Functional diversity in colour vision of fish

By

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Abstract

The overall objective of this thesis was to understand better the mechanisms that shape the diversity in colour vision of fish, and to explore the adaptive significance of this divergence. Among the vertebrates, teleost fish show the greatest diversity in colour vision systems. The cichlid model system illustrates that the visual system of fish may differ among species, sexes, individuals, and life stages of individuals. The large number of available cone opsin genes, which have resulted from multiple opsin gene duplications, facilitates this high degree of variation in the mechanisms of colour vision. In general, cichlids possessed complements of four to five cone pigments, and these complements varied across species, sexes, and individuals. Additionally, lens transmission, cone pigment expression, post-receptorial sensitivity, and retinal circuitry differed across life stages of individuals. My results suggest that the diversification of colour vision across species and across life stages of individuals contributes to sensory adaptations that enhance both the contrast of zooplanktonic prey, and the detection of optical signals from conspecifics. Therefore, both natural and sexual selection may have worked in concert to shape colour vision in fish. Since light is more complex under water than on land, fish required four to six cone classes to reconstruct the colour signals reflected from aquatic objects. This suggests that the large number of cone pigments in fish have likely evolved to enhance the reconstruction of the complex colour-signals in aquatic environments. Taken together, these findings improve our understanding of the variable nature of fish colour vision, and, more generally, help unravel the evolution of photoreceptors and colour vision.
Co-Authorship

Chapter 2: Functional diversity in the colour vision of cichlid fishes
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I designed the experiments, executed the spectral sensitivity measurements, performed all data analysis, and wrote the manuscript; RLL performed the qPCR gene expression profiling; SMG performed the spectral reflectance measurements and edited the manuscript; CWH participated in the design of the study and edited the manuscript.

Chapter 3: Colour vision requires more cone classes in aquatic than in terrestrial environments
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I designed and performed the experiments, analyzed the data, and wrote the manuscript; NFT helped in the design of the experiments and edited the manuscript; CWH and SMG helped in performing the experiments and edited the manuscript.

Chapter 4: Ontogeny in the visual system of Nile tilapia
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I designed the experiments, executed some of the spectral sensitivity measurements, performed all data analysis, and wrote the manuscript; JH and FEH executed the rest of the spectral sensitivity measurements. WAN helped in the development of the spectral sensitivity model and edited the manuscript; CWH participated in the design of the study and edited the manuscript.
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Chapter 1

General Introduction

1.1 Introduction

How do animals see the world? This question has fascinated both researchers and lay people for centuries. However, confined to our sensation and perception, we often fail to fully appreciate the sensory world of other species. Yet, during recent decades, numerous new visual capabilities that are well outside the human perception have been described. For example, many animals are sensitive to the polarization of light and make use of a spectral range that extends into the ultraviolet and infrared spectral regions.

An animal’s visual system is designed to optimize the detection and recognition of objects that are crucial for its survival and reproduction [1, 2]. A primary visual function, thus, is the detection and recognition of objects against a background. Photoreceptors sample the light entering the eye, but they are colour blind and respond only to the amount of energy they absorb [3]. Consequently, detecting objects based solely on brightness differences can fail when the brightness of objects is similar to that of their background. However, apart from rod photoreceptors that function under low-light conditions (scotopic vision), the retina in most vertebrates also contains several classes of cone photoreceptors that function under well-lit conditions (photopic vision), each class being maximally sensitive to a different spectral range. By comparing the brightness differences between several spectral classes of cones (chromatic differences),
objects can be detected even when brightness differences at any given spectral range are absent. Thus, colour vision combines both brightness and wavelength differences to detect objects, and requires retinal and brain circuitry to compare the signals from at least two spectrally distinct cone classes.

In order to understand colour vision, the number and type of colour channels, and how the colour channels interact with one another should be known [4]. To date, however, very little is known about the properties of these colour channels in most of the vertebrates. Fortunately, throughout the years, the characteristics of some of the factors that affect these colour channels have been gradually explored. Among others, these include the transmission of the ocular media, amino acid sequence of opsin proteins, expression of cone opsin genes and pigments, chromophore composition, and retinal circuitry. These factors may lead to different processing of the spectral content of visual signals incident on the eye and may potentially affect colour perception. Both background light and light reflected from objects, which are central for object detection and recognition, depend on the prevailing environmental light. The underwater light environment imposes a great challenge on the vision of aquatic animals [5]. Light under water can be dim and have an irregular spatial distribution [6]. The aquatic medium selectively degrades the light spectrum and spatial frequencies of a pattern [7]. Moreover, surface action in the form of waves refracts light and produces a light field that is highly fluctuating both spatially and temporally [8, 9]. All of these factors create a distinct environment, where aquatic animals forage, escape predators, and communicate visually.
It is therefore not surprising that fish show the greatest diversity in visual systems among the vertebrates [10]. Fish demonstrate a large diversity in the morphology of cone photoreceptors and their spatial organization [11], and they also vary considerably in the spectral location and number of cone classes they possess. For example, deep-sea fish often possess pure rod visual systems [12, 13], whereas fish residing in moderate depths typically possess visual systems with one, two, or three cone classes (mono-, di-, or trichromatic systems), and many shallow-living fish possess visual systems with four cone classes [14]. Diversity in the mechanisms of colour vision of fish can occur at different levels, for example, between species, between individuals of a species, and between life stages of an individual. The objective of this thesis is to better understand the mechanisms that shape the diversity in colour vision of fish, and to explore the adaptive significance of this divergence. The general characteristics of the fish retina and the mechanisms underlying the divergence in the colour vision of fish will be presented below. Additionally, the different levels of divergence in colour vision, i.e., between species, between individuals of a species, and between life stages of an individual, as well as the potential adaptive significance of this divergence will be introduced.

1.2 The fish retina

The vertebrate retina is composed of three layers of neuron cell bodies. The outer nuclear layer (ONL) contains the cell bodies of the rod and cone photoreceptors; the inner nuclear layer (INL) contains the cell bodies of the bipolar, horizontal, and amacrine cells; and the ganglion cell layer (GCL) contains the cell bodies of ganglion cells and displaced
amacrine cells. These cell body layers are interspaced by two layers where synaptic contacts occur: (i) the outer plexiform layer (OPL), where there are synapses between photoreceptors, horizontal cells and bipolar cells, and (ii) the inner plexiform layer (IPL), where bipolar cells and amacrine cells synapse to ganglion cells. See Figure 1.1 for a simple scheme of the organization of the retina. The characteristics and function of the different neurons in the retina will be introduced briefly below. For a detailed description of retinal anatomy and physiology in fish see Djamgoz and Yamada [15] and Djamgoz et al. [16].

1.2.1 Photoreceptors

The outer segment of photoreceptors contains disks filled with visual pigments that absorb light. A visual pigment, in turn, is composed of an opsin protein linked to a chromophore, which is a vitamin A derivative. The absorbance spectrum of visual pigments includes two main peaks: the $\alpha$-band – the peak of maximum absorbance whose spectral location is denoted by $\lambda_{\text{max}}$, and the $\beta$-band – a secondary, short wavelength absorbance peak. The spectral position of these peaks depends on the variation in both chromophore and opsin pigment. Absorption of light isomerizes the chromophore and starts a phototransduction cascade that ultimately leads to perception [17]. Phototransduction in rods and cones is unique. These photoreceptors hyperpolarize to a light stimulus, causing a reduction in the cell's response [18, 19].
Figure 1.1 Organization of the retina

The various nuclear and plexiform layers are presented along with the cell types embedded in each layer. Key: R, rod photoreceptor; C, cone photoreceptor; H, horizontal cell; B, bipolar cell; M, Müller cell; Am, amacrine cell; and G, ganglion cell (modified from Ogden [20]).
1.2.2 Horizontal cells

In fish, horizontal cells (HCs) receive synaptic input from photoreceptors and other HCs of the same functional class [21]. HCs respond to increasing areas of illumination with increasing response, thus indicating extensive spatial summation [22, 23]. Two general types of HCs have been identified. Monophasic HCs hyperpolarize to all spectral stimuli (achromatic response), whereas multiphasic HCs hyperpolarize to some wavelengths and depolarize to others (chromatic response) [24]. HCs provide inhibitory feedback to cone photoreceptors [25, 26] and have a role in light adaptation [27], colour constancy [28] and polarization sensitivity [29].

1.2.3 Bipolar cells

Bipolar cells (BCs) receive synaptic input from photoreceptors and horizontal cells. BCs receptive fields show a concentrically-antagonistic center-surround organization [30, 31]. Two major types of BCs have been identified: ON BCs - depolarizing to a central spot and hyperpolarizing to an annular (peripheral) stimulus, and OFF BCs - hyperpolarizing to a central spot and depolarizing to an annular stimulus [19]. Generally, the receptive field center receives input from photoreceptors while the receptive field surround receives input from HCs. Three chromatic types of BCs have been described in goldfish and carp: (i) non-colour-coded BCs - the receptive field center and surround receive input from a single cone class; (ii) colour-opponent BCs – the receptive field center receives input from a single cone class while the surround receives an inverted-polarity input from two cone classes; (iii) double-colour-opponent BCs –
both the receptive field center and surround receive input from two cone classes, where in the center, the polarity of input from one cone is opposite to that from the other cone, and opposite to that in the surround, i.e., these BCs show opponency both in the receptive field center and surround [32, 33]. In fish retina, BCs of the same morphological and functional type are electrically coupled via gap junctions [34, 35], i.e. there is spatial summation of signals at the level of BCs, which makes the receptive field centers of these neurons larger than their dendritic fields. However, the coupling of BCs is much weaker than that of HCs.

1.2.4 Amacrine cells

Amacrine cells (ACs) are the most abundant group of local circuit neurons in the vertebrate retina [36]. Amacrine cell bodies are situated in the inner nuclear layer or in the ganglion cell layer, while their complex dendritic fields stratify in the inner plexiform layer [36]. Unlike other retinal neurons, most ACs do not have axons. ACs are morphologically diverse and release a large number of neuroactive substances - classical transmitters, neuropeptides and neurohormones [37, 38]. ACs make synaptic and electrical contacts with bipolar cells, ganglion cells and other ACs. Light-evoked responses in ACs can be sustained or transient, with sustained responses being depolarizing or hyperpolarizing [31, 39]. AC responses are thought to extensively modulate chromatic and direction-selective information in the retina. However, due to their complex connectivity, the role of ACs in shaping the visual signals emanating from the retina is poorly understood.
1.2.5 Ganglion cells

Retinal ganglion cells (RGCs) are the output neurons of the retina. Their dendrites receive synaptic inputs from BCs and ACs, and the axons are bundled together to form the optic nerve. The basic RGC receptive field arrangement is antagonistic center-surround organization; similar to BC receptive fields [40]. Light-evoked spike trains in RGCs can be sustained or transient, with sustained responses being depolarizing or hyperpolarizing [41, 42]. Similarly to BCs, three major unit types that differ in their receptive field chromatic organization have been identified: (i) non-colour-coded units, (ii) single-colour-opponent units – only the receptive field center shows colour-opponency, and (iii) double-colour-opponent units – both the receptive field center and surround show colour-opponency [43]. Different RGCs have specialized in detecting diverse aspects of the visual scene, including colour, size, direction and speed. Typically, RGCs that receive substantial input from BCs reflect essential receptive field properties of BCs, whereas RGCs that receive substantial input from ACs show wide and asymmetric receptive fields with direction selectivity.

1.3 Mechanisms contributing to the divergence in colour vision of fish

1.3.1 Diversity in ocular media transmission

The visual process begins with light being transmitted through the ocular media, which includes the cornea, lens, and vitreous and aqueous humors. Thus, the spectral transmission of the ocular media shapes the spectrum of light reaching the retina. In fish, the lens is typically less transmissive to ultraviolet (UV) wavelengths than the cornea and
humors. Consequently, the transmission of the ocular media in fish is largely determined by the transmission properties of the lens. The wavelength of half-maximum lens transmission, T_{50}, serves as an index for the ability of the lens to transmit UV wavelengths and typically ranges between 340 and 400 nm [44-48]. Lens transmission is modulated through deposition of UV-absorbing compounds [48], and was shown to depend on the photic environment of the fish [49], and to vary across species [45] and life stages of individuals [47, 50].

UV sensitivity can be achieved either by having a cone photoreceptor maximally sensitive in the UV range, or via the secondary sensitivity peak (β-band) of any of the longer wavelengths sensitive cones. Yet, regardless of the cones involved, UV sensitivity requires that UV light is transmitted by the ocular media and reaches the retina. UV sensitivity might aid in intraspecific communication [51], detection of zooplanktonic prey [52], and orientation and navigation using the polarization of light [53, 54]. However, transmission of UV wavelengths into the eye comes at a cost. UV radiation causes photo-oxidative damage to retinal tissues [55]. Additionally, inclusion of UV wavelengths in the image-forming light may degrade and blur the retinal image. This is because chromatic aberrations and scattering are strongest at short wavelengths [5]. Fish might cope with photo-oxidative damage by high turnover rate of the membrane discs in the outer segments of photoreceptors [56] and by rapid replacement of degenerated photoreceptor tissues [57]. Moreover, fish may overcome the degradation of retinal image through the use of multifocal lenses that reduce chromatic aberrations [58].
1.3.2 Diversity in amino acid sequence of opsin genes

The spectral absorbance of visual pigments can be modified through amino acid substitutions in the opsin protein. Key sites tend to be in one of the seven transmembrane alpha helices close to the retinal-binding pocket and usually involve changes in amino acid polarity [59, 60]. Single amino acid changes can cause shifts of between 2 and 35 nm in $\lambda_{\text{max}}$, depending on their location and interaction with the chromophore [61, 62].

Four classes of cone opsin genes are found in the most basal vertebrate lineage, the lamprey (Hyperoartia)[63]. These include an ultraviolet-sensitive ($SWS1$), a short wavelength-sensitive ($SWS2$), a rhodopsin-like mid wavelength-sensitive ($Rh2$), and a long wavelength-sensitive ($LWS$) opsin gene [60, 61, 63-66]. In fish, however, cone opsin genes have duplicated and accumulated mutations to produce up to two copies of $SWS1$ [67] and $SWS2$ [68, 69], up to four copies of $Rh2$ [70], and up to six copies of $LWS$ [71, 72]. This classification of cone opsin genes often depends on the degree of sequence similarity and variation in $\lambda_{\text{max}}$. Interestingly, cone opsin genes that are found in single cones ($SWS1$ and $SWS2$) have duplicated less often than those in double cones ($Rh2$ and $LWS$). Recently, however, fish that are exposed to broad light spectrum and often express the $SWS1$ opsin gene were reported to show elevated numbers of potentially functional amino acid substitutions in the $SWS1$ sequence. Similarly, fish that are exposed to a narrow, long wavelength-shifted light spectrum and often express the $LWS$ opsin gene were reported to show elevated numbers of amino acid substitutions in the $LWS$ sequence [73]. This suggests that changes in coding sequence may fine tune cone pigment absorbance at the end of the spectrum utilized (either the short- or long-wavelength end).
1.3.3 Diversity in opsin gene expression

Duplicate opsin genes create the opportunity for tuning the visual sensitivity of fish by differential gene expression. Expression of cone opsin genes is commonly estimated based on reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Different species vary considerably in the complement of cone opsin genes that they express. For example, zebrafish (Danio rerio) express seven out of eight reported cone opsin genes [70, 74] and guppies (Poecilia reticulata) express six out of nine reported cone opsin genes [71, 75]. Variation in cone opsin gene expression is probably best known for African cichlids (Cichlidae), where different species and different life stages of a species express distinct complements of three to five out of seven reported cone opsin genes [69, 73, 76]. Unfortunately, it is currently unknown how cone opsin genes that are expressed at low levels contribute to vision in fish. Consequently, opsin genes that are expressed at ‘low levels’ in fish are typically treated as non-significant without any justification or estimation of the biological significance of the pigments they encode [77].

