.848	
GnRH		SWS1	340-390	-1.314	-0.335	+6.391	-	-	0.948
		SWS2b	400-440	+0.245	+0.164	+1.134	-	-	
		RH2b	460-500	-	+0.415	+0.317	+0.840	-	
		RH2aβ	530-550	-	-	+0.432	+0.635	-	
		RH2aα	560-640	-	-	-	+0.037	+0.776	
10 Week GnRH		SWS1	350-380	-1.022	-	-	+5.800	-	0.956
		SWS2b	400-450	+0.220	+0.058	-	-	+2.658	
		RH2b	460-500	-	-0.024	+0.776	+0.252	-	
		RH2aβ	500-550	-	-	+0.948	-1.444	+1.762	
		RH2aα	550-620	-	-	-0.890	+0.992	+0.163	

curve of the 10 week GnRH treatment, the interaction of only two cone mechanisms, SWS1 and an RH2 (RH2aβ) were sufficient to fit the peak. The SWS2b (short-wavelength sensitive) cone mechanism (blue line segment) was best represented by cone weightings for SWS1, SWS2b and RH2aα.

The mid-wavelength portion of the spectral sensitivity curve (green line segment) required the input of SWS2b, RH2b and RH2aβ cone mechanisms. The long- (orange and red line segments) wavelength portions of the spectral sensitivity curve were represented by cone weighing factors for either two or all three RH2 cone mechanisms.

The specific weights of the cone contributions and nature of the interactions varied for each treatment and adaptation condition (Table 2.4).

2.3.2.1 Analysis of Spectral Sensitivity Using ERG *a*-wave and *d*-wave

To rule out the possibility that GnRH may have affected ERG waveform components other than the *b*-wave, I selected *a*-wave and *d*-wave data from three fish from each control and GnRH treated groups. Figure 2.10 shows a comparison of an ERG waveform of a control and GnRH treated fish. Analysis of the *a*- and *d*-waves of the control and GnRH groups for both white and yellow adaptation conditions did not reveal any additional differences in the ERG waveforms (Figures 2.11 and 2.12). In the *a*-wave data, peaks were present at approximately 370-390 nm and 500 nm, with a trough at 400-420 nm and 530 nm for the spectral sensitivity curve obtained from ERG recording under

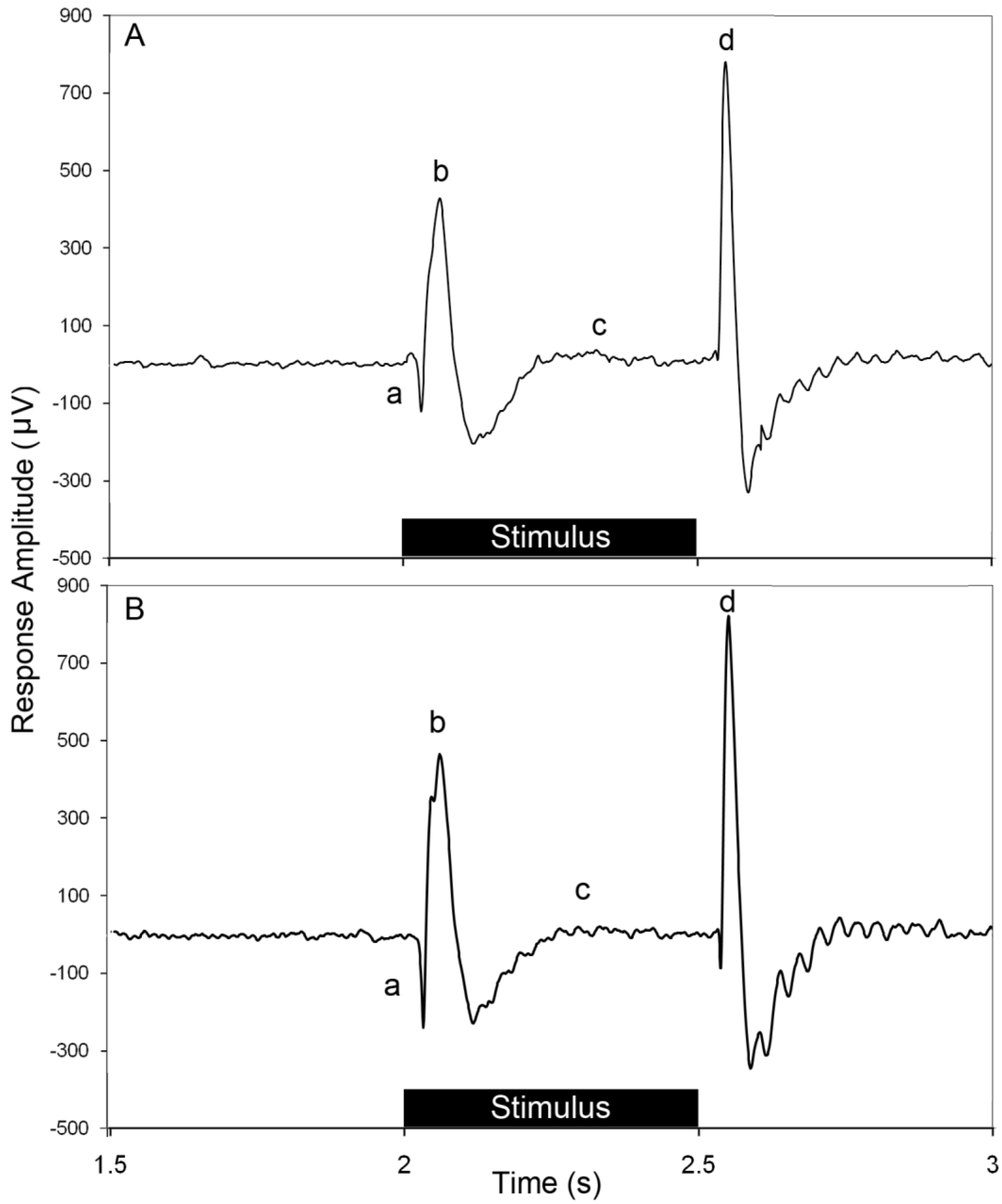

Figure 2.10. Electroretinogram (ERG) waveforms showing similarity of response between a (A) control and (B) a GnRH treated fish for the intubation study. Both waveforms show the *a*-wave (photoreceptor response), *b*-wave (ON bipolar cell response), *c*-wave (pigment epithelium) and *d*-wave (OFF bipolar cell response) at a test wavelength of 530 nm and 16.241 log photon irradiance.

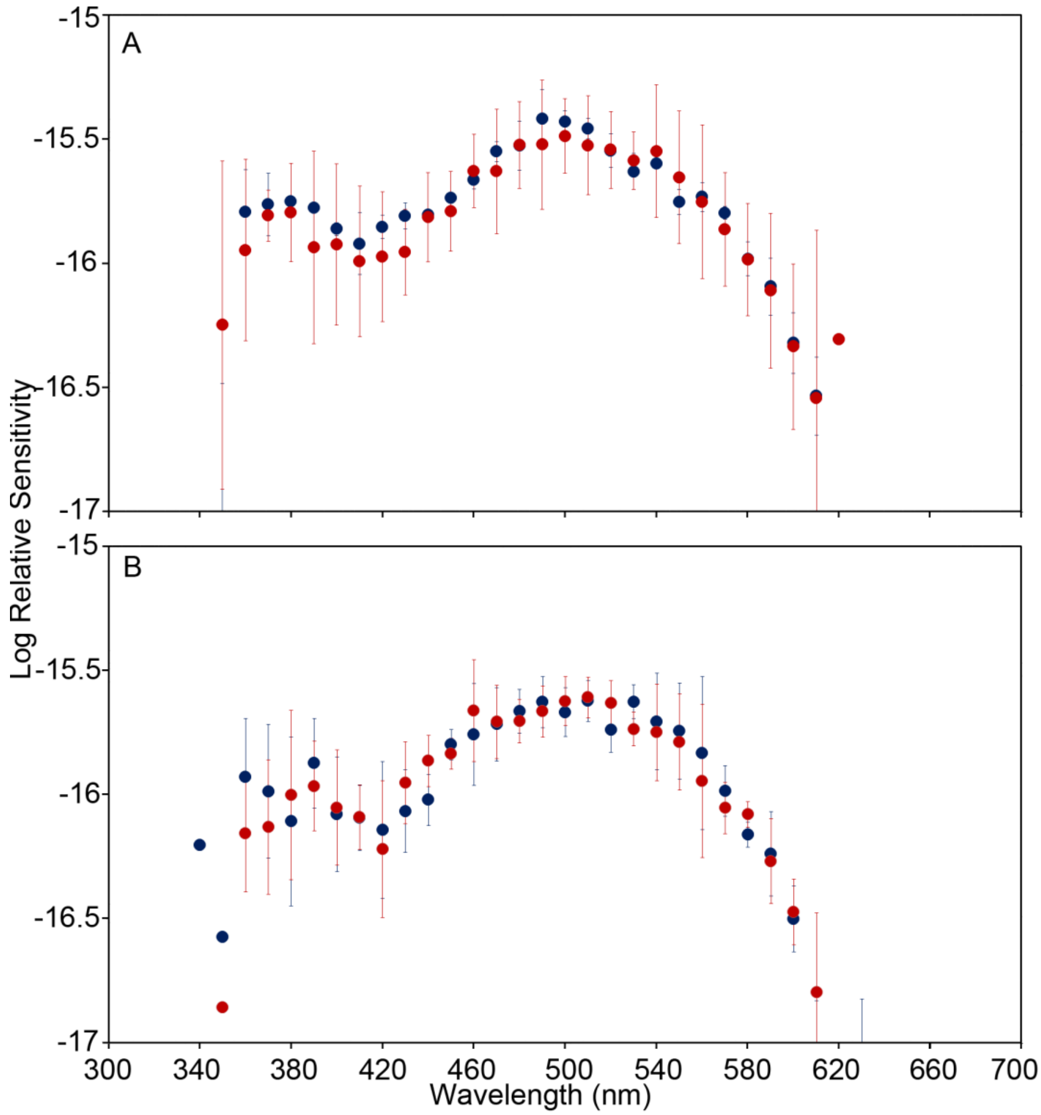

Figure 2.11. Mean relative spectral sensitivity curves for two different background conditions obtained from measurements of the *a*-wave for the intubation study. (A) Log relative sensitivity (1/log photons cm^{-2} s^{-1}) using a white background condition. Blue filled circles represent the control fish (n=3) and the red filled circles represent the GnRH treated fish (n=3). (B) Log relative sensitivity using a yellow background condition. Blue filled circles represent the control fish (n=3) and the red filled circles represent the GnRH treated fish (n=3). Error bars represent ± one standard error of the mean.