1.3.4 Diversity in cone pigment expression

The fish retina typically contains an organized mosaic of photoreceptors. These mosaics may contain single cones, which can be long or short, and double cones, which are composed of large (principle) and small (accessory) members. Generally, the SWS1 and SWS2 cone pigments are found in single cones, and the Rh2 and LWS cone pigments
are found in double cones [11, 78, 79]. The arrangement of the cone mosaic can vary considerably between species [11] and between life stages of an individual [80-83].

The expression of cone pigments is commonly estimated based on the frequency of cone photoreceptor classes measured using microspectrophotometry (MSP) (e.g. [84]) and by fitting visual pigment absorbance templates to spectral sensitivity curves measured using electrophysiological and psychophysical techniques (e.g. [85, 86]). Additionally, the location, but not the expression level, of cone pigments can be determined by using immunohistochemistry techniques [80]. Shallow-water fish were shown to possess two to seven cone pigment classes in their retina. For example, zebrafish (*Danio rerio*) display seven cone pigments [70, 74]; guppies (*Poecilia reticulata*) [87] and killifish (*Lucania goodei*) [88] display five cone pigments; salmonids (Salmonidae) [89, 90] display four to six cone pigments; black bream (*Acanthopagrus butcheri*) [91], goldfish (*Carassius auratus*) [92, 93], and three-spine stickleback (*Gasterosteus aculeatus*) [94] display four cone pigments; coral reef fish display two to four cone pigments [84, 95]; and African cichlids typically display three cone pigments [96, 97], although several species were shown to display up to seven pigments [69]. The adaptive value of this diversity in the number of cone pigments expressed in fish is yet to be explored thoroughly.

### 1.3.5 Diversity in chromophore composition

Shifts in the spectral absorbance of visual pigments in fish can also occur through changes in chromophore composition. Many fish can modulate the relative levels of $A_1$
(retinal) and A₂ (3,4-dehydroretinal) chromophores in their retina [98, 99]. Increasing A₂ proportion shifts the $\lambda_{\text{max}}$ of a visual pigment to a longer wavelength, broadens the spectral bandwidth of absorbance, and decreases the molar extinction coefficient [100-102]. The magnitude of this $\lambda_{\text{max}}$ shift increases for longer $\lambda_{\text{max}}$ values [98, 103-105]. Thus, modulation of A₂ proportion may alter the spectral sensitivity of fish and optimize the signal-to-noise ratio for which temperature may be a limiting factor [106, 107]. Both of these factors might be of adaptive value for fish that are exposed to diverse photic environments and temperatures, such as fish that migrate between habitats. Indeed, shifts in A₂ proportion were found to correlate with seasonal changes in temperature [108-110], as well as in photoperiod, light intensity and wavelength [111-113]. Additionally, thyroid hormones were also shown to modulate the proportion of the A₂ chromophore in fish [90, 114].

1.4 Levels of divergence in colour vision and its potential adaptive significance

1.4.1 Diversity in colour vision between species

Different species may have different visual demands that arise from differences in the light environment, foraging modes, and levels of inter- and intraspecific competition. Species may also differ in their body colour pattern. In many fish species, these colour patterns facilitate recognition of conspecifics and thus have an important role in efficient mate-choice and intra-specific (e.g., male-male) competition. Therefore, to optimize the recognition of conspecifics, fish with different body colour patterns are expected to also differ in their visual sensitivity. These differences in visual demands may promote
diversification in colour vision. Diversification in colour vision between different species was reported to involve variation in ocular media transmission [84], expression of opsin genes [69, 73, 76], amino acid sequence of opsin genes [73], and chromophore composition [115]. For example, sensitivity to UV light depends on the ability of the ocular media to transmit UV wavelengths, and the possession of cones that exhibit sensitivity in the UV spectrum (through either the $\alpha$- or $\beta$-band). Zooplankton strongly absorb UV wavelengths. Consequently, UV sensitivity might enhance the contrast of transparent zooplankton against the water background, and thus, aids in their detection [52, 116]. Thus, UV sensitivity is expected to be advantageous for planktivorous species. However, UV sensitivity might also be used for communication. UV reflections from the body patterns of fish were shown to be used in species-specific recognition [51]. Additionally, UV signals were suggested to increase the contrast within the body pattern, leading to an increase in the attractiveness of males during female mate-choice [117]. Thus, UV sensitivity might be advantageous for species that prey on zooplankton and those that show UV body pattern reflections.

The environment of the viewer and object may affect vision by: (i) providing the illumination, (ii) acting as a transmission medium between object and viewer and (iii) providing the background against which the object is viewed. The characteristics of illumination, light transmission through the medium, and reflectance of objects may vary across environments. Therefore, one may expect the colour vision of species from different environments to diverge. Indeed, the number of cone classes was shown to vary with water depth, as shallow-water fish possess a greater number of cone classes than
deep-water fish [14, 118, 119]. The spectral location of cone pigments was also reported
to vary between shallow- and deep-water fishes, with the latter having cone pigments
shifted toward short wavelengths [120, 121]. Moreover, fish from habitats with a narrow,
long-wavelength-shifted light spectrum were shown to have cone classes that are shifted
toward longer wavelengths as compared to fish from habitats with a broad light spectrum
[69, 122]. Nevertheless, how the characteristics of the photic environment and the
reflectance of objects shape the number and spectral sensitivity of cone photoreceptors
remains largely unknown.

1.4.2 Diversity in colour vision between individuals of a species

The diversification in colour vision between individuals of a fish species was not
studied in detail previously. Recently, however, opsin gene expression was found to vary
between individuals and populations of a single species [123, 124], suggesting the
potential importance of the genetic history as well as other factors such as the natural
habitat and social status in determining the expression of opsin genes. Additionally,
thyroid hormones play a central role in vertebrate development and can modulate the
expression of opsin genes and induce cone loss in fish [114, 125]. The level of thyroid
hormones in fish has been associated with environmental stress and subordinance via the
effect of cortisol [126, 127]. Thus, individuals that differ in their stress level or social
status may utilize diverse opsin gene and pigment complements through modulation of
the levels of thyroid hormones. Finally, a special case of diversity in colour vision
between individuals of a species is the variation in colour vision between the sexes. To date, however, such diversification across sexes has not been reported in fish.

1.4.3 Diversity in colour vision between life stages of an individual

Fish at different life stages may differ in their visual demands. For example, unlike adult fish that employ a wide range of foraging styles, many young fish feed strictly on zooplankton [128-130]. This suggests that UV sensitivity, which helps in detecting zooplanktonic prey, would be more beneficial for young fish than for adult fish. On the other hand, a visual system that allows for high recognition of conspecifics would be highly beneficial for adults, for which mate-choice and intra-specific competition are central factors, but not so for young, sexually immature fish. This suggests that colour vision may vary throughout ontogeny in order to meet the specific visual demands imposed at each life stage. Diversification in colour vision during ontogeny was reported to involve variation in ocular media transmission, expression of opsin genes and cone pigments, and chromophore composition. Ontogenetic changes in lens transmission were reported for several families of coral reef fish [46, 50, 131] and the Nile tilapia (Oreochromis niloticus) [47]. Differential expression of cone opsin genes throughout ontogeny was demonstrated in rainbow trout (Oncorhynchus mykiss) [132], Pacific pink salmon (Oncorhynchus gorbuscha) [133], black bream (Acanthopagrus butcheri) [91] and Nile tilapia [96, 134]. Ontogenetic changes in the expression of cone pigments were reported in the Pollock (Pollachius pollachius) [135], goatfish (Upeneus tragula) [136], winter flounder (Pseudopleuronectes americanus) [137, 138], European eel (Anguilla
anguilla) [139-141], Nile tilapia [96], black bream [91], rainbow trout [80] and several coral reef fish species from the Pomacentridae family [142]. Additionally, shifts in chromophore composition were reported to occur with seasonal variation [110, 143-147] and with migration and metamorphosis [148, 149]. However, previous studies typically have looked at the ontogenetic changes in only one or two elements/processes in the visual system, making it difficult to draw conclusions regarding the overall ontogenetic change in the visual system and its adaptive value.

1.5 Thesis overview

In my doctoral work, I have chosen to examine the functional diversity in the colour vision of cichlid fishes. African cichlids are the most extreme case of adaptive radiation among vertebrates, where hundreds of cichlid species have evolved within the last 10 million years [150-152]. Cichlids show a large diversity in the expression of cone opsin genes and pigments [69, 76, 97, 134], inhabit various habitats [153], feed on diverse food sources [150, 154, 155], and exhibit different breeding and parental care behaviours [156]. Thus, cichlids may offer insights into the various factors shaping visual sensitivity and visual communication. The notion that vision may play a crucial role in guiding cichlid communication and speciation [157-160] makes them an excellent model system for studying the diversity in colour vision.

Seven different cone opsin genes have been documented in cichlids [69, 134]. These genes conform to the four major classes of cone opsin genes that diverged early in vertebrate evolution [60, 61, 63-66] and produce an ultraviolet-sensitive (SWS1), two
short wavelength-sensitive (SWS2a and SWS2b), three mid wavelength-sensitive (Rh2α, Rh2αβ, and Rh2b), and a long wavelength-sensitive (LWS) cone pigments. Cone opsin genes in cichlids can be differentially expressed throughout ontogeny [96, 134] and across closely related species [73]. At any given time, however, cichlids were found to express primarily only three to five of the seven available cone opsin genes [69, 73, 76]. The selective force driving the divergence in the visual system of cichlids is still unclear.

Cichlid males display bright breeding colouration while the colour of females usually appears muted [161, 162]. Male breeding colouration varies greatly between closely related species, but not so much between different genera, suggesting a large role of visual cues in the divergence of closely related species. Additionally, to date, no correlation has been found between habitat and colour usage in cichlids [163-166]. This supports the idea that sexual selection, through female mate-choice, has contributed to the differentiation of male breeding colouration.

In the second chapter of the thesis, I studied the diversity in colour vision across species, sexes and individuals by using females and males of three closely related cichlid species from Lake Malawi. In cichlids, mutations in duplicated cone opsin genes have lead to the evolution of novel spectral classes of cone pigments. The expression of different complements of these cone pigments can potentially generate large-scale variation in spectral sensitivity and colour vision. To examine the variation in the number of cone classes that participate in cichlid spectral sensitivity, I used whole organism electrophysiology and opsin gene expression profiling. To assess the presence of
interactions between different cone classes, I reconstructed spectral sensitivity while employing multiple-cone mechanisms modelling.

In the third chapter of the thesis, I explored the factors that might have driven the increase in the number of cone classes in fish. I raised the possibility that the high complexity of underwater light gave rise to high-dimensional colour vision systems in fish. To test this hypothesis I took a comparative approach and studied the dimensionality of colour vision in fish and humans by examining the spectral complexity of the colour-signals (radiance) reflected from aquatic and terrestrial objects.

In the fourth chapter of the thesis, I studied the ontogeny in the visual system of the Nile tilapia. Retinal neurogenesis in fish facilitates cellular rearrangement throughout ontogeny, potentially allowing for optimization of the visual system to shifts in visual demands. To test this possibility, I examined ontogenetic changes in lens transmission, photoreceptor sensitivity and sensitivity of the outer plexiform layer (OPL), and used these to estimate changes in cone pigment expression and retinal circuitry.

Together, these three data chapters examine the factors that have led to the high dimensional colour vision in fish, and the mechanisms and adaptive significance of the divergence in colour vision of fish across species, across individuals of a species, and across life stages of an individual.
Chapter 2

Functional diversity in the colour vision of cichlid fishes

Abstract

Background

Colour vision plays a critical role in visual behaviour. An animal’s capacity for colour vision rests on the presence of differentially sensitive cone photoreceptors. Spectral sensitivity is a measure of the visual responsiveness of these cones at different light wavelengths. Four classes of cone pigments have been identified in vertebrates, but in teleost fishes, opsin genes have undergone gene duplication events, and thus can produce a larger number of spectrally distinct cone pigments. In this study, we examine the question of large-scale variation in colour vision with respect to individual, sex, and species that may result from differential expression of cone pigments. Cichlid fishes are an excellent model system for examining variation in spectral sensitivity because they have seven distinct cone opsin genes that are differentially expressed.

Results

To examine the variation in the number of cones that participate in cichlid spectral sensitivity we used whole organism electrophysiology, opsin gene expression, and empirical modeling. Examination of over 100 spectral sensitivity curves, from 34 individuals of three species, revealed that: i) spectral sensitivity of individual cichlids was
based on different subsets of 4-5 cone pigments, ii) spectral sensitivity was shaped by multiple cone interactions, and iii) spectral sensitivity differed between species and correlated with foraging mode and the spectral reflectance of conspecifics. Our data also suggest that there may be significant differences in opsin gene expression between the sexes.

**Conclusions**

Our study describes complex opponent and non-opponent cone interactions that represent the requisite neural processing for colour vision. We present comprehensive evidence for pentachromatic colour vision in vertebrates, which offers the potential for extraordinary spectral discrimination capabilities. We show that opsin gene expression in cichlids, and possibly also spectral sensitivity, may be sex-dependent. We argue that females and males sample their visual environment differently, providing a neural basis for sexually dimorphic visual behaviour. The diversification of spectral sensitivity likely contributes to sensory adaptations that enhance the contrast of transparent prey and the detection of optical signals from conspecifics, suggesting a role for both natural and sexual selection in tuning colour vision.
Background

Vision is central to the survival of animals. Visual cues are used for orientation, detecting prey, avoiding predators, and communication. The visual process starts with visual pigments absorbing light and initiating a photochemical cascade that leads to neural signalling, perception, and ultimately visually-mediated behaviour. A common method for studying vision is the measurement of spectral sensitivity. Spectral sensitivity is the relative efficiency of detection of light as a function of wavelength. Spectral sensitivity is used to describe the characteristics of visual pigments found in cone photoreceptors in the retina and it is particularly useful in describing the mechanisms of colour vision [167, 168].

Comparative studies in vertebrates established the presence of four spectrally distinct classes of cone visual pigments, produced by several different opsin genes: SWS1 – ultraviolet/violet-sensitive class (355 - 440 nm); SWS2 – short wavelength-sensitive class (410 - 490 nm); Rh2 – mid wavelength-sensitive class (470 - 530 nm); and LWS – long wavelength-sensitive class (495 - 570 nm) [10, 169]. These opsin genes have been identified in the earliest vertebrate lineage, the jawless fishes or Agnathans (lamprey) [63] and arose through duplications of a single ancestral opsin gene. Mutations in duplicated genes can lead to the evolution of additional spectral classes of cone visual pigments within a class of opsin gene. In fact, in many teleost fishes, cone opsin genes have undergone gene duplication to produce a wide range of opsin genes [170-172]. The expression of different subsets of these genes can potentially generate large-scale variation in spectral sensitivity, and the mechanisms of colour vision in fish [69, 76, 91].
The cichlid fishes of Lake Malawi are an excellent model system for examining large-scale variation in colour vision. Lake Malawi has 700-800 species of cichlids [150-152] that have evolved from a common ancestor in a brief period of evolutionary time (2-4 million years) [173]. Malawi cichlids are notable for their diversity in male nuptial colour patterns, sexual dimorphism in colour patterns, and visual communication processes governing mate-choice [157, 174, 175]. Cichlids have undergone multiple opsin gene duplication events, producing seven classes of cone opsin genes [69, 134]. These seven opsin genes include an ultraviolet-sensitive (SWS1), two short wavelength-sensitive (SWS2a and SWS2b), three mid wavelength-sensitive (Rh2aa, Rh2aβ, Rh2b), and a long wavelength-sensitive (LWS) cone opsin genes. Of particular interest is that Malawi cichlids show differential expression of primarily three of the seven available cone opsin genes [73, 76, 96], with some evidence of differential expression through ontogeny [96]. This raises an intriguing question of how seven opsin genes are maintained in Malawi cichlids. Our central research focus concerns the large-scale diversity in cichlid visual systems and differences in the number of cone classes that participate in colour vision. Here, we examine variation in cone classes contributing to spectral sensitivity in cichlids between individuals, sexes and species, and discuss the adaptive significance of this variation in colour vision.

**Results**

To quantify the number of physiologically functional cone classes, we recorded electroretinograms (ERG) from whole fish preparations of three Malawi cichlids: *Metriaclima zebra*, *Melanochromis auratus*, and *Protomelas taeniolatus*. We measured
the *b-wave* amplitude of ERGs, representative of ON bipolar cell activity, to assess the integrative response of functional cones. We were primarily interested in evaluating the number of cone classes contributing to spectral sensitivity and their corresponding visual pigments, but we also used multiple-mechanism modeling to understand the opponent and non-opponent cone interactions at play that can shape the spectral tuning of cone mechanisms. Our analysis focused on describing variation in spectral sensitivity with respect to individual, sex and species differences.

*Individual differences in spectral sensitivity*

Over 100 spectral sensitivity curves were recorded from a total of 15 females and 19 males of the three species studied. The spectral sensitivity of each individual was evaluated under three different colour background conditions, each aimed at isolating the sensitivity of specific cones (Figure 2.1; See Appendix 1, Figure A1.1 for the background conditions and the quantum catches of the various cone mechanisms). For each individual, we identified the number of sensitivity peaks and their spectral locations for the three background conditions. We then fitted cone pigment absorbance templates [176] to the sensitivity peaks using a least squares method.

Individuals of all three species of Malawi cichlids examined in this study possessed 11 different cone subsets (Table 2.1; see Table 2.2 for specific $\lambda_{\text{max}}$ values and goodness of fit of visual pigment templates). A maximum of 5 different cone classes were present in 41% of fish, 4 different cone classes were present in 56% of fish, and 3 different cone classes were present in only 3% of the fish (1 individual) (Table 2.1). All cone subsets (excluding #5) consisted of at least two pigments corresponding to single
Figure 2.1 Relative spectral sensitivity of Lake Malawi cichlids obtained under different background conditions

(A-C) Relative spectral sensitivity of selected individuals of *M. zebra*, *M. auratus*, and *P. taeniolatus* (left to right). The spectral sensitivity of each species was measured under three background conditions: Long wavelength isolation (LW, red), Control (green), and Short wavelength isolation (SW, blue). In *P. taeniolatus*, LW was replaced by Dim short wavelength isolation (Dim-SW, cyan). In *M. zebra*, the spectral sensitivity curves measured under all conditions show four sensitivity peaks. Whereas, in *M. auratus* and *P. taeniolatus*, the complete set of five sensitivity peaks was revealed across all background conditions. Data points were connected with lines to facilitate the identification of sensitivity peaks.

(D-I) Visual pigment templates (dashed lines) fitted to spectral sensitivity (circles) measured under two background conditions (typically, the examination of two spectral sensitivity curves was sufficient to correlate all sensitivity peaks to cone pigments). *M. zebra*: Control (D) and SW (G), *M. auratus*: LW (E) and SW (H), *P. taeniolatus*: Dim-
SW (F) and SW (I). Visual pigment templates: SWS1 (black), SWS2b (violet), SWS2a (blue), Rh2b (green), Rh2a (orange), and LWS (red). Similar plots were used for all individuals to identify sensitivity peaks and correlate them to cone pigments. See criteria for peak identification in the Methods. See Figure A1.1 for background isolation conditions and quantum catch of cone pigments.
Table 2.1 Cone pigment subsets in *M. zebra*, *M. auratus*, and *P. taeniolatus* (estimated from spectral sensitivity)

<table>
<thead>
<tr>
<th>Subset #</th>
<th># individuals</th>
<th>Visual pigment</th>
<th># cones</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SWS1</td>
<td>SWS2b</td>
<td>SWS2a</td>
</tr>
<tr>
<td><strong>M. zebra</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>382±1</td>
<td>427</td>
<td>492±2</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>380±4</td>
<td>460±2</td>
<td>490±4</td>
</tr>
<tr>
<td><strong>M. auratus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>374</td>
<td>424</td>
<td>485</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>380</td>
<td>463</td>
<td>494</td>
</tr>
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<td>5</td>
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<td>429</td>
<td>497</td>
<td>547</td>
</tr>
<tr>
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<td>525</td>
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<tr>
<td>9</td>
<td>1</td>
<td>457</td>
<td>485</td>
<td>525</td>
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<td><strong>P. taeniolatus</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
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<td>379±1</td>
<td>457</td>
<td>488±3</td>
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<tr>
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<td>487±1</td>
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<td>11</td>
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<td>457</td>
<td>529</td>
</tr>
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</table>

In total, 11 different subsets of cone pigments were identified across the species studied. The number of individuals that exhibited each pigment subset, the number of cone classes included in each subset, and the frequency of each cone pigment subset per species are indicated. The $\lambda_{\text{max}}$ [nm] values of cone pigments that comprised each subset are given (mean±standard deviation).
<table>
<thead>
<tr>
<th>Sex</th>
<th>Fish ID</th>
<th>Visual pigment template</th>
<th>A2 proportion</th>
<th>1st $R^2$</th>
<th>2nd $R^2$</th>
<th>Subset #</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SWS1</td>
<td>SWS2b</td>
<td>SWS2a</td>
<td>Rh2b</td>
<td>Rh2a</td>
</tr>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>M. zebra Female</td>
<td>ZF7</td>
<td>381 (0.62)</td>
<td>427 (0.63)</td>
<td>491 (0.76)</td>
<td>535 (0.90)</td>
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<td></td>
<td>ZF6</td>
<td>381 (0.46)</td>
<td>427 (0.82)</td>
<td>491 (0.70)</td>
<td>535 (0.90)</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>ZF8</td>
<td>383 (0.88)</td>
<td>428 (0.55)</td>
<td>494 (0.73)</td>
<td>540 (0.87)</td>
<td>0.53</td>
</tr>
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<td>459 (0.30)</td>
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<tr>
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<td>ZM2</td>
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<td>457 (0.76)</td>
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<tr>
<td></td>
<td>ZM6</td>
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<td>458 (0.57)</td>
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<td>530 (0.95)</td>
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<tr>
<td></td>
<td>ZM4</td>
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<td>463 (0.64)</td>
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<td>ZM1</td>
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<td>374 (0.84)</td>
<td>424 (0.70)</td>
<td>485 (0.82)</td>
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</tr>
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<td>AF15</td>
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<td>424 (0.75)</td>
<td>485 (0.43)</td>
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<td>AF14</td>
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<td>Rh2a (0.94)</td>
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<td>485 (0.33)</td>
<td>525 (0.46)</td>
<td>561 (0.87)</td>
<td>0</td>
</tr>
<tr>
<td>Sex</td>
<td>Fish ID</td>
<td>Visual pigment template $^a$</td>
<td>$A_2$ proportion $^b$</td>
<td>$1^{st} R^2$ $^c$</td>
<td>$2^{nd} R^2$ $^d$</td>
<td>Subset # $^e$</td>
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<td>----------------</td>
</tr>
<tr>
<td>$P. \text{taeniolatus}$</td>
<td>Female</td>
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<td></td>
<td>TF6</td>
<td>SWS1 428 (0.49) SWS2b 457 (0.89) SWS2a 491 (0.75) Rh2b 533 (0.55) Rh2a 576 (0.95) LWS 0.18</td>
<td>LWS (0.86)</td>
<td>7</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>TF2</td>
<td>SWS1 428 (0.66) SWS2b 457 (0.98) SWS2a 487 (0.84) Rh2b 528 (0.55) Rh2a 571 (0.43) LWS 0.11</td>
<td>LWS (0.63)</td>
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<tr>
<td></td>
<td>TF1</td>
<td>SWS1 428 (0.49) SWS2b 457 (0.97) SWS2a 48 (0.49) Rh2b 571 (0.91) LWS 0.15</td>
<td>LWS (0.98) Rh2a (0.82)</td>
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<tr>
<td></td>
<td>TF4</td>
<td>SWS1 428 (0.48) SWS2b 457 (0.54) SWS2a 529 (0.88) Rh2b 571 (0.72) LWS 0.11</td>
<td>LWS (0.91)</td>
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<tr>
<td></td>
<td>TF7</td>
<td>SWS1 379 (0.81) SWS2b 457 (0.47) SWS2a 487 (0.63) Rh2b 529 (0.54) Rh2a 563 (0.73) LWS 0.06</td>
<td>LWS (0.93)</td>
<td>6</td>
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</tr>
<tr>
<td>Male</td>
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<td></td>
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<tr>
<td></td>
<td>TM1</td>
<td>SWS1 428 (0.68) SWS2b 457 (0.64) SWS2a 491 (0.59) Rh2b 533 (0.52) Rh2a 576 (0.88) LWS 0.19</td>
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<tr>
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<td>TM3</td>
<td>SWS1 428 (0.71) SWS2b 457 (0.08) SWS2a 494 (0.89) Rh2b 529 (0.59) Rh2a 571 (0.98) LWS 0.15</td>
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<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TM8</td>
<td>SWS1 428 (0.59) SWS2b 457 (0.94) SWS2a 491 (0.90) Rh2b 534 (0.49) Rh2a 576 (0.80) LWS 0.23</td>
<td>LWS (0.95)</td>
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<tr>
<td></td>
<td>TM4</td>
<td>SWS1 428 (0.64) SWS2b 457 (0.91) SWS2a 486 (0.84) Rh2b 563 (0.98) LWS 0.03</td>
<td>LWS (0.98) Rh2a (0.96)</td>
<td>9</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>TM6</td>
<td>SWS1 428 (0.42) SWS2b 457 (0.45) SWS2a 487 (0.74) Rh2b 571 (0.94) LWS 0.11</td>
<td>LWS (0.97) Rh2a (0.87)</td>
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<tr>
<td></td>
<td>TM5</td>
<td>SWS1 379 (0.78) SWS2b 457 (0.84) SWS2a 486 (0.50) Rh2b 529 (0.57) Rh2a 563 (0.91) LWS 0.03</td>
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<td>6</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>TM2</td>
<td>SWS1 380 (0.95) SWS2b 457 (0.99) SWS2a 491 (0.67) Rh2b 533 (0.87) Rh2a 576 (0.99) LWS 0.18</td>
<td>LWS (0.99)</td>
<td>6</td>
<td></td>
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</table>

$^a$ $\lambda_{\text{max}}$ (nm) of the visual pigment templates that was fitted to the sensitivity curve ($R^2$ values of the template fits in parentheses). Due to cone interactions, the sensitivity peaks measured were typically narrower than the fitted pigment templates, resulting in relatively low $R^2$ values.

$^b$ Estimated $A_2$ proportion in the retina.

$^c$ The visual pigment template that resulted in the highest $R^2$ value when fitted to the long wavelength limb of the spectral sensitivity curve ($R^2$ value of the template fit in parentheses).

$^d$ The visual pigment template which resulted in the second highest $R^2$ value when fitted to the long wavelength limb of the spectral sensitivity curve ($R^2$ value of the template fit in parentheses). In several cases, the $R^2$ value obtained was negative; indicating that the template fits the data worse than does a horizontal line.

$^e$ The cone pigment subset each individual was assigned to.
cones and two pigments corresponding to double cones. Examining the individuals of each species separately revealed that all individuals in *M. zebra* possessed 4 cone pigments, while 55% of individuals in *M. auratus* and 67% of individuals in *P. taeniolatus* possessed 5 cone pigments (Table 2.1).

The collection of all cone subsets exhibited by either *M. auratus* or *P. taeniolatus* included the complete opsin gene set (Table 2.1). That is, each of these species utilizes the complete set of six cone pigments from this opsin gene set (*Rh2α* and *Rh2β* pooled because of genetic and functional similarity [69, 134]). However, the collection of all subsets exhibited by *M. zebra* encompassed only five cone pigments and excluded the LWS pigment.

**Sex differences in spectral sensitivity**

To evaluate how sex differences contribute to the variation in cone subsets, we calculated the frequency of cone subsets in each species and sex. In all species, individual variation in pigment subsets used was high, and a strong trend for differences between females and males in the identity and frequency of the pigment subsets they utilized emerged (Table 2.3). The degree to which pigment subsets were shared between sexes varied across species. Half of *M. zebra* females shared subset #2 with all conspecific males, 28% of *M. auratus* males shared subset #3 with half the females, and 60% of *P. taeniolatus* females shared subsets #6 and #7 with 71% of males.

The frequency of each cone pigment present in females and males was calculated (Figure 2.2). Sex differences in the frequency of cone pigments were evident in all
Table 2.3 Frequency of cone pigment subsets across species and sex

<table>
<thead>
<tr>
<th>Subset #</th>
<th># Cones</th>
<th>Female (6)</th>
<th>Male (5)</th>
<th>Female (4)</th>
<th>Male (7)</th>
<th>Female (5)</th>
<th>Male (7)</th>
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<td>3</td>
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</tr>
<tr>
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<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>20.0</td>
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<td>14.3</td>
<td>40.0</td>
<td>42.9</td>
</tr>
<tr>
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<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>20.0</td>
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</tbody>
</table>

Frequency of cone pigment subsets in females and males of *M. zebra*, *M. auratus*, and *P. taeniolatus*. Frequency of cone pigment subsets was calculated for each sex and each species based on spectral sensitivity data. The sample size for each of the examined groups is indicated in parenthesis.
Figure 2.2 Frequency of cone pigments across sex

Frequency of cone pigments in females (gray) and males (white) - *M. zebra* (top), *M. auratus* (middle), and *P. taeniolatus* (bottom). Frequency of cone pigments was calculated for each sex and each species based on spectral sensitivity data. For example, 3 out of 4 *M. auratus* females possessed the SWS1 cone pigment in their retina, thus, 75% of *M. auratus* females possessed the SWS1 cone pigment. On the other hand, 3 out of 7 *M. auratus* males possessed the SWS1 cone pigment in their retina, thus, 43% of *M. auratus* males possessed the SWS1 cone pigment. Sex differences in the frequency of cone pigments in *M. zebra* and *M. auratus* are larger than in *P. taeniolatus*.
species, with *M. auratus* and *M. zebra* showing the largest differences. The frequency of *M. auratus* females showing SWS1 pigments was 75% larger than in males, while the frequency of males showing SWS2a and LWS pigments was 71% and 100% larger than in females, respectively. The frequency of *M. zebra* males showing SWS2a cone pigment was 100% larger than in females, while SWS2b cone pigment was found only in females. In contrast, the frequency of cone pigments in *P. taeniolatus* showed less variation across sexes, with pigment frequencies differing in 12-42% between sexes.