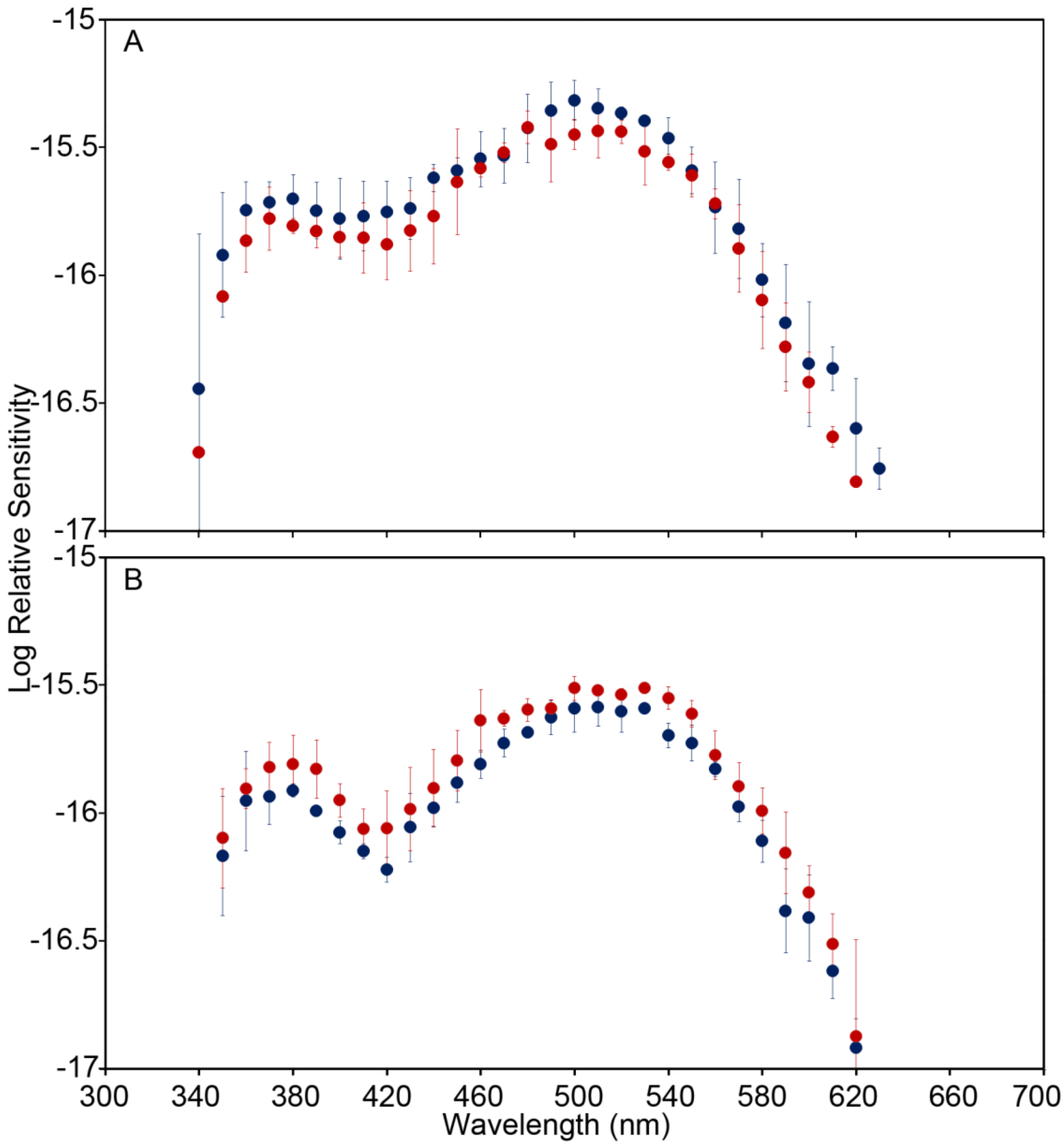

Figure 2.12. Mean spectral sensitivity curves for two different background conditions obtained from measurements of the *d*-wave for the intubation study. (A) Log relative sensitivity (1/log photons cm^{-2} s^{-1}) using a white background condition. Blue filled circles represent the control fish (n=3) and the red filled circles represent the GnRH treated fish (n=3). (B) Log relative sensitivity using a yellow background condition. Blue filled circles represent the control fish (n=3) and the red filled circles represent the GnRH treated fish (n=3). Error bars represent ± one standard error of the mean.

a white background, and peaks at approximately 370-390 nm and 500 nm, with a trough at 410 nm in the yellow background. For the *d*-wave data, sensitivity peaks were present at 370-380 nm and 500 nm, and 370-390 and 500-530 nm, with troughs at 410 nm and 420 nm for the white and yellow spectral sensitivity curves respectively. Because there were no differences in spectral sensitivity between control and GnRH treated fish, no further analysis was conducted on the spectral sensitivity curves in the form of visual pigment template fitting and multiple mechanism model fitting. Although I did not see differences in the spectral sensitivity data in any of the treatments considered, statistically significant differences in gonadosomatic indices (GSI) were apparent, with GnRH treated fish exposed over a three week period having the highest GSI (KW, $H = 8.19$, $p = 0.01$) (Table 2.5).

Table 2.5. Summary of fish mass, gonad mass and gonadosomatic index (GSI) of control and GnRH, and 10 week GnRH fish used in the intubation study.

Treatment Group	N[1]	Fish Mass (g)	Gonad Mass (g)	GSI
Control	10	8.33 ± 0.16	0.132 ± 0.005	1.638 ± 0.070
GnRH	10	7.86 ± 0.12	0.317 ± 0.021	4.670 ± 0.357
10 Week GnRH	3	6.33 ± 0.13	0.082 ± 0.018	1.280 ± 0.267

[1] Only females were included in this analysis as only two males were used in the control group, and two males were used in the GnRH group. Values are expressed as mean ± one standard error.

2.4 Discussion

It is known that GnRH has the potential to mediate changes in sensory systems, especially as it concerns vision and olfaction. However, as demonstrated in this study, the effects of exogenous GnRH administration were not evident at the level of spectral sensitivity measurements, despite previous reports that GnRH can affect visual processing and change the structure and function of optical neurons (Grens et al., 2005; Maruska and Tricas, 2007; Oka, 1992; White et al., 2002). Physiological changes were apparent in my study, as seen in the significant changes in GSI of GnRH treated fish in the intubation study. A regression in gonad size was evident in the 10 week GnRH treated fish relative to GnRH treated fish, which may indicate that the systemic levels of the GnRH administered were metabolized and returned to baseline values within the 10 week period. I took measures to ensure that the functional effects of GnRH were evident within the predicted timeframe by using two modes of GnRH administration and by implementing two testing periods to rule out the possibility of latent effects of GnRH on the retina. The motivation for using the 10 week time point in the intubation study was that modifications in opsin gene expression exert a change in visual pigment with cone outer segment renewal, but membrane turnover in the outer segment requires time for new membrane to be produced and added. A recent study by Jonnal et al., (2010), shows that human cones elongate 2.2 to 2.7 μm per day (fish cone outer segments can be up to 10-40 μm in length) but the rate of turnover in fish cones can be quite variable and dependent on a range of environmental parameters (Bassi and Powers, 1990). Thus I configured the testing time points to correspond roughly to the time constraints of outer segment renewal processes. While there is a remote possibility that the testing timeframe employed in this study missed the time point where GnRH exerted an effect on spectral sensitivity, this was unlikely since I did observe significant changes in the GSI of GnRH treated fish at the time of testing and I did consider these issues related to outer segment renewal.

M. zebra are capable of colour vision, which is mediated by a subset of five opsin genes, *SWS1*, *SWS2b*, *RH2b*, *RH2aβ*, and *RH2aα*, with sensitivity ranging from approximately 340-640 nm. These data were in accordance with previously obtained data from our lab (Sabbah et al., 2010), although it is important to note that my results and those of Sabbah et al. (2010) diverge from previous work that used indices of opsin gene expression that showed Malawi cichlids generally express only three opsin genes (Jordan et al., 2006; Parry et al., 2005). The spectral sensitivity curves presented in this paper have sensitivities falling in the ranges of 372-386 nm (SWS1 cone), 426-430 nm (SWS2b cone), 489-497 nm (RH2b cone), 526-547 nm (RH2aβ cone) and 537-561 nm (RH2aα cone) with opponent and non-opponent cone interactions that act to shape spectral sensitivity, as demonstrated by the good fits provided by the multiple mechanism model. Typically there was a positive contribution from the cone mechanisms exhibiting the greatest sensitivity for a particular modeling, whereas other mechanisms either contributed positively or negatively. The good fits described by the multiple mechanism model demonstrate that neural network connections among cones were present that allow for the discrimination of differing wavelengths of light providing the basis of colour vision in this species. The spectral ranges determined by template fitting and modelling were in accordance with the λ_{max} values determined by MSP and expression for this species (λ_{max} values - SWS1=368 nm, SWS2b=423 nm, RH2b=484-488 nm, RH2aβ=519 and RH2=528-533 nm)(Parry et al., 2005).

During mate choice, the recognition of visual signals and courtship cues from conspecifics is critical. Although GnRH treatment via prolonged-release cholesterol implant or intubation did not demonstrate any obvious differences in spectral sensitivity from controls, the effects of GnRH on cichlid vision may have occurred at a higher level of processing such as in the CNS at the level of the optic tectum or the telencephlon. ERG recordings were designed to capture information from an early stage processing in the outer plexiform layer of the retina.