We also examined the effect of sex on the pattern of cone opsin gene expression. We focused our analysis on *M. auratus* and *P. taeniolatus*, the species that exhibited the lowest and highest degree of common cone pigment subsets between the sexes, respectively. Variation in opsin gene expression between females and males in *M. auratus* was larger than in *P. taeniolatus* (Figure 2.3). Specifically, in *M. auratus*, the expression of *Rh2b* and *Rh2a* opsin genes differed across sexes (*t*-test, df = 8, \(P<0.0002\) and \(P<0.005\), respectively). In contrast, in *P. taeniolatus*, no significant differences in the relative opsin gene expression were detected between sexes (*t*-test, \(P>0.2\) for all genes). See Table A1.1 for detailed *t*-test results and Table A1.2 for primer specifications for cone opsin genes. The frequency of cone pigments and opsin gene expression are not quantitatively comparable. In the calculation of pigment frequency, each individual is scored as either 1 (pigment present) or 0 (pigment absent), whereas gene expression can assume any value between 0 and 1. Moreover, the expression level of an opsin gene does not necessarily dictate its contribution to sensitivity since patterns of convergence of cones onto retinal interneurons and the network processing of these neurons [15] are
Figure 2.3 Relative cone opsin gene expression across sex

Relative expression of cone opsin genes (mean±SEM) in females (gray) and males (white) - *M. auratus* (left) and *P. taeniolatus* (right). Female and male *M. auratus*, but not *P. taeniolatus*, differed significantly in the expression of the Rh2b and Rh2a opsin genes. See Table A1.1 for detailed *t*-test results and Table A1.2 for primer specifications for cone opsin genes. Opsin gene expression was measured for a subset of the individuals for which spectral sensitivity was measured as well as for several additional individuals.
critical to governing visual responsiveness. Our results show that females and males differ in the cone opsin gene expression profiles. Our results also suggest that females and males differ in cone pigment subsets and in the frequency of cone pigments. However, due to sample size limitation imposed by the complexity of spectral sensitivity measurements, the statistical significance of the sex differences observed in cone pigment subsets and in the frequency of cone pigments cannot be evaluated at this time. It is important to note, however, that the variability of all three properties of the visual system of cichlids was consistently larger in *M. auratus* than in the other two species.

**Species differences in spectral sensitivity**

In general, different cichlid species used different cone subsets (Table 2.3). Closer examination reveals that females of the three species did not use the same cone subsets, but 43% of male *M. auratus* shared three cone subsets with *P. taeniolatus* males.

The frequency of cone pigments varied across species (Figure 2.4A). The frequency of Rh2a (s.d. = 14%) and Rh2b (s.d. = 5%) showed the smallest variation and did not differ significantly between species (Fisher exact test, \( P = 0.091 \) and \( P = 1 \), respectively). In contrast, the frequency of LWS (s.d. = 53%) and SWS1 (s.d. = 38%) showed the largest variation and differed significantly between species (Fisher exact test, \( P < 0.001 \) for both pigments). See Figure 2.4 caption for detailed statistics. The frequency of SWS1 was highest in *M. zebra* (100%), lower in *M. auratus* (54%), and lowest in *P. taeniolatus* (25%). The frequency of LWS in *P. taeniolatus* (100%) and *M. auratus* (81%) was higher than in *M. zebra* (0%).
Figure 2.4 Frequency of cone pigments and spectral reflectance of the colour pattern of cichlids

(A) Frequency of cone pigments in *M. zebra*, *M. auratus*, and *P. taeniolatus*. Visual pigments: SWS1 (black), SWS2b (violet), SWS2a (blue), Rh2b (green), Rh2a (orange), and LWS (red). Cone pigment frequency varied across species, with LWS and SWS1 showing the largest frequency variation between species. The frequency of the SWS1, SWS2a, and LWS differed significantly across species (*P* = 0.0005, *P* = 0.002, and *P* = 0.00001, respectively). The frequency of the SWS2b, Rh2a, and Rh2b did not differ significantly across species (*P* = 0.05, *P* = 0.09, and *P* = 1, respectively). Error rate was set to *α* = 0.0083 following Bonferroni correction for 6 hypothesis tests.

(B) Average normalized spectral reflectance (*n* = 10; black lines) of the color pattern of *M. zebra* (solid line), *M. auratus* (dashed line), and *P. taeniolatus* (dotted line). The normalized absorbance of LWS visual pigment (red line) and the normalized sidewelling irradiance measured at 5 m depth in Lake Malawi (blue thin line) are also depicted.
The frequency of the SWS1 cone pigment across species qualitatively correlated with planktivory. *M. zebra* may be highly planktivorous, feeding on both phytoplankton (mainly diatoms) and zooplankton (Cladocerans and copepods) [177]. *M. auratus* is omnivorous, consuming algae and a wide range of non-algal dietary items such as cyclopoid copepods [177, 178]. *P. taeniolatus* is almost strictly herbivorous and feeds on the biocover detached from rocks, mainly comprising of algae and diatoms [73, 179].

The frequency of the LWS cone pigment across species qualitatively correlated with the proportion of long wavelength reflectance in the colour pattern of conspecific males (Figure 2.4B). As a first order approximation, the quantum catch of a LWS cone pigment was calculated for the spectral reflectance of the species used in this study. The normalized quantum catch was highest in *P. taeniolatus* (100%), lower in *M. auratus* (80%), and lowest in *M. zebra* (17%).

**Colour vision in Lake Malawi cichlids**

Spectral sensitivity curves determined using different colour background conditions were used to evaluate mechanisms of colour vision. Colour vision requires the possession of at least two differentially sensitive cones that interact through opponent and non-opponent processes to enable wavelength discrimination [4]. Our results show that 97% of individuals possessed 4-5 cone classes. To study cone interactions, we used a multiple-cone mechanism (MCM) model, employing strict criteria (see Methods), to reconstruct spectral sensitivity under different background conditions. To do this we selected three individual fish that exhibited the most frequent cone subset for a given
species. These subsets were: #2, #3, and #7 occurring in 73%, 36%, and 42% of the *M. zebra*, *M. auratus*, and *P. taeniolatus* individuals, respectively.

Spectral sensitivity was successfully reconstructed using the MCM model ($R^2 = 0.83-0.99$ for all species and background conditions) (Figure 2.5). Cone interaction was expressed as an additive (non-opponent) or subtractive (opponent) contribution to the modeled spectral sensitivity and we summarize these cone interactions in Table 2.4 (see Table A1.3 for more comprehensive information that details the specific coefficients and goodness of fit of the model). Typically, the cone class exhibiting the highest sensitivity for a spectral range contributed positively to the modeled spectral sensitivity, whereas other cone classes contributed either positively (non-opponent) or negatively (opponent) to the modeled spectral sensitivity. The successful reconstruction of spectral sensitivity curves further validated our analysis, and the distribution of the cone pigment subsets. We identified and characterized cone interactions representative of retinal neural network processing, essential to colour vision.

**Discussion**

*Pentachromatic colour vision in Lake Malawi cichlids*

Ninety seven percent of fish examined in this study employed cone subsets of 4-5 pigments, with most *M. auratus* and *P. taeniolatus* exhibiting five functional cone classes. The spectral sensitivity of all three species of cichlids was shaped by both opponent and non-opponent cone interactions and thus, demonstrated the potential for tetra- and pentachromatic colour vision in these species. Although the
Figure 2.5 Spectral sensitivity of cichlids reconstructed using the multiple-cone mechanism model

Measured (circles) and modeled (lines) relative spectral sensitivity for subsets #2 (M. zebra; left), #3 (M. auratus; middle), and #7 (P. taeniolatus; right). The low sensitivity at 340-380 nm in subset #7 corresponds to $\beta$-band absorbance of the Rh2b, Rh2a, and LWS pigments and was not modeled. Relative spectral sensitivity of each species was measured under three background conditions: Long wavelength isolation (LW, red), Control (green), and Short wavelength isolation (SW, blue). In P. taeniolatus, LW was replaced by Dim short wavelength isolation (Dim-SW, cyan).
<table>
<thead>
<tr>
<th>Spectral range (nm)</th>
<th>Single cones</th>
<th></th>
<th></th>
<th></th>
<th>Double cones</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>$K_{SWS1}$</td>
<td>$K_{SWS2b}$</td>
<td>$K_{SWS2a}$</td>
<td>$K_{rhh2b}$</td>
<td>$K_{rhh2a}$</td>
<td>$K_{LWS}$</td>
<td>$K_{SWS1}$</td>
<td>$K_{LWS}$</td>
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<td></td>
<td>$K_{SWS1}$</td>
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</tr>
<tr>
<td></td>
<td>400-480</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>480-540</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td></td>
<td>540-620</td>
<td>-</td>
<td>+</td>
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<td>540-640</td>
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<td>420-460</td>
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<td>460-540</td>
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<tr>
<td></td>
<td>540-580</td>
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</table>

$K_{SWS1} - K_{LWS}$ represent the weights of the relative contribution of each cone mechanism to spectral sensitivity used in the multiple-cone mechanism model. Weights can be positive (non-opponent) or negative (opponent). The spectral ranges listed in this table represent discernable peaks (or cone mechanisms) in the spectral sensitivity curves and each peak was fitted by the multiple cone mechanism model.
presence of five (or more) cone classes was previously reported in microspectrophotometry studies [69, 88], this is the first demonstration that five cone mechanisms were used by a single individual. In this regard, designating the dimensionality of colour vision requires the essential evidence that shows not only that the different cone mechanisms participate in spectral sensitivity but also that the different cone mechanisms show multiple neuronal interactions. *P. taeniolatus* and *M. auratus* showed five distinct cone classes spectrally compressed in a range of 146 nm (subset #7) and 187 nm (subset #3), respectively. The spectral compression of this sensitivity provides the basis for extraordinary spectral discrimination, which could be important in visual communication. To better understand how pentachromatic colour vision would be beneficial to cichlids we need to consider factors at play in the visual environment of cichlids where visual communication takes place - (i) the high species diversity of cichlids in Lake Malawi (700-800 species) [152, 178]; (ii) the large within-genera variation in the colour pattern of fish and the small within-genera variation in their size and shape [164, 166, 180], and (iii) the prevalence of visual cues driving mate-choice [158, 181] and intrasexual competition [182]. Visual communication would depend on exquisite sensory performance in this visual environment since cichlids are continually challenged to make fine scale discriminations of complex colour patterns. This visual system capability would be particularly important in guiding visual behaviour related to mate-choice, where the detection and recognition of conspecific optical signals is critical. These visual adaptations could be fundamental to mate-choice fidelity and contribute to the maintenance of biodiversity in the cichlid communities of Lake Malawi.
It is important to point out that our findings differ from the notion that Lake Malawi cichlids primarily exhibit three cone pigments [69, 97]. Recent reports show a significant variation in the number of opsin genes expressed among Malawi species [77], with several species (one of which is \textit{P. taeniolatus}) expressing more than three cone opsin genes [73]. Furthermore, microspectrophotometry studies have revealed classes of cones in low abundance in the retina of various species that are of unknown significance [69, 97]. Our study suggests that these rare cone classes contribute to the spectral sensitivity of Lake Malawi cichlids.

\textit{Sex differences in the colour vision of Lake Malawi cichlids}

Our study provides some evidence for sex differences in opsin gene expression and cone pigment frequency for cichlids. While the sample size in electrophysiology studies tends to be low, a possible pattern of differentiation between males and females emerged. The large diversity of cone pigment subsets found in each species was in part qualitatively related to sex differences. This, together with qualitative sex differences in pigment frequency and significant differences in cone opsin gene expression suggests the possibility for sex differences in the visual system of some Lake Malawi cichlids. Our results suggest that the most prominent sex differences occurred in \textit{M. auratus}. We argue here that males and females may have quite different visual requirements in visual communication scenarios [183, 184]. \textit{M. auratus} differs from the two other species in two aspects: (i) \textit{M. zebra} and \textit{P. taeniolatus} males hold a permanent territory while females choose among potential mates [156]. \textit{M. auratus} males, on the other hand, assume a territory only temporarily around breeding events [178]; (ii) \textit{M. zebra} and \textit{P. taeniolatus}
males are conspicuous while the colour pattern of females is dull, whereas both females and males *M. auratus* are conspicuous and exhibit colourful, but very different, body patterns [178].

We propose a possible explanation for the observed distribution of sex differences across species. Considering the significant role of visual cues in female mate-choice [158, 181] and male-male competition for territory [182], both females and territorial males of *M. zebra* and *P. taeniolatus* would likely benefit from possessing colour vision tuned to provide the best recognition ability of conspecific males. In contrast, *M. auratus* males assume a territory only temporarily, and thus are less dependent on recognition of conspecific males to increase their mating success. Additionally, not holding a permanent territory potentially allows *M. auratus* males to choose between females. Indeed, in the cichlid *Astatotilapia flavijosephi*, males defend territories only during the breeding season and actively choose between females [185]. Furthermore, the investment of *M. auratus* females in their colour pattern adds support to the hypothesis that *M. auratus* males choose between females. Thus, the distribution of sex differences in the visual system across species might be associated with territorial behaviour. However, the adaptive role of sexual dimorphism in colour vision of Lake Malawi cichlids requires further examination.

*Adaptive significance of species differences in the colour vision of Lake Malawi cichlids*

The variation in cone pigment frequency across species reflects visual adaptations that both enhance the contrast of transparent prey and detect signals important for mate-
choice, suggesting that both natural selection and sexual selection played a role in shaping the spectral sensitivity of cichlids.

The frequency of the SWS1 cone pigment across species qualitatively correlated with planktivory. UV photoreception mediated by the SWS1 cone pigment enhances the contrast of transparent zooplankton against the water background, and thus, aids in their detection [52, 116]. However, UV sensitivity does not confer any advantage over longer wavelength sensitivity in the detection of opaque food items such as loose algae. Thus, UV sensitivity is expected to be highly advantageous for the planktivorous *M. zebra*, less advantageous for the omnivorous *M. auratus*, and least advantageous for the herbivorous *P. taeniolatus*, which is in agreement with our findings. Thus, the presence of the SWS1 pigment in the retina of cichlids has likely a significant adaptive value. In this regard, the expression of the *SWS1* opsin gene was recently reported to be highest among species foraging on zooplankton, phytoplankton, and algae, and lowest among species foraging on fish or benthic invertebrates [73]; however, the relationship between opsin gene expression and spectral sensitivity in cichlids is still largely unknown. UV photoreception is also tightly linked to polarization sensitivity and this may provide yet greater contrast enhancement of transparent prey [186-188]. Our lab is currently examining the polarization sensitivity in cichlid fishes and its role in visual behaviour.

The frequency of the LWS cone pigment across species was quantitatively correlated with the proportion of long wavelength reflectance in the colour pattern of conspecific males. While the possession of the LWS cone pigment by *M. auratus* and *P. taeniolatus* may facilitate the detection of long wavelength signals of conspecifics, this
would not be the case for *M.* zebra that do not have prominent long wavelength signals, which is in agreement with our findings. Therefore the presence of the LWS pigment in *M. auratus* and *P. taeniolatus* has a significant adaptive value. Interestingly, the detection of UV reflections from the colour pattern of these fish species may be mediated through the β-band of the LWS cone pigment (Figure 2.4B), thus potentially eliminating the need for the possession of the SWS1 pigment in order to detect UV signals from conspecifics.

Both natural and sexual selection work sequentially to contribute to the divergence of Lake Malawi rock-dwelling cichlids [174]. In the first episode of cladogenesis, competition for trophic resources resulted in the differentiation of trophic morphology and the diversification of the visual system to allow the utilization of different foraging styles. The correlation of the frequency of the SWS1 cone pigment across species to planktivory (this study), and the recent report that the expression of the *SWS1* opsin gene correlates with planktivory [73], support the idea of diversification of the visual system based on competition for trophic resources. In the second episode of cladogenesis, sexual selection contributed to the differentiation of male nuptial colouration and the accompanying diversification of the visual system to allow high recognition ability of conspecifics. The correlation of the frequency of the LWS cone pigment across species to the proportion of long wavelength reflectance from male colour patterns supports the idea of visual system diversification based on the diverse male nuptial colouration.
The retention of cone opsin genes in Lake Malawi cichlids

None of the individuals examined possessed all available six cone pigments (Based on analysis of spectral sensitivity; Rh2α and Rh2β pooled). However, the large number of pigments utilized and the high diversity in pigment subsets within a species, adds support for the retention of the complete set of cone opsin genes in Malawi cichlids.

Several factors may contribute to the observed pigment diversity within a species. First, a significant genetic component of opsin expression was reported [124] where individuals with different genetic history may utilize different pigment subsets. Second, thyroid hormones (TH) are important in vertebrate development and can modulate the opsin gene expression and induce cone loss in fish [114, 125]. The level of TH in fish has been associated with environmental stress and subordinance via the effect of cortisol [126, 127]. Thus, individuals differing in their stress level or social status may utilize diverse pigment subsets through TH level modulation. Thirdly, social visual cues in fish modulate the activity of GnRH neurons [189]. Since GnRH has been shown to affect retinal neurons [189] it is possible that it may be responsible for changes in opsin gene expression and spectral sensitivity in fish.

Conclusions

The cichlid model system illustrates that the visual system of fish may differ across individuals, sexes and species. The large number of available cone opsin genes facilitates this variation in the spectral sensitivity of fish. We show that even rare cone populations and opsin gene expression at low levels contribute to the spectral sensitivity of fish. Our results suggest that the diversification of colour vision across species
contributes to sensory adaptations that both enhance the contrast of transparent prey and
the detection of optical signals of conspecifics. Therefore, both natural and sexual
selection may work in concert to shape spectral sensitivity in fish. Taken together, our
findings have important implications for understanding the variable nature of fish colour
vision and the selective forces shaping detection and recognition capabilities.

Methods

Animals and holding conditions

Three species of Lake Malawi cichlids were used in this study: Metriaclima zebra
(blue top manda), Melanochromis auratus, and Protomelas taeniolatus (Old World
Exotics, Florida). M. zebra and M. auratus belong to a rock-dwelling evolutionary
lineage - the mbuna, whereas P. taeniolatus is a member of the non-mbuna lineage.
These species are sexually dimorphic and occur sympatrically in the rocky habitat in
Lake Malawi. The males have distinctive nuptial colour patterns and they use the rocky
habitat differently with respect to reproductive behaviour [178]. Adult fish were held in
our aquatic facility tanks under 12h:12h light:dark photoperiod at 25±1°C. Facility
lighting featured enhanced full spectrum fluorescent lamps (UV-Blue actinic and
BlueMax lamps; Full Spectrum Solutions). All experimental and animal care procedures
were approved by Queen’s University Animal Care Committee, under the auspices of the
Canadian Council for Animal Care.
Electroretinogram (ERG) experimental apparatus

The general design of the optical system and recording apparatus has been described previously [95, 168]. Two background channels each with a 250W quartz-halogen lamp (Osram) were used to provide constant background, to which test fish were light adapted. Long- and short-pass interference filters (Fused Silica, optical density OD = 2, Edmund optics), bandpass interference filters (Edmund optics), broadband colour filters (Schott) and reflective neutral density filters (Edmund optics), were used to manipulate the spectral irradiance of each background channel. Light from the two background channels was guided to the electrophysiology rig using a bifurcated optical fiber (fused silica, numerical aperture NA = 0.22; Fiberoptic Systems).

The stimulus channel used a 300W xenon arc lamp system (Thermo Oriel). The optical path consisted of a monochromator (Instruments SA), Inconel quartz neutral density wedge (OD = 0-4.0; Melles-Griot), shutter (Uniblitz), 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 produce a stimulus beam (dia. 0.5 cm at the plane of the fish eye) contained within the background beam (dia. 1 cm). This setup ensured that the chromatically adapted portion of the fish retina is also the one stimulated.

Preparation of fish

Prior to ERG recordings, fish were immersed in a solution of 150 mg l⁻¹ tricaine methanesulfonate (MS-222) until the fish reached stage III anesthesia [190]. Intramuscular injections of a general anaesthetic, Maranil (0.1 mg g⁻¹ body mass) and an...
immobilizing agent, pancuronium bromide (0.04 mg g^{-1} body mass) were administered at several sites. Test fish were then placed in a holding cradle in a Faraday cage. Experimental fish were irrigated with aerated fresh water (20°C, flow rate ~3 ml s^{-1}) and their body was covered with a moist cloth.

**ERG recording procedure**

ERG recordings started at least 1 hour following the onset of the light phase and concluded before the onset of the dark phase to avoid any effects related to circadian rhythm [168, 191, 192]. A glass electrode (1.5 mm outer diameter; 1 mm inner diameter, borosilicate glass, World Precision Instruments - WPI) that was pulled to exhibit a tip diameter of 80-125 μm (P-97 Flaming/Brown Micropipette puller; Sutter Instruments) was loaded with saline (0.684 M sodium chloride) and inserted into a saline filled chlorided AgCl half-cell (A-M systems Inc.). The electrode tip was positioned using a micromanipulator on the dorsal-nasal surface of the right eye. A ground electrode was attached to the caudal fin and a chlorided-silver reference electrode was placed on the head of the test fish. Fish were chromatically adapted for 1 hour prior to experiments. The stimulus duration was 500 ms with an inter-stimulus interval of 5 s. An isolated bio-amplifier (ISO-80, WPI) amplified the ERG signal and filtered the signal using bandpass filter settings (5 Hz low-pass; 100 Hz high-pass). The amplified signal was analyzed with a 16-bit A/D data acquisition system (Micro 1401; CED). A custom designed software analysis module determined the *b-wave* amplitude that corresponds to the response of ON bipolar cells [193, 194]. Spectral sensitivity was measured in 20 nm increments, from 320
to 700 nm, using a staggered wavelength presentation to prevent adaptation to a specific spectral region.

**Analysis of electroretinograms**

A response versus intensity (RI) curve was generated for each wavelength examined. To determine the sensitivity at a given wavelength, the empirical Naka-Rushton function was least squares fitted to the RI curve with a slope parameter of 1 [195, 196]. The log irradiance level corresponding to half response (LogK) was determined, from which the sensitivity was calculated by taking the reciprocal of this value. Typically, the upper asymptote of the Naka-Rushton function could be reached for all wavelengths. There were a few exceptions at wavelengths shorter than 360 nm and longer than 600 nm. At these wavelengths, the value of the maximum response parameter (R_max) was set to that of the neighbouring examined wavelength prior to fitting. A log relative sensitivity curve was constructed by normalizing the log absolute sensitivity values to the maximum sensitivity. This procedure was repeated for each individual under each of the three background conditions.

**Background isolation conditions**

Four background conditions were designed and used for isolating cones dominating certain spectral regions. A long-wavelengths-isolation condition (LW) was used for isolating cone mechanisms most sensitive to long-wavelengths; dim and intense short-wavelengths-isolation conditions (Dim-SW and SW) aimed at isolating cone mechanisms most sensitive to short-wavelengths; and a relatively spectrally flat
background condition was used as a control. To design and carefully control the level of light adaptation of the respective cone mechanisms, a quantum catch model was used:

\[
Q_i = \int_{300}^{800} A_i(\lambda)E(\lambda)d(\lambda)
\]  

(1)

where \(Q_i\) (photons cm\(^{-2}\) s\(^{-1}\)) denotes the quantum catch of cone mechanism \(i\) \((i = 1, 2, \ldots, 6)\), \(A_i(\lambda)\) represents the visual pigment absorbance coefficient of cone mechanism \(i\) at a wavelength \(\lambda\) (nm), and \(E(\lambda)\) denotes the photon irradiance of the background light field at a wavelength \(\lambda\).

**Characterization of background conditions**

The irradiance provided under the various background conditions was characterized by measuring the spectral irradiance of the background beam. Spectral irradiance was measured using a spectroradiometer (QE65000, Ocean Optics) connected to a 2 m optical fiber (QP600-2-UV/VIS, Ocean Optics) that was fitted with a cosine corrector (CC-3-UV, Ocean Optics). The spectroradiometer utilized a 1024x58-element square silicon CCD array and was configured with a 25 \(\mu m\) slit and a variable blaze wavelength grating (HC-1, groove density = 300 mm\(^{-1}\), Ocean Optics) resulting in an effective spectral resolution of 1.9 nm (FWHM) between 200 and 950 nm. The fiber end was held approx. 5 cm away from the emergence plane of the background beam, ensuring sampling of the entire beam diameter. The spectroradiometer setup was calibrated for absolute irradiance using a NIST calibrated Halogen–Deuterium dual light source (200-
1000 nm, DH-2000-CAL, Ocean Optics). In cases where spectral variation exceeded the
dynamic range of the spectroradiometer and thus, a reliable measurement could not be
obtained, the irradiance delivered under the background condition was calculated by
multiplying the measured output of each light source by the spectral transmission of the
filters used in producing the background condition.

Analysis of spectral sensitivity curves

Identification of functional cones

Two criteria were devised for the identification of cone mechanisms. A sensitivity
peak was considered to be a cone mechanism if it satisfied the following conditions: (i)
the sensitivity peak appeared under at least two background isolation conditions, and (ii)
the sensitivity peak exhibited changes that were directly related to changes in the spectral
composition of the background conditions. For instance, a sensitivity peak in the
ultraviolet range (UV; 340-400 nm) was identified as a UV cone when sensitivity
increased under a short wavelength isolation background, or as the $\beta$-band of mid and
long wavelength cones when sensitivity decreased under a short wavelength isolation
background.

Correspondence between visual pigments and cone mechanisms

To relate visual pigments with cone mechanisms, absorbance templates of visual
pigments were fitted to spectral sensitivity curves using a least-squares fit. This technique
was previously used in numerous studies and repeatedly has been shown to allow for the
identification of the cone classes in the retina of animals [197-199]. In this regard,

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interactions between different cone classes produce sensitivity peaks that are narrower than the absorbance templates of visual pigments, resulting in a somewhat reduced goodness of fit [197, 198]. Visual pigment absorbance templates [176] were constructed for the opsin genes previously reported in cichlids: note the $\lambda_{\text{max}}$ of A1-reconstituted visual pigment is provided in parentheses SWS1 (368 nm), SWS2b (423 nm), SWS2a (456 nm), Rh2b (484 nm), Rh2aα (519 nm), Rh2aβ (528 nm), and LWS (560 nm) [69, 134]. In the case of Rh2aα and Rh2aβ, the spectral overlap of the two visual pigment curves necessitated calculating an average $\lambda_{\text{max}}$ of 523 nm. The 523 nm $\lambda_{\text{max}}$ absorbance spectrum was used for subsequent analysis and is hereafter referred to as Rh2a. To generate visual pigment templates we combined absorbance spectra for the A1 and A2 chromophores (A1 – equations 1,2,4,5a,5b; A2 - equations 1,4,6a,6b,8a,8b) [176]. The proportion of the A2 state was presented using a fraction parameter, $a$ (ranging 0-1) and therefore, the absorbance spectra of a given cone type exhibiting an A2 proportion of $a$ was calculated as: $A = A_1 \cdot (1-a) + A_2 \cdot a$. The $\lambda_{\text{max}}$ of each cone type exhibits a defined wavelength shift as the A2 proportion changes [105]. This shift was also taken into account when generating the visual pigment templates for varying A2 proportions.

Typically, the long-wavelength peak in spectral sensitivity was located between 560 and 600 nm, and thus it could correspond to either the Rh2a (523-560 nm; $\lambda_{\text{max}} A_1 - \lambda_{\text{max}} A_2$) or the LWS (560-626 nm; $\lambda_{\text{max}} A_1 - \lambda_{\text{max}} A_2$) visual pigments. Therefore, to correlate the long-wavelength peak with a visual pigment absorbance curve, it was necessary to determine the A2 proportion in the retina of each individual. Visual pigment absorbance templates for the Rh2a and LWS pigments were fitted to the long-wavelength
limb of the spectral sensitivity curve. The least-squares fit was performed while leaving the A2 proportion and a magnitude coefficient unrestricted, allowing the software to find a visual pigment template with an A2 proportion that best describes the long wavelength limb. Estimating the A2 proportion based on the long wavelength limb is the best possible approach since: (i) the $\lambda_{\text{max}}$ shift between the A1 and A2 states is largest for long wavelength-sensitive cones; and (ii) the spectral sensitivity is based on one cone class and thus is not affected by opponent interactions. For each individual, assuming that the A2 proportion is similar for all visual pigments, the A2 proportion estimate was used for fitting all visual pigment templates to the spectral sensitivity curve. Specifically, for each of the sensitivity peaks identified, the absorbance template (out of the six possible absorbance templates) that corresponded best to the $\lambda_{\text{max}}$ of the sensitivity peak was manually chosen to be fitted to the sensitivity peak; the goodness of fit ($R^2$) was determined for each pair of sensitivity peak and absorbance template. This procedure was repeated for all sensitivity peaks included in each of the sensitivity curves. All absorbance templates were corrected for the spectral transmission of the fish lens ($P. taeniolatus$ [44]; $M. zebra$ and $M. auratus$ – measured [167]).

**Modeling cone interactions**

A multiple-cone mechanism (MCM) model [198] was used to determine the relative contribution of each cone mechanism to each spectral sensitivity curve by assigning weights, which can be positive (excitatory) or negative (inhibitory) for each cone mechanism. This "upper envelope" model, that was previously used in primates [200, 201] and fish [198], assumes that the spectral sensitivity of the eye is determined by
the cones that are most sensitive over the spectral region of concern. This linear model takes the general form: \( S_{\lambda_1,\lambda_2}(\lambda) = \sum k_i \cdot A_i(\lambda) \), where \( S_{\lambda_1,\lambda_2}(\lambda) \) denotes the modeled spectral sensitivity in the spectral range enclosed by \( \lambda_1 \) and \( \lambda_2 \) while accounting for cone interactions; \( A_i(\lambda) \) represents the absorbance of the visual pigment template, corresponding to cone \( i \) at a wavelength \( \lambda \) (nm); \( k_i \) denotes the weight representing the contribution of cone \( i \). The MCM model was least-squares fitted to different portions of the spectral sensitivity curves separately, where the spectral range between two sensitivity notches was set as the \( \lambda_1 - \lambda_2 \) range [198, 200, 201]. The cone mechanisms that were determined for each individual in the previous analysis step (Correspondence between visual pigments and cone mechanisms) were the only cone mechanisms that were assumed to interact, and thus, were included in the MCM model. Only two or three cone mechanisms were chosen manually from the list of cone mechanisms of each individual for fitting over each spectral range. Therefore, for each spectral range, the MCM model essentially estimated the weights of two to three predetermined cone mechanisms that would allow for the best fit to the spectral sensitivity curve. That is, the number of free parameters in the model was two or three, while the number of data points used to fit the model was always larger than three. For modeling spectral sensitivity, we selected the cone interaction that: (i) included the minimum possible number of cones; (ii) allowed for the best fit under all background conditions; and (iii) ensured the same type of cone interaction (either opponent or non-opponent) under all background conditions. The weights assigned to each cone, in addition to the \( R^2 \) values (total amount of variance accounted for by the model across the spectrum) were determined. Log
relative sensitivity was transformed to percent relative sensitivity and normalized between zero and one prior to fitting [202].