Terminal GnRH fibers synapse near amacrine cells in the inner plexiform layer. As certain types of amacrine cell are thought to play a critical part in motion detection (Werblin et al., 1988), it may be that effects on colour vision were not the main target of GnRH in the retina. As the courtship displays of cichlids as well as many other teleost species often contain highly dynamic movements, such as the high frequency quivering of displaying male *M. zebra*, it is possible that GnRH acts to enhance the detection of these signals.

Studies by Fernald and colleagues have demonstrated that GnRH receptors are distributed in the visual and olfactory systems of cichlids, and that receptor and GnRH neuron distribution can vary with social status, sex, and reproductive state (Grens et al., 2005; Maruska and Fernald, 2010; White et al., 2002). *In situ* hybridization experiments comparing the receptor distributions of GnRH-R between GnRH treated and untreated fish, as well as quantifying changes in GnRH-R receptor expression may help elucidate the specific targets of the peptide.

It may be necessary to examine stimuli presented in a reproductive context that may be under the influence of GnRH. For instance, I may see more significant changes in body colouration, including in the intensity of the signal and the spatial distribution on the body of the fish, and in the expression of opsin genes in the skin. As male colouration plays a critical role in female mate choice (Kidd et al., 2006), it may be that GnRH enables males to enhance their nuptial colouration. Males with brighter more contrasting colour patterns are often selected (Kidd et al., 2006). Male-male interactions over territories and females may also be under the influence of GnRH, since dominant male cichlids express higher levels of GnRH in their brains (White et al., 2002).

The fish used in the current experiment remained visually isolated from each other in order to control for changes in GnRH expression, as there is evidence to support social regulation of GnRH. However, it may be that a social component is in fact necessary in order to effectively activate the GnRH pathways. GnRH ligands act via cognate receptors to initiate a cascade of changes in many tissues, including contributing to the release of gonadotropins from the pituitary, gonadal maturation, and as discussed, regions involved in sensory integration including the visual and olfactory systems. Previous studies have presented evidence that odour cues can increase visual sensitivity in the early morning (when the fish is presumably least sensitive to light) mediated through the olfatoretinal centrifugal connections provided by the TN-GnRH system (Maaswinkel and Li, 2003; Ramakrishnan and Wayne, 2009) demonstrated that visual cues from conspecifics can modulate the firing of GnRH neurons.

The overall result from this study was that GnRH does not cause a direct change in spectral sensitivity over a three or ten week period. It may be that the effects were indirect, acting via a mediator such as thyroid hormone, alternatively it is also possible that colour vision was not a target at all. The mechanisms by which GnRH may affect visual perception, remains as an open question. Numerous hormones and neurotransmitters have been identified in the GnRH regulatory pathway, adding a high level of complexity to the mechanisms by which GnRH causes physiological and neurological changes.

Chapter 3

Does GnRH treatment affect opsin gene expression of the retina in zebra cichlids (*Metriaclima zebra*)?

3.1 Introduction

In teleosts, hormones mediate changes in sensory systems. For instance, in the case of thyroid hormone (TH) in salmonid fishes it has been shown to initiate changes in the retinal cone mosaic and opsin gene expression that are comparable to those that occur during smoltification, such as topographically specific degeneration of UV sensitive cone photoreceptors (Allison et al., 2006; 2003; Raine and Hawryshyn, 2009; Raine et al., 2010). In addition, the expression of the *RH2a* and *RH2b* opsin genes in the mid-wavelength sensitive (MWS) cones can be either up-regulated or down-regulated by exogenous treatment with thyroid hormones (Temple et al., 2008a).

Studies have shown that GnRH, a hormone that can mediate reproductive effects on a physiological and behavioural level, plays a role in modifying sensory systems in fishes (Kawai et al., 2009; Maruska and Tricas, 2007; Stell et al., 1984; Stell et al., 1987; Behrens et al., 1993; Eisthen et al., 2000; Park and Eisthen, 2003; Umino and Dowling, 1991). Gonadotropin releasing hormone 3 (GnRH3) is localized in the terminal nerve (TN) ganglia (White et al., 1995) and projects to retinal and olfactory epithelia (Soga et al., 2005; Stell, 1987; Grens et al., 2005). Specifically, TN-GnRH connections coming from the optic nerve are found near several cells in the retina, including dopaminergic interplexiform cells, ganglion, bipolar, amacrine and horizontal cells (Grens et al., 2005; Munz et al., 1982; Stell et al., 1984; Zucker and Dowling, 1987). The interconnected nature of the retinal neural network enable GnRH-immunoreactive fibers to form connections from the photoreceptors to the telencephalon (Behrens et al., 1993). Horizontal cells, which provide a negative feedback onto cones, seem to be under the influence of GnRH (Behrens et al., 1993; Umino and Dowling, 1991). Candidates for mediators of indirect effects of GnRH include dopamine, via the release from the dopaminergic interplexiform cells (Behrens et al., 1993) and thyroid hormones (TH) through a process that is not yet clear, but may involve retinal TH regulation and result in elevated 3,5,3'-triiodothyronine (T3) levels (Plate et al., 2002).

In this study, I evaluated the effect of GnRH3 treatment on opsin gene expression in the retina using two techniques: quantitative real-time RT-PCR, which measures mRNA levels expressed by opsin genes in retinal tissues; and *in situ* hybridization, which labels the opsin gene mRNA in cone photoreceptors providing information on the identity of cone types and their spatial arrangement in the cone mosaic. My prediction was that GnRH treatment would affect opsin gene expression through differential expression. Zebra cichlids (*Metriaclima zebra*) possess seven distinct opsin genes: *SWS1*, *SWS2b*, *SWS2*a, *RH2*b, *RH2aβ*, *RH2aα*, and *LWS* (Carleton et al., 2008), and express five of the above genes (*SWS1*, *SWS2b*, *RH2*b, *RH2aβ*, and *RH2aα*) (Parry et al., 2005). I was also interested in assessing the degree of correlation between the qRT-PCR and *in situ* hybridization data. In general, my analysis showed that GnRH treatment resulted in a shift in opsin gene expression in the retinal cone mosaic.

3.2 Methods

3.2.1 Preparation of Retinal Samples

Tissues used for gene expression and *in situ* hybridization were obtained from experimental animals described in Chapter 2, Section 2.2.1. Upon completion of ERG measurements, the fish was dark adapted for at least 30 minutes after which it was sacrificed by means of cervical transection. Both eyes were enucleated and hemisected along an anterior–posterior axis, and retinas were removed under deep red illumination. The right retina was placed in RNAlater (Ambion) for use in gene expression studies, and the left retina was removed and fixed overnight in 4 percent paraformaldehyde in PBS (phosphate buffered saline) and stored in 100 percent methanol at -20^{o}C to be used for *in situ* hybridization and confocal microscopy. GnRH treated and control samples were compared using the following procedures.

3.2.2 Opsin Gene Expression

3.2.2.1 RNA Extraction and cDNA Synthesis

Total RNA was extracted from whole retinas using Absolutely RNA Miniprep Kit as per manufacturer's instructions (Stratagene, La Jolla, CA, USA) and quantified by reading the absorbance at 260 nm using a spectrophotometer (Cary 300 Bio, Varian, Santa Clara, CA, USA). The quality of the RNA sample was verified by gel electrophoresis on a non-denaturing 1.0 percent agarose gel 1× TBE (Tris/Borate/EDTA) buffer, run with a benchtop 50-bp RNA ladder (Promega, Madison, WI, USA), and viewed using GelRed on a gel imager (AlphaImager, Cell Biosciences, Santa Clara, CA, USA). RNA was reverse transcribed to first strand cDNA by using random primers and Superscript II First-Strand Synthesis Supermix for qRT-PCR (Invitrogen, Carlsbad, CA, USA) at 50^{o}C for 30 minutes for samples extracted from fish used for the GnRH implant experiment. GoScript Reverse Transcription System (Promega) was used instead of Superscript II for samples extracted from fish used in the GnRH intubation experiment at 42^{o}C for 60 minutes. When possible, total cDNA was synthesized using 250 ng total retinal RNA. For samples with lower RNA concentrations, 4 µl of template was used and final concentrations calculated. Note that results from both systems were comparable.

3.2.2.2 Quantitative Real-Time RT-PCR (qRT-PCR)

Opsin gene expression was examined using quantitative real-time RT-PCR (qRT-PCR) to assess the differences in opsin gene expression levels between GnRH treated and control groups. SYBR® Green qPCR Master Mix (Agilent Technologies) was used for cDNA synthesized from retinas in the cholesterol implant study, and Plexor™ qPCR Systems (Promega) was used for samples obtained in the GnRH intubation study. All samples were run on 96-well non-skirted polypropylene PCR plates capped with optical strip caps (Agilent Technologies) in a real-time quantitative system MX3000P™ (Agilent Technologies). Non-template controls (NTC) and non-reverse transcribed (NoRT) reactions were included on the qRT-PCR plate.

3.2.2.2.1 qRT-PCR of Cholesterol Implant Samples Using SYBR® Green

Each reaction contained 1× Brilliant SYBR Green Master Mix, 200 mM of both forward and reverse primers, 0.05 µl of ROX passive reference dye and 1 µl cDNA template diluted 4-fold in molecular biology grade (MOBI) water, for a total reaction volume of 25µl. Primers used were identical to those used in a previous study (Sabbah et al., 2010) and are available in Appendix E. The thermal profile for the reaction was 95°C for 10 minutes, followed by 40 cycles of 95°C for 25 seconds, 55°C for 25 seconds and 72°C for 25 seconds.

Using SYBR® Green technology, product accumulation results in an increase in fluorescence, proportional to the amount of initial cDNA template added to the reaction mixture. An amplification threshold was set within the early exponential phase of amplification. Since the SYBR ® Green dye binds non-specifically to double-stranded DNA, a dissociation curve was generated at the end of each qRT-PCR run to ensure that no products other than the gene of interest were amplified during the thermocycling program, indicated by the manifestation of only one distinct DNA peak that dissociated at a consistent temperature.

3.2.2.2.2 qRT-PCR of GnRH Intubation Samples Using Plexor™ Technology

Each 25 µl reaction contained 12.5 µl 2x Plexor™ Reaction Mix, 50 µM of each forward and reverse primers, 1.5 mM $MgCl_2$, and MOBI water to complete the master mix to a final volume of 20 µl, plus 5µl of the cDNA template (previously diluted 1:5 in MOPS buffer (3-(N-morpholino) propanesulfonic acid). Primers were designed using the Plexor™ Primer Design Software (available online through Promega) as per manufacturer's recommendations (see Appendix E for primer sets used for quantifying opsin gene expression). The thermal profile for the reaction was 95^{o}C for 2 minutes, followed by 40 cycles of 95^{o}C for 5 seconds and 55^{o}C for 35 seconds, 55^{o}C for 15 seconds and 95^{o}C for 5 seconds. Using Plexor™ Technology, the accumulation of product results in a decrease in fluorescence which was proportional to the amount of initial cDNA template added to the reaction mixture. An amplification threshold was set within the early exponential phase of amplification.

3.2.2.3 Analysis of qRT-PCR Data

The number of cycles required to cross the amplification threshold value (critical cycle number, Cq) was used to determine an initial target concentration from the beginning of the reaction. Genes having higher levels of expression have smaller critical cycle numbers than genes with lower expression. Gene expression was determined as a fraction of the total cone opsin genes expressed for an individual according to the following formula:

$$\frac{T_i}{T_{all}} = \frac{(1+E_i)^{-Cqi}}{\Sigma(1+E_i)^{-Cqi}} \qquad \text{Equation 3}$$

Where T_i/ T_{all} was the relative gene expression ratio for a given gene to the total cone opsin genes expressed, E_i was the PCR amplification efficiency for each gene, and C_{qi} was the fractional cycle number for each gene (Carleton et al., 2000).

All samples were analysed in duplicate and the Cq values averaged for expression levels of six opsin genes (*SWS1*, *SWS2a*, *SWS2b*, *RH2a*, *RH2b* and *LWS*). *RH2A*α and *RH2A*β were considered together due to their genetic and functional similarities (Parry et al., 2005; Spady et al., 2006) and quantified as a single gene using primers that amplify regions conserved between both targets.

Prior to performing statistical analyses, normality and homogeneity of variance of the data were evaluated using Shapiro-Wilk's and Levene's tests respectively. If the assumptions for a parametric test were met, a one-way analysis of variance (ANOVA) was used for comparison, while both an ANOVA and a Wilcoxon Rank-Sum test was used if the assumptions were violated. In instances where both parametric and non-parametric tests were used, similar findings were found, indicating that the ANOVAs were robust to violated assumptions, and were therefore used to report statistical results.

As these statistical tests require a continuous variable (with no fixed limits), the percentages of gene expression were transformed by taking the arcsin value of the square root of the percentage value (Quinn & Keough, 2002). As multiple statistical tests were conducted, the probability level ($\alpha = 0.5$) was corrected using Bonferroni correction for six hypothesis tests corresponding to six opsin genes, whereby an α-level of 0.0083 was used ($\alpha = 0.5/6 = 0.0083$) (Quinn & Keough, 2002). Statistical analysis was completed in JMP 8.0.1 (SAS Institute, Cary, NC, USA).

3.2.3 Distribution of Opsin Gene Expression in the Retinal Cone Mosaic

In situ hybridization and riboprobe synthesis procedures were modified from protocols outlined in Barthel & Raymond (2000). Targets selected for localization with *in situ* hybridization (RH2a and RH2b) were based on evidence of significant changes observed by opsin gene expression in *M. zebra* retina, and *in situ* hybridization experiments were only carried out for retinas obtained from fish in the GnRH intubation experiment. As in opsin gene expression profiles, the RH2Aα and RH2Aβ were considered together due to their similarities in amino acid sequences and functionality (Parry et al., 2005; Spady et al., 2006).

3.2.3.1 Riboprobe Synthesis

Riboprobes were prepared from gel purified PCR amplified segments of cDNA samples synthesized with Superscript II Reverse Transcriptase (Invitrogen) using total RNA extracted from tilapia (*Oreochromis niloticus*) retinas as a template. Purified segments were inserted into pGEM-T-Easy plasmids (pGEM®-T-Easy Cloning Kit, Promega). Vectors were transformed into *E. coli* JM109 competent cells (Promega). Positive clones (white colonies) were selected in Luria-Bertoni (LB) media containing 100 μg mL^{-1} ampicillin (Sigma, St-Louis MO, USA), 100 mM isopropyl β-D-1-thiogalactopyranoside (IPTG**)** and 50 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-**β**-D-galactopyranoside (X-Gal). Selected clones were grown in LB media containing ampicillin at 37°C overnight, and plasmids were extracted using Fast Plasmid Mini® (Eppendorf, Hamburg, Germany) according to the manufacturer's protocol. Plasmids were linearized by means of restriction enzymes (ApaI and SacI) and riboprobes were prepared using T7 RNA polymerase (Gibco-BRL, Gaithersburg, MD, USA) or SP6 RNA polymerase (Amersham-Pharmacia, Arlington Heights, IL). Antisense riboprobes were either digoxigenin (DIG)-labeled or biotin-labeled (Roche Biochemicals, Basel, Switzerland).

3.2.3.2 Tissue Fixation

Retinas excised from the left eyes of *M. zebra* were fixed overnight at 4^{o}C in 4 percent paraformaldehyde in 100 mM PBS pH 7.4. After fixation the tissue was rinsed twice in PBS (pH 7.4) with 5 percent sucrose at 20^{o} C for 5 minutes. The retina was then rinsed briefly with 70 percent methanol, dehydrated in 100 percent methanol for 5 minutes, and then stored at -20^{o}C in fresh 100 percent methanol.

3.2.3.3 Prehybridization and Hybridization

The tissue was rehydrated in a graded series in methanol:PBST (PBS with 0.1% v/v Tween 20) at ratios of 3:1, 1:1, and 1:3, for 5 minutes each, and subsequently rinsed four times with 100 percent PBST, for 5 minutes each. The rehydration was followed with proteinase K treatment at 10 μg ml^{-1} for 30 minutes for mid-wavelength sensitive (RH2a and RH2b) probes. After digestion with proteinase K, the retina was rinsed with PBST, washed for 5 minutes in fresh PBST and fixed once again in 4% paraformaldehyde /PBS for 20 minutes at 20^{o} C. Residual fixative was removed by rinsing the sample 5 times for 5 minutes each with PBST, and subsequently prehybridized with hybridization buffer for 2 hours at 56^{o}C with gentle rotation. Probes were diluted (1:400) in Hybridization buffer and boiled at 90^{o}C for 5 minutes and kept at 60^{o}C until added to the sample. Samples were incubated at 56^{o}C overnight in a hybridization oven.

3.2.3.4 Post Hybridization

After overnight incubation, the tissue was rinsed in 2x concentrated SSC (Standard Saline Citrate) followed by high stringency washes at 56^{o}C in 2x SSC with 50% formamide, twice for 10 minutes in 2x SSCT (0.01% Tween 20) and twice for 30 minutes in 0.2x SSCT. The sample was then rinsed at room temperature in 0.2x SSC:PBST (0.01% Tween 20) for 10 minutes each at ratios 3:1, 1:1 and 1:3, and transferred to a microcentrifuge tube and incubated in blocking reagent for 1.5 hours. The blocking reagent was then removed and replaced with anti-DIG/AP-conjugated antibody (1:100 in blocking reagent) and incubated overnight at 4^{o}C with gentle agitation.

The antibody was rinsed out of the tissue with Maleic Acid Buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) three times for 15 minutes each. The sample was subsequently stained with 200 μl of FastRed (1 tablet dissolved in Tris-HCl pH 8.0) for 1 hour at 20^{o}C in the dark. The colour reaction was monitored by means of a dissection microscope until completion. The reaction was stopped with TE (tris/EDTA) buffer, and the excess washed with PBST three times for 5 minutes each. The sample was then incubated with the second antibody, anti-biotin/HRP-conjugated antibody (1:100 blocking buffer) for 30 minutes at room temperature.

This incubation was followed by five 15 minutes washes in PBS, and incubated with TSA (tyramide signal amplification) solution (diluted 1:50 with amplification reagent). Next, three washes at room temperature in PBS were completed for 15 minutes each. Lastly, the tissue was mounted on a slide using Prolong Gold Antifade Reagent (Invitrogen), photoreceptor side up, and fluorescent signals were detected using confocal laser scanning microscopy (CLSM) (Zeiss LSM 710, Toronto, Canada).

3.2.3.5 Confocal Microscopy and Image Analysis

DIG-labeled riboprobes were detected using anti-digoxigenin antibody conjugated to alkaline phosphatase (anti-DIG/AP) (Roche Biochemicals). FastRed (Roche Biochemicals) was used to produce a fluorescent signal detected using a confocal microscope with a standard rhodamine filter set. Biotin labeled riboprobes were identified using and anti-biotin antibody conjugated to peroxidase and a fluorescein tyramide amplification reaction system (TSA) (Perkin Elmer, Boston, MA, USA) and viewed with a standard fluorescein filter set. DAPI was included as a counter-stain to allow visualization of the nucleus.

The beam paths were configured using the Smart Setup configuration tool provided with the LSM Software Zen 2008 (Zeiss). The dyes fluorescein, rhodamine and DAPI were selected to analyse the samples. Each retinal preparation was scanned using excitation wavelengths of 405 nm for DAPI 488 nm for fluorescein and 543 nm for FastRed (Argon laser). The selected emission filters were 409-484 nm, 404-544 nm, and 571-685 nm for DAPI, fluorescein and FastRed respectively. The tissues were scanned with a 63x oil immersion Plan-Apochromat objective (1.4 numerical aperture). Pinhole size was adjusted to reduce the background fluorescence (range 42 to 197 μm). Images were captured and pseudocoloured using the LSM Software Zen 2008 (Zeiss) based on the dye used (blue for samples treated with DAPI, red for samples treated with FastRed and green for samples treated with fluorescein) and were cropped and adjusted for brightness and contrast using Adobe Photoshop 12.0 (Adobe Systems, Mountain View, CA, USA) but no changes were made to image content.