**Limitation of analysis procedures**

Several stages of our analysis of spectral sensitivity functions may introduce errors into the estimates of sensitivity peaks, cone pigments, and cone interactions. First, to determine the sensitivity at a given wavelength, we fitted the Naka-Rushton function to RI curves. In a few instances, the upper asymptote of the Naka-Rushton function could not be reached for UV and long wavelengths, and therefore, the value of the maximum response parameter (R_{max}) was set to that of the neighbouring examined wavelength prior to fitting. This procedure may introduce some error into our estimates of the sensitivity at UV and long wavelengths. Second, fitting absorbance templates of visual pigments to spectral sensitivity functions is a standard procedure that has been used to relate between sensitivity peaks and cone pigments in numerous studies [197-199]. Nonetheless, such a procedure typically involves fitting over a restricted spectral range, typically corresponding to only 4-5 data points. Additionally, interactions between different cone classes produce sensitivity peaks that are narrower than the absorbance templates of visual pigments, resulting in a reduced goodness of fit. Thus, the small number of data points used for fitting as well as the contribution of cone interactions to the spectral sensitivity function may compromise the reliability of our estimates of cone pigments. Third, fitting the multiple cone mechanisms model to characterize the interactions between different spectral classes of cones was also performed over a restricted spectral range, i.e., the spectral range enclosed between two sensitivity notches. Thus, the small
number of data points used for fitting in this analysis stage may compromise the reliability of our cone interaction estimates. Taken together, the potential errors associated with each of the analysis steps may have accumulated and propagated throughout the analysis. Consequently, at this time, we cannot eliminate the possibility that some of the variation observed in cone pigments across species, sexes, and individuals arose from accumulation and propagation of these errors.

**Preparation of retinal samples**

Upon completion of ERG recordings fish were dark adapted for 1 h and then killed by cervical transection. Under deep red illumination (>650 nm), both eyes were enucleated and hemisected along an anterior–posterior axis. The neural retina was then dissected free of pigmented epithelium. Each isolated retina was preserved in 0.5 ml RNAlater (Ambion), stored at 4°C for the first 24 hours, and then kept at -80°C until further processing. The sex of the fish was determined using the aceto-carmine technique [203, 204]. Fish were dissected and their gonads were removed, stained with aceto-carmine and viewed under a dissecting microscope to determine the fish sex. All fish were sexually mature adults showing well developed eggs or sperm and were at least 9 months old. Cichlids typically reach sexual maturity at 6 months from release [205, 206].

**Relative gene expression by qPCR**

Quantitative real time polymerase chain reaction (qRT-PCR) was used to quantify the relative levels of mRNA expression corresponding to the various cone opsin genes. Unless specified differently, all procedures described below were performed following
the manufacturer’s protocols. Total RNA was extracted from retinas (Absolutely RNA Miniprep Kit, Stratagene) and its amount and quality were determined by reading the absorbance at 260 nm and calculating the absolute 260/280 absorbance ratio using a benchtop spectrophotometer (Cary 300 Bio, Varian). Total cDNA was synthesized using 250 ng total retinal RNA and a Superscript III first-strand synthesis SuperMix (Invitrogen) at 50°C for 30 minutes. See Table A1.2 for primer sequences for the amplification of the cDNA opsin genes in cichlids (Rh2αa and Rh2αβ combined). All primers were analyzed with Primer-Blast primer design tool (NCBI) for product size, melting temperature, GC content (%), and sequence specificity. The specificity of all primer pairs was tested by amplifying target sequences present within the cDNA synthesized from retinal total RNA. Amplification was carried out in a Mastercycler® gradient 5331 (Eppendorf) using the following concentrations: 2.5 mM MgCl₂, 200 μM dNTPs, 100 μM of Forward and Reverse primers, 0.05 Units GotTaq® Flexi DNA polymerase (Promega), 1 x PCR buffer, and 1 μl of cDNA template, in a final volume of 25 μl. The PCR cycling profile consisted of an initial hold 92°C 2 min, 40 cycles (92°C 25 s, 55°C 25 s, 72°C 25s), and a final extension at 72°C 5 min. To verify that the amplified product obtained from each primer pair consisted of a single band and was of the correct size, amplified DNA products were separated in a 2.0% agarose gel 1 x TBE buffer, visualized by GelRed on a gel imager (AlphaImager), and were compared with a Bench top 100bp DNA ladder (Promega).

Quantitative PCR analysis of individual retinal cDNA samples was carried out for each opsin gene using Brilliant SYBR green qPCR Master Mix (Stratagene) in a real-
time quantitative system MX3000P™ (Stratagene). Each 25 μl reaction contained 1x Brilliant SYBR Green Master Mix, 200 mM of both forward and reverse primers, 0.05 μl of ROX passive reference dye, and 1 μl of four times diluted cDNA. The reaction mix was placed in 96-well non-skirted polypropylene PCR plate, and capped with optical strip caps (Stratagene). The plate was briefly centrifuged and eye inspected for the presence of bubbles. The thermocycle program was 95°C for 10 min, followed by 40 cycles of 95°C for 25 s, 55°C for 25 s, and 72°C for 25 s. Controls included a reaction lacking cDNA template (NTC) and a non-transcribed reaction for genomic DNA contamination (No-RT). All samples were run in triplicate, and fractional cycle values (Cq) were averaged. Amplification efficiencies per sample per target were calculated from the slope of the amplification curve in the exponential phase using the LinRegPCR 11.0 Software [207]. Relative gene expression was determined for the six opsin genes as a fraction of the total cone opsin genes expressed for an individual [134]:

\[
\frac{T_{g,i}}{T_{all,i}} = \frac{(1 + E_{g,i})^{-C_{q_{g,i}}}}{\sum (1 + E_{g,i})^{-C_{q_{g,i}}}}
\]

(2)

where \(T_{g,i}/T_{all,i}\) is the relative gene expression ratio for gene \(g\) normalized by the total cone opsin genes expressed for individual \(i\), \(E_{g,i}\) is the amplification efficiency for gene \(g\) and individual \(i\), and \(C_{q_{g,i}}\) is the fractional cycle value for gene \(g\) and individual \(i\). Finally, relative gene expression was averaged for each species and sex.
Underwater irradiance measurements

Underwater spectral irradiance at a horizontal line of sight was measured in July 2008 at a near shore site at Cape Maclear on the northwestern part of the Nankumba Peninsula, Lake Malawi (14° 1' 0" S 34° 51' 0" E; local time: 11:00 – 11:30, average solar zenith angle within the water = 25.6°). The sampling site, Otter point, is exposed to wind and wave-action and exhibits a rocky bottom that subsides with depth and becomes a sandy bottom at ~15 m of depth [178]. Irradiance was measured using a thermoelectrically cooled spectroradiometer (QE65000, Ocean Optics) connected to a 30 m optical fiber (ZPK600-30-UV/VIS, Ocean Optics) that was fitted with a cosine corrector (CC-3-UV, Ocean Optics). See specification of the spectroradiometer in the Characterization of background conditions section. Holding the irradiance probe, a SCUBA diver attained position at a depth of 5 m, and readings were saved on a computer placed on a boat (positioned as far as possible and never between the diver and the sun to prevent shading). The spectroradiometer setup was calibrated for absolute irradiance following the same procedure described in the Characterization of background conditions section.

Spectral reflectance measurements

Spectral reflectance of the colour pattern of fish was measured using a spectroradiometer (USB2000, Ocean Optics) connected to one arm of a 2 m bifurcated optical fiber (BIF600-2-UV/VIS, Ocean Optics). The other arm of the fiber was connected to a light source (DH-2000-BAL, Ocean Optics). The spectroradiometer utilized a 2048-element linear silicon CCD array and was configured with a 50 μm slit.
and a grating (groove density = 600 mm\(^{-1}\); blaze wavelength = 400 nm; grating #2, Ocean Optics) resulting in an effective spectral resolution of 2.06 nm (FWHM) between 200 and 950 nm. The light source integrated two lamps, Tungsten-Halogen and Deuterium, providing a high and spectrally balanced output between 200 and 1000 nm. The common end of the bifurcated fiber was fitted with a flat black reflectance probe exhibiting a 3 mm diameter tip cut at an angle of 45°. Prior to each measurement session, the light source was allowed to warm up for at least 40 minutes. Then, a measurement of a Spectralon diffuse reflectance standard (WS-1-SL, Ocean Optics) was taken as 100% reflectance, and a dark measurement was taken as zero reflectance. Fish were immersed in 500 ml lake water containing 2 ml of 1:10 clove oil:ethanol solution immediately after capture until the fish reached stage III anesthesia [190]. Fish were held submerged in lake water and spectral reflectance was measured at 10 points along the trunk of the fish (one individual per species). Care was given to sealing the reflectance probe against the fish skin to reduce stray light. Readings were acquired and saved on a laptop computer. Reflectance measurements of each species were averaged in order to estimate the proportion of long wavelength reflectance. However, this approach can serve only as a first order approximation for the proportion of long wavelength reflectance because the area of colour patches, represented by the various reflectance measurements, may differ.

To calculate the quantum catch of the LWS cone pigment we used the spectral radiance reflected from the colour pattern of the species studied (eq. 1). In this case, however, \(E(\lambda)\) was substituted by the product of the sidestwelling irradiance at depth of 5 m, \(E_h(\lambda)\) times the spectral reflectance of the colour pattern of the fish, \(R(\lambda)\). Absorbance
template for the LWS cone pigment was generated while assuming A$_2$ proportion of 0.5 [176].

**Statistical analysis**

To study the effect of sex on the relative opsin gene expression, we performed multiple $t$-tests where an experiment-wise error rate of 5% was corrected to 0.83% ($\alpha = 0.05/6 = 0.0083$) following Bonferroni correction for 6 hypothesis tests [208] that correspond to 6 opsin genes ($Rh2a\alpha$ and $Rh2a\beta$ were pooled because of genetic and functional similarity [69, 134]). Prior to performing statistical analyses, normality of all data was confirmed using the Kolmogorov-Smirnov test, and homogeneity of variance was confirmed using the Cochran’s $C$ test [209]. To study the effect of species on the frequency of cone pigments, we performed multiple Fisher exact tests (two-tailed) with a corrected experimental error ($\alpha = 0.0083$; see above). Statistical analysis was performed using the STATISTICA® ($t$-test) and R 2.11.1. (Fisher exact test) software.
Chapter 3

Colour vision requires more cone classes in aquatic than in terrestrial environments

Abstract

Colour vision, mediated by cone photoreceptors, is crucial for the survival of animals. Humans are equipped with three cone classes, yet many animals have high-dimensional colour vision systems with more cone classes. One such example is teleost fish, where cone opsin genes have duplicated and undergone mutations to produce numerous opsin genes that can be functionally expressed. What, then, has driven the increase in the number of cone classes in these visual systems? Colour vision depends on environmental light, which is more complex under water than on land, having been spectrally filtered by the aqueous medium. This raises the intriguing possibility that high-dimensional colour vision in fish has evolved in response to the higher spectral complexity of underwater light. We compared the dimensionality of colour vision in humans and fish by examining the spectral complexity of the colour-signal reflected from objects into their eyes. Here we show that fish require four to six cone classes to reconstruct the colour-signal of objects as accurately as humans. This holds for diverse marine and freshwater ecosystems, suggesting that the diversity of cone opsin genes and high-dimensional colour vision in fish evolved to enhance the reconstruction of complex colour-signals in aquatic environments. Moreover, we find that fish cones can detect
small fluctuations across wavelengths better than human cones. This, together with our finding that the colour-signal of aquatic objects exhibits a greater proportion of these spectral fluctuations than terrestrial objects, suggests that cone photoreceptors evolved to recover spectral information at high-resolution from the colour-signals of relevant objects. These findings help explain the evolution of cone photoreceptors and colour vision, which in turn could change our understanding of animal behaviour and ecology.
Introduction

How animals sense the world has fascinated people for centuries. However, confined to our own sensation and perception, we often fail to fully appreciate the sensory world of other species. The challenge of every sensory system is to optimize the sensitivity and number of receptors to allow for capturing and ultimately distinguishing between stimuli that are crucial for survival (e.g. [210]). However, it remains largely unknown what the ideal sensitivity tuning of receptors is, how many receptors are enough, and how the environment and the range of available stimuli shape these receptor characteristics. Visual systems, and specifically, colour vision systems, are ideal for investigating the factors that shape receptor characteristics. Photoreceptor sensitivity is well conserved across vertebrates and invertebrates (determined by the interaction of an opsin protein and a chromophore) [176, 211], and the visual stimuli available in the environment of animals can be readily characterized (e.g. [9, 212-214]).

Colour vision is commonly used in foraging, predator avoidance, and inter- and intra-specific communication [210, 215]. Colour vision discriminates variation in the spectrum of light from changes in brightness and therefore requires the neural circuitry to compare the signals from at least two spectrally distinct cone photoreceptor classes [216]. Moreover, a colour vision system seeks to recover the reflectance of relevant objects, under varying lighting conditions. This is achieved by decomposing the colour-signal (spectral radiance) arriving at the eye from an object into the spectral reflectance of the object and the spectral irradiance in the environment. Considering the broad absorbance spectra of cone photoreceptors and the limitations they impose on spectral resolution, it
was suggested that more than three cone classes may not be able to add enough spectral resolution to outweigh the costs of an additional cone class [217, 218]. Why, then, do many reptiles, birds, and fish use four or five cone classes [86, 89, 93, 95, 168, 215, 219-222]? Birds and diurnal reptiles often possess coloured oil droplets in their eyes. These screening pigments narrow the spectrum of photoreceptors, and thus render visual systems with four cone classes more efficient [215, 223, 224]. To date, however, the question of high-dimensional colour vision in fish, that lack coloured oil droplets, is still unsolved. The aquatic light environment imparts a great challenge on the vision of fish, which in turn, show the greatest diversity in visual systems among vertebrates [10]. The numerous available cone opsin genes in teleost fish species, which have resulted from multiple opsin gene duplications [68, 69, 71, 91, 134], facilitates the expression of diverse complements of three to seven cone pigments [69, 70, 74, 87, 89-94, 96, 97]. Many of these species were demonstrated to possess four [86, 89, 93, 95, 168, 219, 221], and recently, even five distinct cone classes that interact with one another [225].

A factor that may shape the dimensionality of colour vision is the complexity of colour-signals of objects found in the environment. This complexity of colour-signals accounts for variation between colour-signals of different objects as well as the variation in each colour-signal across wavelengths. Additionally, the complexity of colour-signals depends on the spectral reflectance of objects and the prevailing light in the environment (spectral irradiance). Indeed, the spectral sensitivity of cone pigments and the number of different cone classes participating in colour vision were shown to vary with the width and shape of the spectrum of environmental light [14, 69, 118-122]. However, the role of
the complexity of environmental light and the available range of colour-signals in shaping the dimensionality of colour vision remains unknown.

We raised the possibility that the high complexity of aquatic environmental light has contributed to high-dimensional colour vision in fish. The complexity of the colour-signal, and thus the number of photoreceptors required to reconstruct it, depend on the complexity of the irradiance in the environment and the reflectance of objects. Aquatic irradiance is expected to be more complex than terrestrial irradiance, having been spectrally filtered by the aqueous medium. Therefore, the number of photoreceptors required for reconstructing the aquatic irradiance and the colour-signal of aquatic objects is expected to be greater. In this study, we evaluated the dimensionality of colour vision of fish in comparison to humans and old-world primates, by examining the complexity of the colour-signals of aquatic and terrestrial objects. We find that fish require four to six cone classes to reconstruct the colour-signals reflected from objects. This is because environmental light, which alters the colour-signals, is more complex and contains more spectral fluctuations underwater than on land. We show that fish cones are better suited than human cones to detect these spectral fluctuations, and present a model for explaining the variation in the dimensionality of colour-vision across vertebrates.