3.3 Results

Quantitative RT-PCR and *in situ* hybridization were carried out for zebra cichlid fish treated with GnRH and controls using two different administration techniques, a prolonged release cholesterol implant and intubation with a GnRH solution. Significant results were obtained only for the *RH2b* and *RH2a* opsin gene expression in the GnRH intubation study.

3.3.1 qRT-PCR

3.3.1.1 Cholesterol Implant Study

Figure 3.1 shows that both control and GnRH implanted fish expressed *SWS1* (7.6 and 20.4 percent respectively), *SWS2b* (2.6 and 5.9 percent) *RH2b* (32.4 and 25.4) and *RH2a* (56.3 and 46.5) with minimal expression of SWS2a (0.5 and 0.9 percent) and LWS (0.6 and 0.9 percent). However, there were no significant differences in opsin gene

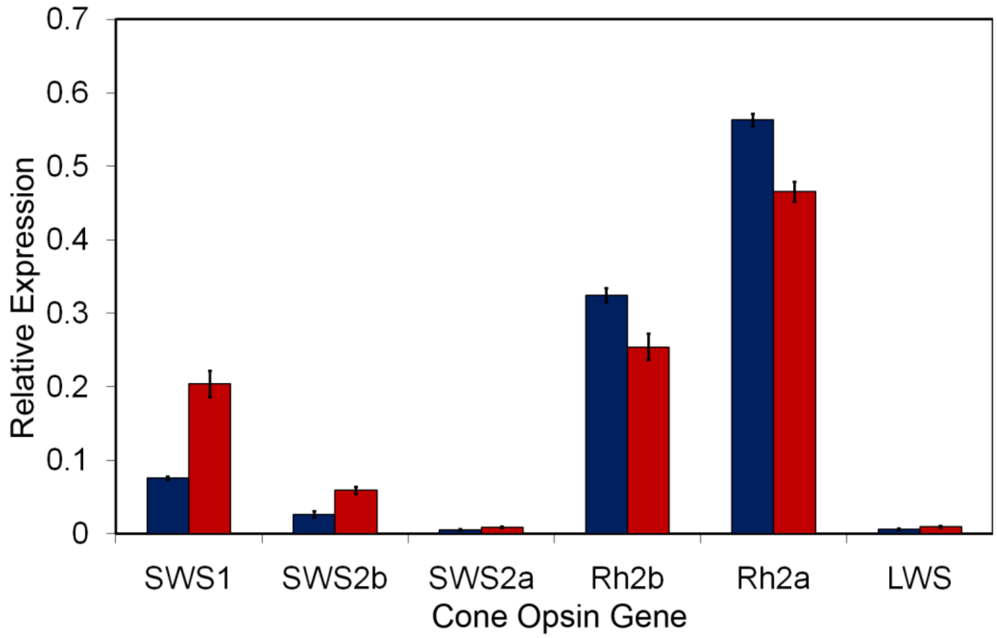

Figure 3.1. Relative opsin gene expression for *Metriaclima zebra* retinas obtained from fish that were treated with GnRH using a cholesterol implant. Opsin gene expression was assessed by qRT-PCR for the target genes *SWS1*, *SWS2b*, *SWS2a*, *RH2b*, *RH2a* and *LWS*. Blue filled bars represent the control fish (n=4) and the red filled bars represent the GnRH treated fish (n=6). Error bars represent ± one standard error of the mean.

expression levels of the target genes between the two groups (ANOVA, $p > 0.05$ for all genes). There did appear to be a trend towards an upregulation of the UV and short wavelength sensitive opsin genes (*SWS1* and *SWS2b*), and downregulation of mid and long-wavelength sensitive opsins (*RH2b* and *RH2a*) but no conclusive differences were evident.

3.3.1.2 GnRH Intubation Study

Consistent with the cholesterol implant study, both control and GnRH fish showed expression of *SWS1* (10.0 and 10.5 percent, respectively), *SWS2b* (23.2 and 29.6 percent) *RH2b* (29.8 and 42.1 percent) and *RH2a* (36.8 and 17.8 percent) opsin genes, with little or no expression of *SWS2a* or *LWS* (Figure 3.2). However, after 3 weeks of GnRH treatment, there was a significant increase in *RH2b* opsin gene expression (ANOVA, $F_{22}= 9.02$, $p = 0.006$) and decrease in *RH2a* expression (ANOVA, $F_{22}= 13.17$, $p = 0.002$), while expression levels of *SWS1* (ANOVA, $F_{22}= 0.03$, $p = 0.857$) and *SWS2b* (ANOVA, $F_{22}= 2.44$, $p = 0.132$) did not significantly differ.

3.3.2 *In situ* Hybridization and Confocal Microscopy

Based on my qRT-PCR data for the intubation study that showed significant changes in the expression of the *RH2* suite of opsin genes, my *in situ* hybridization study focused on the labeling patterns for *RH2a* and *RH2b*. Consistent with the qRT-PCR data,

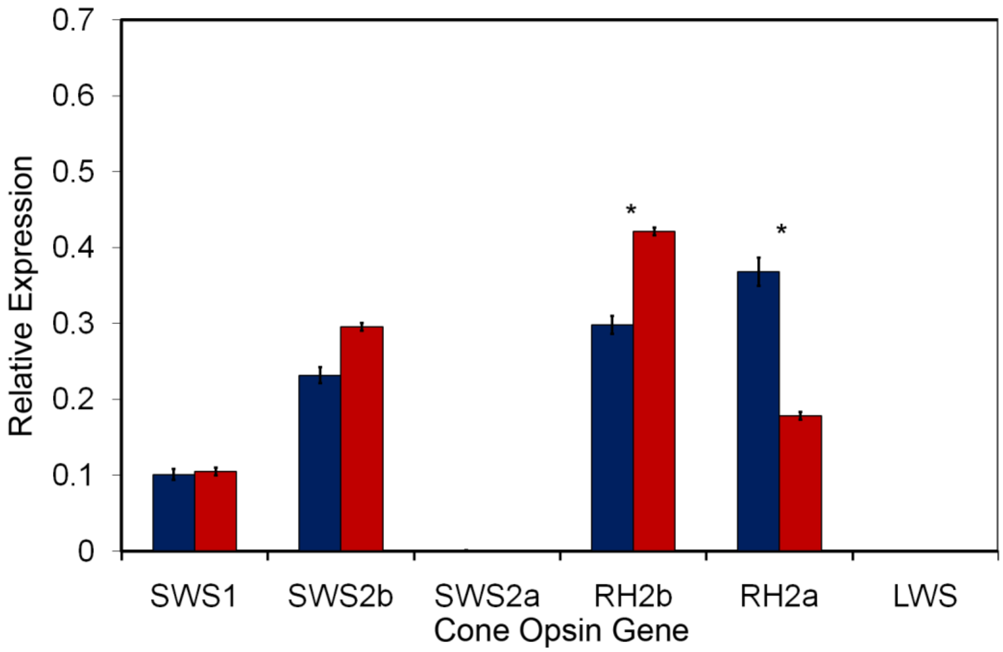

Figure 3.2. Relative opsin gene expression for *Metriaclima zebra* retinas obtained from fish in the GnRH intubation study. Opsin gene expression was assessed by qRT-PCR for *SWS1*, *SWS2b*, *SWS2a*, *RH2b*, *RH2a* and *LWS*. Blue filled bars represent the control fish (n=11) and the red filled circles represent the GnRH treated fish (n=13). Error bars represent ± one standard error of the mean. The symbol * denotes a statistically significant difference in expression ($p < 0.0083$ after Bonferroni correction).

whole mount *in situ* preparations showed that, relative to control animals, GnRH treated and 10 week GnRH fish showed an increase in *RH2b* mRNA distribution while *RH2a* expression was much less pronounced (Figure 3.3). Figures 3.3 show the localization of the signal confined to the double cones in the square mosaic, with no labeling of the central signal cones. Negative controls for each treatment condition containing no probe did not reveal any labeling (data not shown).

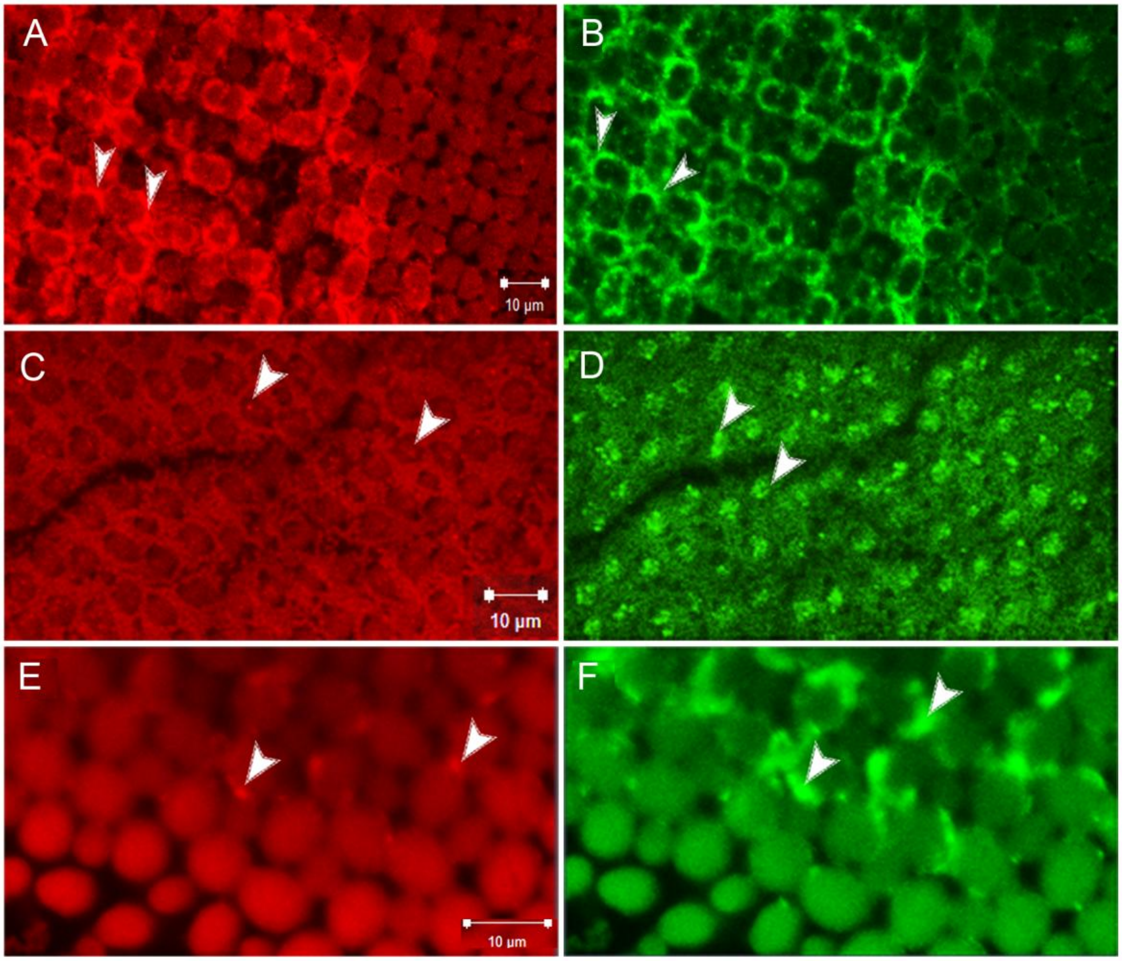

Figure 3.3. *In situ* hybridization labeling of double cones expressing the mid-wavelength sensitive opsin genes in control fish. Target opsin genes *RH2b* (green - fluorescein fluorescent dye), *RH2a* (red -fast red fluorescent dye) of control fish (A-B), GnRH fish (C-D) and 10 week GnRH fish (E-F) in tangential sections; (A) tangential section from control fish showing double-cones in the square mosaic labeled for *RH2a* (red); (B) tangential section from control fish showing double-cones in the square mosaic labeled for *RH2b* (green); (C) tangential section from GnRH fish showing labeling of double cones expressing *RH2a*; (red) (D) tangential section from GnRH fish showing labeling of double cones expressing *RH2b* (green); (E) tangential section from 10 week GnRH fish showing double-cones in the square mosaic labeled for *RH2a* (red); (F) tangential section from 10 week GnRH fish showing double-cones in the square mosaic labeled for *RH2b* (green). Arrowheads highlight location of fluorescent signal.

3.4 Discussion

The results of this study demonstrate that GnRH acts directly on the retina, resulting in a shift in opsin gene expression in the mid wavelength sensitive opsin genes showing an upregulation of *RH2b*, and downregulation of *RH2a*. Specifically, qRT-PCR analysis of retinas from GnRH treated fish in the intubation study showed statistically significant differences in *RH2* opsin gene expression, however, this difference was not apparent for the cholesterol implant study. Shifts in mid wavelength sensitive opsin gene expression have also been seen in salmonid fishes treated with thyroid hormone, but in this case treatment upregulates *RH2a* and downregulates *RH2b* opsin gene expression (Temple et al., 2008a). Furthermore, GnRH treatment of sockeye salmon has been shown to increase retinal T3 levels (Plate et al., 2002) and these increases in T3 probably play a role in mediating shifts in opsin gene expression.

The changes seen in the qRT-PCR gene expression values from the GnRH intubation experiment were further corroborated with data obtained from *in situ* hybridization of retinal tissue in which the *RH2b* riboprobe labeling was much more evident than *RH2a* riboprobe labeling in GnRH treated and 10 week GnRH treated individuals relative to controls. Differences in distribution of opsin gene expression in the cone mosaic were still evident in the 10 week treatment group, even though GSI values had returned to lower levels. It was possible the circulating systemic levels of exogenous

GnRH that targeted the reproductive organs may have been degraded after this period, but may still have been present in the retina.

Fish from both studies predominantly expressed four cone opsins with the greatest expression exhibited by the *RH2* isoforms. The overall expression of *SWS1* relative to *SWS2b* was greater in the cholesterol implant samples, whereas the opposite was true for samples from the intubation study. This may be due to sex ratio differences between the two studies, where a greater number of males (10 of 10 were male), were used for the implant study, while more females (19 of 23 were female) were available for the intubation study. Sex differences in gene expression for this species has been previously shown by Sabbah et al. (2010), specifically, only half of the females examined shared the same visual pigment subsets with all males. The males in this study expressed *SWS2a* instead of *SWS2b* which was only expressed by females. Male *M. zebra* are brightly coloured and hold territories which they defend from other males, whereas drab looking females visit these territories in order to choose a mate. It would therefore be advantageous for the visual spectrum of males to be fine-tuned towards the recognition of conspecifics, whereas females would benefit from having spectral sensitivity that are more fine-tuned to discriminate between competing males.

The use of a prolonged-release implant as a means of systemic delivery for GnRH seemed to be ineffective in exerting changes in opsin gene expression. However, the intubation methodology resulted in GnRH treated fish showing significant changes in opsin gene expression along with significant changes in GSI. It was possible that the dosage used for administration via a cholesterol implant was not physiologically relevant, although previous research, using this treatment paradigm with similar dosages, found significant changes in reproductive physiology in black sea bass and rainbow trout treated with GnRH cholesterol implants (Crim et al., 1988; Watanabe et al., 2003). The form of GnRH3 used in the cholesterol implants was a native peptide, which may have been subject to more rapid degradation in comparison to the synthetic GnRH3 peptide used in the intubation study. In addition, the GnRH intubation solution contained components designed to maximize absorption of GnRH3. It may be that the stress associated with the implant procedure elevated cortisol levels causing decreases in GnRH (Chandran et al., 1994; White et al., 2002) and GnRH-receptor mRNA expression (Fox et al., 1997; Maya-Nunez and Conn, 2003). The overall switch to the shorter wavelength sensitive *RH2b* isoform in GnRH treated fish in the intubation study, would suggest an increased sensitivity to wavelengths in the blue-green range of the spectrum, but this was not evident in my spectral sensitivity data. Perhaps if the GnRH treatment was extended and maintained over the full 10-week period, the shift in spectral sensitivity may have been clearly represented.

Examining visually-mediated sexual behaviour such as mate-choice might highlight behavioural differences related to GnRH treatment, such as assays which look at female preference for conspecific males (control vs. GnRH). The rapidity of selection, time-spent, intensity of courtship displays and number of eggs laid are all factors that may be under GnRH control. GnRH may influence male body colour patterns, increasing reflectivity of colours in specific regions of the spectrum. Current spectral reflectance measurements of *M. zebra* show a peak in the short- to mid-wavelength range that may be relevant for visually-guided behaviour (Sabbah et al., 2010). Salience of optical signaling at the time of courtship may be a factor that ensures mate choice fidelity by increasing the conspicuousness of males to females thus acting to minimize hybridization. Sexual selection through female mate choice based on visual cues has often been described as a mechanism driving speciation in cichlids, and visual cues are often sufficient to stimulate courtship (Jordan et al., 2003).

Chapter 4

General Conclusions and Future Directions

An integrative approach to the study of the visual systems of fish is required to further our understanding of the hormonal influences of GnRH on the complex processes that take place during visual communication. The visual system and visually-guided behaviours of zebra cichlids (*Metriaclima zebra*) have made them an ideal species for this line of investigation. The central aim of this study was to assess whether GnRH treatment causes changes in the visual systems of cichlids at the level of the retina either directly or indirectly through changes in opsin gene expression. I approached this project on two levels: evaluating functional assessments of spectral sensitivity by electroretinogram recording; and evaluating relative opsin gene expression of the seven opsin genes by quantitative real-time RT-PCR and by *in situ* hybridization.

The major findings of this study show that while no obvious differences in spectral sensitivity were apparent, differences at the level of opsin gene expression, namely upregulation of *RH2b* and downregulation of *RH2a*, proved to be statistically significant. The expression level of opsin genes however, does not necessarily translate into changes in opsin protein production, the visual pigment mediating spectral sensitivity. Recent proteome and transcriptome quantifications of single cells in *Escherichia coli* found that mRNA levels for particular genes did not correlate with protein levels for the same genes (Taniguchi et al., 2010). Taniguchi and colleagues (2010) described the stochastic nature of gene expression, referring to the fact that mRNA is transcribed in random bursts, and quickly degraded, while levels of translated proteins can remain high for longer periods of time. They imply that mRNA levels are an indication of recent events while protein expression corresponds to the cell's previous molecular history. In light of these findings, it is possible that a longer GnRH administration regime may have been necessary for the changes in opsin gene expression to be reflected at the level of the ERG recordings. In this regard, immunohistochemical labeling of visual pigments as opposed to mRNA labeling in *in situ* hybridization, may have provided us with expression levels that corroborate the spectral sensitivity data. Alternative explanations for the separation between gene and protein include disruption in protein translation, which can be provoked by metabolic compromises (Parvez and Riederer, 2006), epigenetic effects (for instance chromatin remodeling and DNA methylation), which can arise through post-translational modification of the amino acid sequence and methylation causing areas of DNA to be less available for transcription (Huang et al., 2010; Qureshi and Mehler, 2010).

The mechanism by which GnRH exerts physiological and neurological changes at the level of the retina is indeed a very complex process. I can only speculate on the reasons for the discrepancies between the spectral sensitivity and gene expression data sets. I need to conduct further studies on the underlying mechanisms of GnRH on the visual system. The next step in evaluating the effect of GnRH on the visual system should include extending and maintaining GnRH treatment for a period of 10 weeks. While I did see changes in opsin gene expression after 3 weeks of GnRH treatment, the time required for sufficient outer segment turnover, that is necessary for new visual pigment to be incorporated into the cone outer segment, may be on the order of 10 weeks.

My initial premise was that cichlid mate choice depends on effective visual communication between the signaler and receiver and that GnRH may act on the visual system to elevate visual sensitivity, especially at times of increased visual behaviour related to mate choice. The presence of GnRH releasing fibers in the retina and the many different changes GnRH can bring about in retinal neurons motivated me to specifically examine enhancement of visual sensitivity, the receiver properties in the visual communication network. Equally interesting would be an examination of the enhancement of body coloration assessed by spectral reflectance measurements, the sender properties of the visual communication network. Dominant males cichlids typically have very pronounced coloration in comparison to subdominant males and these differences in coloration correlate with increased expression of GnRH receptors as a result of elevated GnRH levels (Korzan et al., 2008; White et al., 2002). There is a need to examine the coupling between GnRH, spectral quality of body coloration, and the mechanisms that regulate body coloration within the context of dynamic social behaviour.