Results

To evaluate the number of independent sensors (e.g., cone photoreceptors) that are required for reconstructing the irradiance, reflectance, and colour-signal (radiance) spectra, a principal component analysis (PCA) was employed [226, 227]. We focused on the reflectance of objects that are crucial for the survival of fish or humans - objects that
are important for visual foraging and visual communication. For fish vision, we analyzed the reflectance of algae that many fish feed in and upon, and the reflectance of the body pattern of fish [228]. For human and primate vision, we analyzed the reflectance of fruits and the reflectance of the skin and fur of old-world primates. The reflectance of fruit is of particular interest since it was argued that humans and primates evolved trichromatic colour vision to provide acute discrimination between fruits of different degrees of ripeness and to allow for enhanced detection of fruits against the background of leaves [229-231]. Celestial irradiance, and the reflectance and colour-signal of terrestrial objects were analyzed while accounting for the visible spectrum of humans (390-685 nm). Whereas, aquatic irradiance, and the reflectance and colour-signal of aquatic objects were analyzed while accounting for the visible spectrum of fish (323-685 nm), and again while accounting for the visible spectrum of humans, to test the effect of visible spectrum. The visible spectrum of fish extended into the ultraviolet (UV) spectrum and was calculated while accounting for an ultraviolet-transmissive lens and photoreceptors of A1 chromophore, commonly found in many freshwater and marine fishes [115]. See Methods for visible spectrum estimation, and Figure A2.1 and Supplementary Methods in Appendix 2 for irradiance and reflectance spectra used. For each collection of empirical spectra, we computed the linear model that best fits the collection. Then, we fitted each spectrum in the collection by a linear model of one through ten principal components (PCs) and determined the goodness of fit (see Methods and Figure A2.2).

Irradiance, reflectance, and colour-signal spectra were expressed as linear models. An additional PC was required for reconstructing the aquatic irradiance (n = 80) as
compared to celestial irradiance ($n = 400$) (Figure 3.1A), regardless of the visible spectrum used (Table A2.1A). Thus, the increase in the number of PCs required for reconstruction was a characteristic of aquatic irradiance. One to two additional PCs (dependent on the proportion of spectra in a collection reaching the variance criterion) were required for reconstructing the reflectance of aquatic objects ($n = 351$) as compared to the reflectance of terrestrial objects ($n = 676$) (Figure 3.1B). This difference in the number of PCs decreased to zero to one PC when the visible spectrum of humans was used (Table A2.1B). Thus, the increase in the number of PCs required for reflectance reconstruction partly arose from extending the visible spectrum into the UV spectrum. One to three additional PCs were required for reconstructing the colour-signal of aquatic objects ($n = 28,080$) as compared to the colour-signal of terrestrial objects ($n = 270,400$) (Figure 3.1C). This difference in the number of PCs decreased to one to two PCs when the visible spectrum of humans was used (Table A2.1C). Thus, the increase in the number of PCs required for colour-signal reconstruction arose mainly from characteristic differences between celestial and aquatic irradiance but also from extending the visible spectrum into the UV. Clearly, the exact number of PCs required for reconstruction depends on the variance criterion used (the acceptable sampling error) and the proportion of spectra in a collection that can be reconstructed. However, it is the difference between terrestrial and aquatic vision in the number of PCs required for reconstructing the colour-signal, which is important. Thus, fish would require four to six cone classes to reconstruct the colour signal of relevant objects to the degree that humans achieve with three cone classes.
Figure 3.1 Between four and six cone classes are required for reconstructing the colour-signal of aquatic objects.

The goodness of fit, $R^2$, for linear models of one through ten principal components (PCs), for irradiance, reflectance, and colour-signal spectra is presented. The number of PCs required for signal reconstruction was defined as the number of PCs required to exceed a variance criterion of $R^2=0.99$ (horizontal dashed line). We calculated the number of PCs required for reconstructing 50% (larger plots) and 95% (insets) of the spectra in a collection. (A) Irradiance: Reconstruction of 50% of spectra required 1 PC for celestial irradiance but 2 PCs for aquatic irradiance. (A, inset) Reconstruction of 95% of spectra required 2 PCs for celestial irradiance but 3 PCs for aquatic irradiance. (B) Reflectance: Reconstruction of 50% of spectra required 3 PCs for terrestrial objects but 4 PCs for aquatic objects. (B, inset) Reconstruction of 95% of spectra required 4 PCs for terrestrial objects but 6 PCs for aquatic objects. (C) Colour-signal: Reconstruction of 50% of spectra required 3 PCs for terrestrial objects but 4 PCs for aquatic objects. (C, inset) Reconstruction of 95% of spectra required 5 PCs for terrestrial objects but 8 PCs for aquatic objects. (D) Aquatic irradiance for diverse water types: Reconstruction of 50% of spectra required 2 PCs for freshwater eutrophic, freshwater oligotrophic, and marine eutrophic aquatic irradiance.
The aquatic irradiance data analyzed above were collected from Lake Malawi, a clear, oligotrophic lake [214, 232]. To test whether the reported findings hold for diverse water types, analysis was repeated on irradiance datasets taken from diverse ecosystems (Figure 3.1D). An additional PC was required for reconstructing the aquatic irradiance in comparison to celestial irradiance, no matter if aquatic irradiance was collected from freshwater eutrophic ($n = 240$), freshwater oligotrophic ($n = 80$), or marine eutrophic ($n = 131$) systems. This shows that the larger number of cone classes required for reconstructing the colour-signal of aquatic objects is a characteristic of the aquatic environment.

Knowledge of the complexity of the colour signal arriving at the eye is indispensable; however, it is important to consider how the visual system would affect this colour signal. As a matter of fact, the broad spectra of cone photoreceptors are band-limited functions that pass little energy at frequencies beyond a given band-limit [217], i.e., the cone photoreceptors pass on very little energy above a given band-limit or frequency. Consequently, the cone photoreceptors do not sample small fluctuations between wavelengths; they smooth the signal, and therefore, lose information. As a result, the relation between the number of cone classes that fish and humans require for reconstructing the band-limited colour-signal might differ from that required for reconstructing the raw, unfiltered colour-signal. To study the frequency characteristics of cone photoreceptors in fish and humans, cone absorbance spectra were decomposed into their discrete Fourier components, each having a frequency expressed as cycles per wavelength (note, that this frequency refers to the abscissa of the Fourier transform of the
spectrum rather to the frequency of light, sometimes called the comb frequency [217])
(Figure 3.2A-D). The band-limit of a spectrum was defined as the frequency above which
only 1% of the cumulative energy was found [227, 233].

The band-limit of cone photoreceptors decreased when going from short (narrow
bandwidth) to long (broader bandwidth) wavelength-sensitive cones. The band-limits of
fish cones spanned a wider range of frequencies and the band-limits of the three short-
 wavelength cones in fish were higher than those in humans (fish: 7.22-16.01 cycles/μm,
humans: 8.39-14.45 cycles/μm) (Figure 3.2E,F; Table A2.2). The Nyquist sampling
theorem determines the number of samples (e.g., cone photoreceptors) required to
reconstruct a signal up to a certain frequency. The relation between the band-limit of a
signal $f$ (cycles/μm) and the number of significant independent samples $n$ required to
reconstruct the signal is: $n = 2Wf$ where $W$ stands for the width of the visible spectrum
(μm) [234]. That is, the band-limit of photoreceptors is directly related to the minimal
number of photoreceptors that are required for reconstruction of the signal up to a
frequency that equals the band-limit of photoreceptors. The number of samples required
to reconstruct the low-pass portion of the colour-signal that is passed by cones in fish
(5.23 to 11.60) was higher by up to three samples (dependent on cone class) than in
humans (4.96 to 8.53); results for humans are consistent with past studies [217, 227].
Thus, fish would require four to six cone classes (an additional one to three cone classes
in comparison to humans) to reconstruct the band-limited colour signal of relevant
objects.
Figure 3.2 Fish cones, compared to human cones, recover higher frequencies from the colour-signal received.

(A,B) Absorbance spectra of cone photoreceptors in humans and fish. (C,D) Absorbance spectra of cone photoreceptors expressed as band-limited functions. The discrete Fourier transform (DFT) was calculated while accounting for the visible spectrum of humans and fish. (E,F) Cumulative energy of absorbance increased quickly with frequency. Band-limits of the S-, M-, and L-cone photoreceptors in humans equaled 14.45, 9.57, and 8.39 cycles/µm, respectively. Band-limits of the SWS1 through LWS cone photoreceptors in fish ranged from 16.02 to 7.23 cycles/µm, where SWS1, SWS2b, and SWS2a are short wavelength-sensitive cones, Rh2b, Rh2aα, Rh2aβ are rhodopsin-
like mid wavelength-sensitive cones, and LWS is a long wavelength-sensitive cone. The band-limit of spectra was defined as the frequency above which only 1% of the cumulative energy was found [227, 233] (dashed line).
Given the filter properties of their photoreceptors, fish could potentially recover higher frequencies from the signal received (higher band-limits). However, this would be advantageous only if the band-limit of the colour-signal of aquatic objects is higher than those of fish cones. To investigate whether this is the case, irradiance, reflectance, and colour-signal spectra were expressed as band-limited functions (Figure A2.3) and their cumulative energy as a function of frequency was calculated (Figure 3.3). The band-limit of the colour-signal of aquatic objects was significantly higher than that of terrestrial objects (Figure 3.4A,B; left axes); the greater proportion of high frequencies in the colour-signal of aquatic objects arose from a greater proportion of high frequencies in the aquatic irradiance (Figure 3.4C) rather in the reflectance of aquatic objects (Figure 3.4D). This indicates a correlation between the band-limits of cones and those of the colour-signal of objects, i.e. the ability of fish cones to recover higher frequencies coincided with the higher frequencies found in the colour-signal of aquatic objects. Additionally, apart from the shortest-wavelength photoreceptors (S-cone in humans and SWS1 in fish), which adequately sampled the colour-signal of most relevant objects, photoreceptors undersampled the colour-signal of approximately half of relevant objects (Figure 3.4A,B; left axes). Thus, the colour-signal of objects contains frequencies that are higher than those that can be recovered by photoreceptors. In conclusion, the capability of cone photoreceptors of fish to recover high frequencies from the colour-signal confers an advantage and allows for enhanced reconstruction of the colour-signal.
Figure 3.3 Cumulative energy of irradiance, reflectance, and colour-signal spectra increases quickly with frequency.

Cumulative energy was calculated for (A) irradiance, (B) reflectance, and (C) colour-signal spectra.
Figure 3.4 Aquatic colour-signals contain more high-frequency energy and require more samples to reconstruct than terrestrial colour-signals.

(A, B, left axes) Band-limit of colour-signal of aquatic objects was significantly higher than that of terrestrial objects (fish-fur: randomization test (RT), \( P<0.001 \), confidence interval 2.5-97.5\% - CI\text{fish}=9.518-10.968, CI\text{fur}=6.236-6.762, \( n\text{fish}=24,320, n\text{fur}=77,600 \); algae-fruit: RT, \( P<0.001 \), CI\text{algae}=9.405-10.483, CI\text{fruit}=8.768-9.146, \( n\text{algae}=3,760, n\text{fruit}=192,800 \)). Photoreceptors undersampled the colour-signal of objects. Humans: band-limit of M- and L-cones exceeded that of the colour-signal of less than 75\% and 50\% of fruit tissues, respectively. Fish: band-limits of SWS2a through LWS exceeded that of the colour-signal of less than approximately 50\% of algae and fish tissues. (A, B, right axes) The number of samples required to reconstruct the colour-signal of aquatic objects was larger than that of terrestrial objects. (C) Band-limit of aquatic irradiance was significantly higher than that of celestial irradiance (RT, \( P<0.001 \), CI\text{aquatic}=5.833-6.187, CI\text{celestial}=5.001-5.091, \( n\text{aquatic}=80, n\text{celestial}=400 \)). (D) Band-limits of reflectance of fruit and algae were significantly higher than those of primate fur and fish pattern (fruit-fur: RT, \( P<0.001 \), CI\text{fruit}=7.885-8.178, CI\text{fur}=5.711-6.236, \( n\text{fruit}=482, n\text{fur}=194 \); algae-fish pattern: RT, \( P<0.001 \), CI\text{algae}=7.239-9.196, CI\text{fish}=4.892-5.265, \( n\text{algae}=47, n\text{fish}=304 \)); thus, the reflectance of integumentary tissues is constrained differently than that of plant and algae.
tissues. Band-limit of reflectance of terrestrial objects was significantly higher (RT, \(P<0.001\), CI\(_{fur}=5.703-6.239\), CI\(_{fish}=4.891-5.266\), \(n_{fur}=194\), \(n_{fish}=304\)) than, or did not differ (RT, \(P=0.574\), CI\(_{fruit}=7.881-8.174\), CI\(_{algae}=7.256-9.171\), \(n_{fruit}=482\), \(n_{algae}=47\)) from that of aquatic objects; thus, the greater proportion of high frequencies in the colour-signal of aquatic objects arises from the greater proportion of high frequencies in the aquatic irradiance. (A-D) The visible spectrum of fish (humans) was taken into account for the analysis of aquatic (celestial) irradiance, and the reflectance and colour-signal of aquatic (terrestrial) objects. Reanalysis of aquatic irradiance, reflectance and colour-signal data with the visible spectrum of humans yielded similar results (not presented). Box: mean (dashed), median (solid), 25\(^{th}\) and 75\(^{th}\) percentiles; whiskers: 10\(^{th}\) and 90\(^{th}\) percentiles; points: 5\(^{th}\) and 95\(^{th}\) percentiles.
Discussion

This study significantly advances our understanding of the evolution of visual function and the visual ecology of animals. First, the correspondence between the band-limits of cone photoreceptors and those of the colour-signal of objects suggests that the filtering properties of photoreceptors evolved to allow for recovery of high frequencies from the colour-signal of relevant objects. Second, the number of cones required for reconstructing the colour-signal of aquatic objects was larger than that of terrestrial objects, regardless of whether the unfiltered colour-signal or the band-limited colour-signal passed by the cones was considered. Therefore, our results, based on two independent approaches, show that fish would require four to six cone classes for reconstructing the colour-signal of aquatic objects at the accuracy level achieved by humans viewing terrestrial objects. Third, the need for a larger number of cone classes for colour signal reconstruction was an attribute of the aquatic environment, and was true for diverse marine and freshwater ecosystems. This suggests that the large diversity of cone opsin genes and high-dimensional colour vision in fish are of adaptive significance, and have likely evolved to enhance the reconstruction of the complex colour-signal in aquatic environments. Trichromacy might be satisfactory for human colour vision, but it is not so for fish vision. Many freshwater and some marine fish have been shown to functionally express four cone classes [10], while a recent study has reported the functional expression of five cone classes in some cichlid fish [222]. However, the efficiency of colour reconstruction in fish has yet to be explored thoroughly.
Our findings are consistent with the current framework for the evolution of the number of cone classes in vertebrates [10]. Four classes of cones are found in the most basal vertebrate lineage, the lamprey (Hyperoartia) [63]. All four cone opsin classes were retained by many bony fishes (Osteichthyes) that include the ray-finned fish (Actinopterygii) and the lobe-finned fish (Sarcopterygii). However, opsin gene duplications that occurred early in the evolution of ray-finned fish (230 million years ago or later; after their divergence from the lobe-finned fish) had increased the number of cone opsin genes [68, 69, 71, 91, 134]. Thus, lobe-finned fish (represented today by the lungfishes and coelacanths), that later gave rise to the first tetrapods and to all terrestrial vertebrates [235-237], have fewer cone opsin classes compared to teleost fish, an infraclass of ray-finned fish. While four cone opsin classes were retained in lobe-finned fish, diurnal reptiles and birds [61], many amphibians, nocturnal reptiles and mammals have lost at least one cone opsin class. For example, amphibians express only three cone classes [238-243]; nocturnal snakes such as the boas and pythons [244, 245] as well as most placental and marsupial mammals, and monotremes (mammals that lay eggs) express only two cone classes; and most marine mammals express only one cone class [246]. The only significant gain of opsin genes in mammals was the duplication of the LWS gene in primates that led to trichromacy [247].