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Appendix A

Details on Fabrication and Implantation of Cholesterol Pellets

Cholesterol pellet production was adapted from procedures described in (Lee et al., 1986).

A.1. Pellet Mixture Preparation

This procedure was designed to make six cylindrical pellets with an 80-85% cholesterol composition. A stock solution of GnRH (Sigma-Aldrich) was made by adding 1 mg of GnRH to 16.7 ml of 50% ethanol. In order to have a dosage of approximately 0.100 μg GnRH per gram body mass, I measured and averaged the weights of several *Metriaclima zebra*, and determine the average weight to be 15 g. Therefore, I added 1.5 μg GnRH per pellet, which translated to 0.5 ml of the GnRH/ethanol stock solution being added to 300 mg cholesterol (Sigma-Aldrich). This mixture was allowed to dry for 1 hr at 37^{o}C. Once dry, 3 drops of molten coconut oil (Fisher-Scientific) were added to the powder. The powder was then transferred to the pellet mold.

A.2. Pellet Mold and Pellet Production

A mold was created by cutting two pieces of plexiglass into 7 cm by 7 cm squares that were 0.93 cm deep. One piece was used as the base for the mold while the other had 16 evenly spaced holes drilled through the plexiglass using a 2.34 mm (3/32”) drill bit.

The tops of the holes were subsequently beveled using a 3.52 mm (9/64") drill bit. Due to portions of the plexiglass fracturing during drilling, only six of the holes were used. The powdered GnRH mixture (recipe outline below) was packed into the holes using a flat ended rod (1.87 mm diameter x 2.9 cm long) and gentle hammering. The pellets were then hammered out of the mold and stored at $4^{o}C$.

A.3. Implantation Device and Procedure

An implantation device (implanter) was created by cutting a glass Pasteur pipet with a drill bit but with a diamond disk-tip so that the front portion of the pipet had an opening of 2.5 mm (the width of the pellets). A second Pasteur pipet with a fire sealed tip was then inserted into the first pipet to act as a plunger to push out the pellet.

Prior to implantation, the fish were anesthetized with tricaine methanesulphonate, (MS-222; 200 mg L^{-1}) until level IV anesthesia was reached (Jolly et al., 1972). The pellet was loaded into the first pipet and an incision was created in the peritoneal cavity of the fish using a scalpel. The tip of the first pipet was inserted into the incision, and the pellet was subsequently pushed into the cavity of the fish. The incision was then sealed with an adhesive.

Appendix B

Fabrication and Administration of Intubation Solutions

Intubation solutions and procedures were adapted from Solar et al., 1990.

B.1. Intubation Solutions

Stock solutions of 1 mg ml^{-1} sGnRH (Ova-RH, Syndel Laboratories, Vancouver B.C.) in 0.65% saline, 5mg ml^{-1} L-lysophosphatidylcholine (Sigma, St-Louis MO., U.S.A.) and 100 mg ml^{-1} sodium bicarbonate were prepared prior to making the intubation solution. In order to administer 10 μl of intubation solution containing 1 μg GnRH per gram body mass fish, I mixed 5 μl of the L-lysophosphatidylcholine stock solution, 25 μl of the sodium bicarbonate stock solution, 250 μl of the GnRH stock solution and topped up to 2.5 ml with 2220 μl 0.65% saline. The final concentrations of the active components were 0.01 mg ml^{-1}, 1 mg ml^{-1}, and 0.1 mg ml^{-1} respectively. The control solution was made in the same fashion without addition of the hormone.

The sodium bicarbonate and L-lysophosphatidylcholine were added to elevate gastric pH and increase gastro-intestinal absorption (Tagesson et al., 1985) as cited in (Solar et al., 1990).

B.2. Intubation Device and Procedure

An intubation device was fashioned out of a 3 ml syringe with either a 19 G or 20 G needle with the tip smoothed down attached to a piece of polythene tubing (approximately 15 cm long, 0.86 mm i.d., 1.52 mm o.d.; SIMS Portex Ltd.).

Appendix C

Electroretinogram Experimental Set-up

The ERG electrophysiological apparatus consists of three optical channels (Coughlin and Hawryshyn, 1995; Lisney et al., 2010; Parkyn and Hawryshyn, 1993), including two background channels and a stimulus channel, which project to the position of the fish inside a Faraday cage. The background channels were both set up in the same fashion and were equipped with 250W, 24V ELC halogen projector bulbs with built-in parabolic reflector (EJH Spectro) that provide the light source, which was focused through a liquid-filled bifurcated fiber optic cable (fused silica, numerical aperture =0.22; Fiberoptic Systems, Simi Valley, CA, USA). Intensity and spectral characteristics of the light were be manipulated via Inconel-coated neutral density filters (Corion) and interference filters (Corion).

The stimulus channel light was provided by a 300 W Xenon arc lamp (Oriel) which was also focused into a liquid-filled fiber optic probe. The optical setup was made up of a monochrometer (Instruments SA, Metuchen, New Jersey, USA) to control the spectral characteristics, an Inconel quartz neutral density wedge (0-4.0 neutral density; CVI Melles Griot, Albuquerque, NM, USA) to control the stimulus intensity, a shutter (Uniblitz, Rochester, NY, USA), and optical filters to block spectral sidebands and UV lenses to match the numerical aperture of the liquid light pipe (fused silica; NA = 0.55; Fiberoptic Systems). The background and stimulus optical fibers were fitted to a beam splitter to integrate the stimulus and background lighting. This ensures that the same area of the fish's eye that was being adapted was also the same being stimulated.

C.1. Electrodes

Electrolyte-filled glass electrodes were used for ERG recordings from the optic nerve at the level of the cornea. Electrodes were pulled from borosilicate glass capillaries (World Precision Instruments, Sarasota Florida) using a Sutter P-97 Flaming-Brown micropipette puller (Sutter Instruments Company, Novato, CA) and with a tip diameter of ~75-100 μm. I ensured the tip of the electrode was smooth and cut at an approximately 60^o to 90^o angle using a dissecting microscope and micrometer slide.

The electrodes were filled with an electrolyte solution (0.69 M NaCl) and inserted into a microelectrode holder (half-cell) (A-M Systems, HRS Scientific, Montreal, QC). The electrodes were positioned on the right eye of the fish using a micromanipulator. In addition to a glass recording electrode, a Teflon coated chlorided-silver reference electrode was placed on the head of the test fish, and a ground clip was attached to the caudal fin.

The electrodes were connected to an ISO-80 bioamplifer (World Precision Instruments, Sarasota, FL, USA) and signals were digitized with Cambridge Electronic Design (CED; Cambridge, UK) 1401+ data acquisition hardware and Signal 4.0 software (CED). Retinal responses were recorded differentially, amplified 1,000 times with a cut-off bandwidth of 5–100Hz and digitally filtered using Infinite Impulse Response (IIR) filters in the Signal 4.0 software to remove 60 Hz, 180 Hz and high-frequency noise.

Appendix D

Background Adaptation Conditions and Quantum Catch of Photoreceptors

D.1. White Light Adaptations

The background channels identified in Appendix C were used to adapt the eye of the fish to white-light (broad spectral conditions), ensuring that all cone mechanisms were expressed in the spectral sensitivity curves.

D.1.1. Cholesterol Implant Study

For the study administering GnRH via cholesterol implants, the white light adaptation was produced using a combination of a 700 nm short-wavelength pass (SP) interference filter and 2.0 and 1.0 neutral-density (ND) Inconel filters (Coherent Inc., Santa Clara, CA, USA). This produced a power spectrum in the wavelength range between 300 and 700 nm. Table D.1. lists the filters used in creating the background conditions.

D.1.2. GnRH Intubation Study

For the study involving GnRH treatment by means of oral intubation, the white light adaptation condition was produced by using a combination of a 550 nm long-wavelength pass (LP) interference and 1.0 ND filters (Edmund Optics, Barrington, NJ, USA). Table D.1. lists the filters used in creating the background conditions.

D.2. Chromatic Adaptation

Coloured backgrounds were used to manipulate cone mechanism. The principle is based on sensitivity being approximately inversely proportional to the background intensity. Thus, chromatic adaptation allows for selective manipulation of cone mechanism.

D.2.1. Cholesterol Implant Study

A long-wavelength adaptation condition (to elevate the sensitivity of the short-wavelength cone mechanisms) using a filter set including 500 nm LP, 550 nm LP and 550 nm LP (Edmund Optics) were employed. See Table D.1 for filters used to create background conditions.

D.2.2. GnRH Intubation Study

A bright yellow background was used as a mid to long-wavelength adaptation condition for the fish treated via intubation. The filter set used included two 550 nm LP filters (Edmund Optics). See Table D.1 for filter used to create the background conditions.

Table D.1 Filters used for the background adaptation conditions: spectrally broad background (white) and a long-wavelength depressing background (yellow). See Figure D.1 for spectral energy distribution plots.

Administration Technique	Background Condition	Background Channel: Channel 1	Background Channel: Channel 2
Cholesterol Implant	White	2 .0 ND[1]B, 700 nm SP[2] Z	1.0 ND A
	Yellow	500 nm LP[3] A, 550 nm LP B	550 nm LP A
GnRH Intubation	White	1.0 ND	550 nm LP Edmund
	Yellow	550 nm LP B	550 nm LP Edmund

[1] ND = Neutral density
[2] SP = Short pass filter
[3] LP = Long pass filter

D.3. Quantum Catch and Spectral Energy Distributions of Background Channels

White (broad spectrum) and yellow (long-wavelength depressing) adaptation conditions were used in order to enable all available cone mechanisms to be visible and to isolate cone mechanisms in the short-wavelength end of the spectrum, respectively. A quantum catch model was used to determine the level of light adaptation of the respective cone mechanisms based on the following formula:

$$Qi = \int_{300}^{800} A_1(\lambda) E_i(\lambda) d(\lambda) \qquad \text{Equation 4}$$

Where *Qi* (photons cm^{-2} s^{-1}) denotes the quantum catch of cone mechanism *i* (i = SWS1, SWS2b, RH2b, RH2aβ and RH2aα), *Ai*(λ) represents the visual pigment absorption coefficient of cone mechanism *i* at a wavelength λ (nm), and *Ei* (λ) denotes the photon irradiance of the background light field at wavelength λ. The spectral irradiance measurements were obtained in a previous study in our lab (Sabbah et al 2010). The irradiance from each of the background conditions was calculated by multiplying the measured output of each light source by the spectral transmission of the filters (measured using a spectrophotometer (Cary) used in producing the background condition.

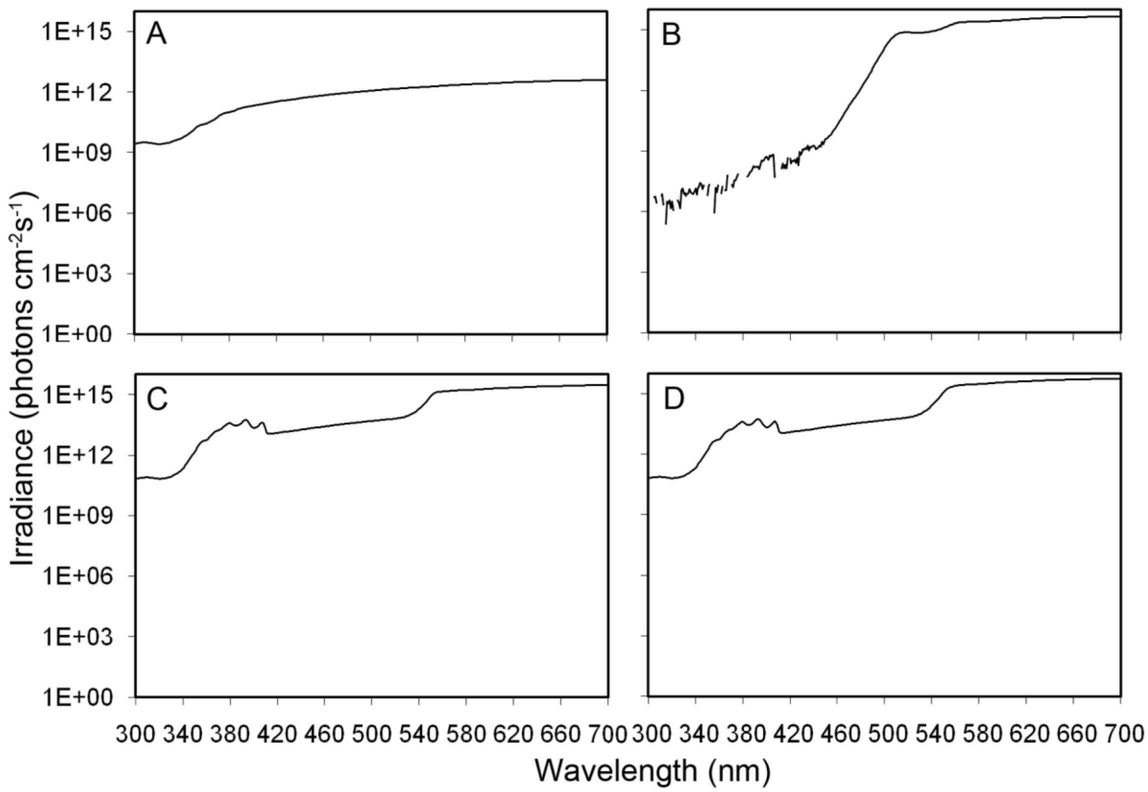

Figure D.1. Spectral irradiance of adaptation conditions used for electroretinogram recordings (ERG). See table D.1 for filter sets used. (A) A white broad spectrum background condition used for the cholesterol implant study to visualize all available cone mechanisms. (B) A yellow long-wavelength depressing background used for the cholesterol implant study. (C) A white broad spectrum background condition used for the GnRH intubation study to visualize all available cone mechanisms. (D) A yellow long-wavelength depressing background used for the GnRH intubation study.

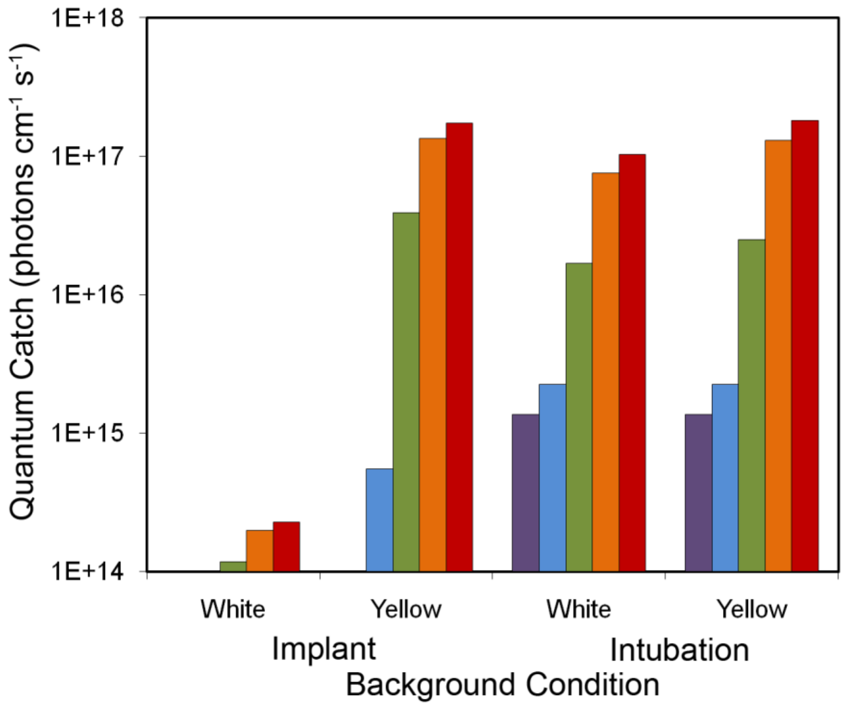

Figure D.2. Quantum catch of five opsins genes expressed in *Metriaclima zebra* (SWS – purple, SWS2b – blue, RH2b – green, RH2aβ – orange, RH2aα – red) under for the white and yellow adaptation conditions for cholesterol implant and GnRH intubation studies. Cone quantum catches were calculated by setting the proportion of A2 to 50 percent.

Appendix E

Primer Sets for qPCR

Table E.1. SYBR® Green primer specifications for cone opsin genes**.**

Target	Primer	Sequence	Primer Length (bp)	GC (%)	Melting Temperature (Tm, ^{o}C)	PCR Template
SWS1	UV F3	5'- ACATCCCTGAAAGTCTGGGC -3'	20	60	55.6	AF191222
	UV R2	5'- AGCAGCTGGGAGTAGCAGAA -3'	21	55	55.0	
SWS2b	Blue2 F2b	5'- GCTTGTGGTCTCTTGCTGTGG -3'	20	57	56.0	AF317674
	Blue2 R2b	5'- CCAAACAGAGGTGGAAGTGC -3'	20	55	53.3	
SWS2a	Blue1 F1a	5'- GCAGAGAGGGAAGTGACCAG -3'	20	60	54.0	AF247114
	Blue1 R1a	5'-AGCCTTTGAGAAACAGGACG -3'	20	50	52.3	
RH2b	PaG-Rh2b-F12	5'- GCCTTGTCATTACTGGATTC -3'	20	45	48.4	DQ088645
	PaG-Rh2b-R2	5'-AGCAAGAACCACAAGAGACC -3'	20	50	52.0	
RH2a	Grren1 F4	5'- CTGAGAAGGAAGTGACCCGT -3'	20	55	53.2	AF247122
	Green1 R3	5'- AGCACGTAGATAACAGGGTTGT -3'	22	45	53.5	
LWS	Red F1a	5'- TGAGGGTCCCAATTACCA -3'	18	50	48.6	AF247126
	Red R0	5'-GCCCTCAAAGATACACATTGG -3'	21	48	51.2	

[1] Length in basepairs.
[2] Accession numbers of genes used to assess specificity of primers.

Table E.2. Plexor™ primer specifications for cone opsin genes.

Target	Primer	Sequence	Primer Length (bp)	GC (%)	Melting Temperature (Tm, ^{o}C)	PCR Template
SWS1	SWS1 FAM F	5'-TGTGCTCTGGAGAGTGCTGTG-3'	21	57	66.0	AF191222
	SWS1 FAM R	5'-FAM™-iso-dC-CGAACGGTTTACAA ATGACCAGGT-3'	24	46	65.0	
SWS2b	SWS2B Cy5 F	5'-ATGAAACCTACGTGATGTTCCTCT TC-3'	26	42	65.1	AF317674
	SWS2B Cy5 R	5'-Cy5™-iso-dC-GCAGAAGACGATGG TGGAGAAAG-3'	23	52	65.0	
SWS2a	SWS2A HEX F	5'-GGTTTTTTGCAGTATTTGCCTCA-3'	30	39	64.6	AF247114
	SWS2A HEX R	5'-HEX™-iso-dC-GTTGTTGTATTTGTTG TTTGTGGTGTACCA-3'	23	37	61.1	
RH2b	RH2B HEX F	5'-AACAACAAGACAGGACTCGTGAG-3'	23	48	64.9	DQ088645
	RH2B HEX R	5'-HEX™-iso-dC-GGCAAAACATATAGA AAGCAAGCAATCTG-3'	29	38	65.2	
RH2a	RH2A Cy5 F	5'-TCTGTGCAATTGAGGGATTCATGG-3'	24	46	65.0	AF247122
	RH2A Cy5 R	5'-Cy5™-iso-dC-CAGAGAACTTGA AGCTTCCCATGG-3'	24	50	65.2	
LWS	LWS FAM F	5'-GGGCTATGTTGTCTCAGTTTGTG TATT-3'	27	41	65.1	AF247126
	LWS FAM R	5'-FAM™-iso-dC-GACATTTCCAAAAGGT TTGCACACA-3'	25	40	64.7	