Our results suggest that opsin gene duplications and the resultant increase in the number of cone classes in teleost fish were associated with the pressures exerted by the aquatic environment to allow for accurate reconstruction of the colour-signal of aquatic objects. The retention of only three of the four ancestral cone opsin classes in amphibians
likely was associated with partial exposure to the aquatic environment and the subsequent relaxation of the pressures it exerts. The retention of the four ancestral cone opsin classes in diurnal reptiles and birds was associated with little exposure to the aquatic environment as well as with the development of coloured oil droplets within the inner segment of their cone photoreceptors [10, 215, 223, 224]. By narrowing the spectrum of photoreceptors, these screening pigments allow cones to recover higher frequencies from the signal received and thus, increase the number of samples required for reconstructing the band-limited colour-signal that is passed by cones (assuming a constant span of the visible spectrum). Thus, the inclusion of coloured oil droplets renders visual systems with four cone classes in those reptiles and birds more cost effective. However, narrowing the cone spectrum might be accompanied by a costly loss in overall sensitivity, which only diurnal animals that live in well-lit environments can afford [248, 249]. The further reduction in the number of cone classes in nocturnal reptiles and mammals was associated with no exposure to the aquatic environment as well as the loss of coloured oil droplets as a result of a nocturnal phase that presumably characterized the early evolution of mammals [248].

The requirement for four to six cone classes for the reconstruction of colour-signals of aquatic objects holds for shallow water that is characterized by irradiance of high levels and a broad spectrum. However, the gradual decrease in irradiance and narrowing of the irradiance spectrum with increasing water depth [6], would favour colour vision systems with gradually decreasing number of cone classes. Eventually, the dim and monochromatic irradiance encountered at deep water would favour one
photoreceptor (either cone or rod) maximally sensitive about the wavelength of maximum transmission of irradiance. This prediction is supported by previous studies. The number of cone classes in fish was shown to vary with water depth, as shallow-water fish possess a greater number of cone classes than deep-water fish [14, 118, 119]. The spectral location of cone pigments was also reported to vary between shallow- and deep-water fish, with the latter having cone pigments shifted toward short wavelengths, closer to the wavelength of maximum irradiance transmission [120, 121]. Our prediction is also supported by the tendency of aquatic mammals such as the cetaceans (whales and dolphins) and pinnipedians (seals, sea-lions, and walruses), that frequently forage in deep water, to show monochromatic visual systems [250-252].

The proposed model indicates that the level of exposure to the aquatic environment, and in aquatic vertebrates, also the water depth, have played a role in the evolution of the number of cone classes. Nonetheless, other factors could also have contributed to the evolution of the number of cone classes and the dimensionality of colour vision. For example, the variation between colour-signals of objects in the immediate surroundings of the animal and the visual tasks at hand may have had a role in shaping colour vision. The proposed model can serve as a framework for investigating the role of such factors. Importantly, our results show that fish would require four to six cone classes to reconstruct the colour-signal of relevant objects at an accuracy level comparable to that of humans. This does not imply that all fish are expected to possess such a large number of cone classes; fish might possess a smaller number of cone classes, but with the inevitable loss in accuracy of signal reconstruction.
Methods

**Irradiance, reflectance, and colour-signal data sets**

Aquatic and celestial spectral irradiance as well as spectral reflectance of aquatic and terrestrial objects were measured or adopted from previous studies [214, 253-257] (Supplementary Methods in Appendix 2). To calculate colour-signals, spectral reflectance of each terrestrial object was multiplied by every single celestial irradiance spectrum, and spectral reflectance of each aquatic object was multiplied by every single aquatic irradiance spectrum. Objects were assumed to be viewed from a short distance, thus light attenuation through air or water was neglected.

**The visual system of fish**

Absorbance spectra [176] for cone pigments in fish were constructed based on the seven cone pigments reported in cichlid fish - $\lambda_{\text{max}}$ of A$_1$-reconstituted visual pigment is provided in parentheses: SWS1 (368 nm), SWS2b (423 nm), SWS2a (456 nm), Rh2b (484 nm), Rh2aa (519 nm), Rh2aβ (528 nm), and LWS (560 nm) [69, 134]. The spectral range spanned by these pigments is common to many freshwater and marine fish families [10, 84, 118, 258]. Absorbance spectra were generated for cone pigments with either A$_1$ (retinal) or A$_2$ (3,4-dehydroretinal) chromophores, where the $\lambda_{\text{max}}$ shift associated with changes in chromophore composition was accounted for [105]. Absorbance spectra of cone pigments were corrected for lens transmission. With the spectral limits of wavelength discrimination in fish variable and largely unknown (but see [259]), the visible spectrum of fish was calculated as the range enclosed between the wavelengths at
which the absorbance of the shortest-wavelength cone (SWS1) and the longest-wavelength cone (LWS) were at 1% of maximum absorbance. The visible spectrum of fish shifted and varied in width with varying chromophore composition and lens transmission. The visible spectrum (VS) was calculated for four key combinations of chromophore and lens transmission: (i) A₁ retina and a UV-transmissive lens, VS: 323-685 nm, (ii) A₁ retina and a non-UV-transmissive lens, VS: 357-685 nm, (iii) A₂ retina and a UV-transmissive lens, VS: 323-767 nm, and (iv) A₂ retina and a non-UV-transmissive lens, VS: 357-767 nm. Thus, the width of the visible spectrum of fish might range between 362 and 444 nm. To correct the absorbance spectra of cone pigments for a UV-transmissive lens, we used the lens transmission of a Lake Malawi cichlid, *Metriaclima zebra* whose wavelength at half-maximum lens transmission (T₅₀) was 350 nm [222]. To correct the absorbance spectra of cone pigments for a non-UV-transmissive lens (T₅₀ = 400 nm), we used the lens transmission of a Lake Malawi cichlid, *Melanochromis auratus* [44]. These T₅₀ values are commonly found in many freshwater and marine fishes [44, 46]. For all analyses presented, unless specified differently, the visible spectrum and spectra of cone photoreceptors in fish were calculated while accounting for an ultraviolet-transmissive lens and photoreceptors of A₁ chromophore (case i). Aquatic irradiance at shallow depths is not expected to limit the visible spectrum of fish that exhibit A₁ retina. The aquatic irradiance analyzed in this study attained values of 10^{10} to 10^{13} photons cm^{-2} s^{-1} nm^{-1} across the visible spectrum of fish exhibiting A₁ retina. These irradiance values are well within the photopic range of many fish, e.g., cyprinids [202], salmonids [197], and cichlids [222].
The visual system of humans

Absorbance spectra [176] for the S-, M-, and L-cone photoreceptors in humans were constructed based on absorbance templates for the A₁ chromophore using $\lambda_{\text{max}}$ values of 420, 530, and 558 nm, respectively [260, 261]. Absorbance spectra were corrected for lens [262, 263] and macula [264] transmission. The visible spectrum of humans was calculated as the range enclosed between the wavelengths at which the absorbance of the S-cone and the L-cone were at 1% of maximum absorbance, and ranged between 390 and 685 nm. For consistency, construction of cone absorbance spectra and estimation of the visible spectrum were performed similarly in both fish and humans. Note, however, that the cone absorbance spectra generated for humans were quantitatively similar to the commonly used sensitivity spectra [261] (Figure A2.4).

Characterizing spectra as linear models

Irradiance, reflectance, and colour-signal spectra were expressed as the weighted sum of $d$ fixed principal components (PCs) [227],

$$S(\lambda) = \sum_{i=1}^{d} \sigma_i P_i(\lambda)$$

(1)

where $P_i(\lambda)$ denotes principal component $i$ at a wavelength $\lambda$ (nm) and $\sigma_i$ denotes the weight of each PC and determines the spectrum of concern, denoted $S(\lambda)$. $S$ may stand for the spectrum of irradiance $E$, surface reflectance $R$, or colour-signal $L$. To fit a linear model to a collection of $n$ empirical spectra we used principal component analysis (PCA).
All spectra were normalized to be of length 1 to avoid differential weighting of different spectra in determining the choice of principal components [227]. To eliminate the need for subtracting the mean value (across spectra) prior to performing PCA, analysis was performed on an augmented data set that has mean zero [227]. This data set was generated by appending to the set of empirical spectra $S_1(\lambda),\ldots,S_n(\lambda)$ the negations of these spectra: $-S_1(\lambda),\ldots,-S_n(\lambda)$. Then, to fit a given empirical spectrum $S_j(\lambda)$ to a given $d$-dimensional linear model, we used a least-squares fitting procedure which returned the multiple correlation coefficient ($R^2$) as a measure of goodness of fit [265]. It is unknown how accurately animals reconstruct spectra. Thus, the number of PCs required for signal reconstruction was defined as the number of PCs required to exceed a variance criterion of $R^2=0.99$ [227]. Additionally, the proportion of spectra in a collection that are biologically necessary to reconstruct is unknown. Therefore, we calculated the number of PCs required for reconstructing 50% (median) and 95% (5th percentile) of the spectra in a collection.

*Characterizing spectra as band-limited functions*

To identify component frequencies in spectra, the discrete Fourier transform (DFT) was applied using the Fast Fourier Transform (FFT) algorithm using Matlab (Matlab R2009a, The Mathworks, Natick, MA, USA). DFT was calculated for each of the cone absorbance, irradiance, reflectance, and colour-signal spectra examined. Many spectra had considerable levels of energy at both ends of the light spectrum. Any value difference between the two ends of a spectrum may introduce spurious, high-frequency components into the Fourier power spectrum. A Hanning window was used to attenuate
these artefacts [227, 233]. In order to increase the Fourier frequency resolution to 0.2 cycles/μm, cone absorbance, irradiance, reflectance, and colour signal spectra were zero-padded [233].

**Comparison between PCA and DFT and validation of calculations**

Both approaches used, expressing spectra as linear models using principal component analysis (PCA) and expressing spectra as band-limited functions using discrete Fourier transform (DFT), attempt to estimate the complexity of the signal, but they do it in different ways. The PCA examines the statistics (dimensionality) of a whole collection of spectra while the DFT examines each individual spectrum separately. The advantage of the DFT is that it accounts for the filtering properties of cone photoreceptors, and therefore, allows for estimating the complexity of the filtered, band-limited, signal.

To verify our calculations, we analyzed the full spectra set of 462 Nickerson–Munsell chips [266] that were analyzed previously [227]. To verify our PCA calculations, the first ten principal components of the Munsell data set were calculated. Variance accounted for by the model, $R^2$, was in agreement with previously reported values for the 400-700 nm range [227], where 0.9756, 0.9960, 0.9980, 0.9993, and 0.9996 of median variance was recovered with two to six principal components, respectively. To verify our DFT calculations, the band-limits of all spectra included in the Munsell data set were calculated. Band-limits were in agreement with previously reported values for the 380-770 nm range [227], where 0.9459, 0.9859, 0.9990, and 0.9998 of median energy was recovered at frequencies of 3.3, 5, 10, and 15 cycles/μm, respectively.
Several data sets analyzed in this study included spectra measured at a resolution of 1 nm, while others included spectra measured at a spectral resolution of 4 nm. For consistency, low-resolution spectra were spline interpolated to every 1 nm prior to analysis. To test the effect of spectral interpolation on our PCA calculations, the variance accounted for by the model, $R^2$, was calculated for a selected data set (Lake Malawi irradiance) measured at a spectral resolution of 1 nm, and again after this data set was sampled every 4 nm and interpolated back to 1 nm. The variance accounted for by the model did not differ between the original and interpolated data sets, where 0.9833, 0.9965, 0.9994, 0.9998, 0.9999, 0.9999, and 1.0000 of median variance were recovered with the first seven principal components. Similarly, to test the effect of spectral interpolation on our DFT calculations, the band-limits of spectra included in the original and interpolated data sets were calculated and were found similar (randomization test, $P = 0.986$, confidence interval 2.5-97.5% - CI$_{original}$=5.833-6.187, CI$_{interpolated}$=5.832-6.186, $n = 80$).

Statistical analysis

Band-limit values for each collection of empirical spectra did not follow normal distribution (Kolmogorov-Smirnov test) and their variance differed across collections (Leven’s test). Additionally, reflectance and colour-signal spectra within each collection were not independent. Therefore, to compare the mean band-limit of two collections of empirical spectra, we used a randomization test, with the difference between the means of the two collections as a test statistic. The observed test statistic was compared to the null distribution estimated from 10,000 replicates, where band-limits were randomly
permutated while maintaining the original sample sizes [267]. Non-parametric percentile-based bootstrapping (10,000 replicates) was used to estimate the 95% confidence intervals around the mean of band-limits for each collection of spectra [268]. Statistical analyses were performed using R 2.13.0 (The R Foundation for Statistical Computing).
Chapter 4

Ontogeny in the visual system of Nile tilapia

Abstract

Retinal neurogenesis in fish facilitates cellular rearrangement throughout ontogeny, potentially allowing for optimization of the visual system to shifts in habitat and behaviour. To test this possibility, we studied the developmental trajectory of the photopic visual process in the Nile tilapia. We examined ontogenetic changes in lens transmission, photoreceptor sensitivity, and sensitivity of the outer plexiform layer (OPL), and used these to estimate changes in cone pigment expression and retinal circuitry. We observed an ontogenetic decrease in ultraviolet (UV) photoreceptor sensitivity, which resulted from reduction in the SWS1 cone pigment expression, and was associated with reduction in lens transmission at UV wavelengths. Additionally, OPL sensitivity to both UV and long wavelengths decreased with age, likely reflecting changes in photoreceptor sensitivity and retinal circuitry. This novel remodelling of retinal circuitry occurred following maturation of the visual system but prior to reaching adulthood, and thus may facilitate optimization of the visual system to the changing sensory demands. Interestingly, the changes in OPL sensitivity to long wavelengths could not be predicted by the changes observed in lens transmission, cone pigment expression, or photoreceptor sensitivity. Thus, emphasizing the importance of considering knowledge of visual sensitivity and retinal processing when studying visual adaptations and attempting to relate visual function to the natural environment. This study advances our
understanding of ontogeny in visual systems and demonstrates that the association between different elements of the visual process can be explored effectively by examining visual function throughout ontogeny.
Introduction

Ontogeny, the developmental history of an organism throughout its lifetime, is a key program that affects all organisms. During ontogeny, organisms may shift habitat, food source, and behaviour. The teleost fish retina is an important model system for studies of ontogeny, neural plasticity, and neurogenesis. Unlike in mammals and other vertebrates [269], the teleost eye grows throughout life, and new neurons, generated by retinal stem cells are continuously added to the growing retina [270, 271]. This neurogenesis in the fish retina facilitates cellular rearrangement throughout ontogeny, potentially allowing for optimization of the visual system to shifts in visual demands.

Ontogenetic changes in several elements and processes in the visual system of fish have been described. The visual process begins with light being transmitted through the ocular media to the retina. In fish, the transmission of the ocular media is typically determined by the transmission of the lens [84], which has reported to change during ontogeny in several fish species [46, 48, 50, 131]. Within the retina, visual pigments absorb light and initiate a phototransduction cascade that leads to neural signalling and ultimately behaviour. Visual pigments are light-sensitive molecules found in the outer segments of photoreceptors and consist of a vitamin-A-based chromophore bound to an opsin protein. Four cone opsin classes have been identified in vertebrates: ultraviolet-sensitive (SWS1), short-wavelength sensitive (SWS2), rhodopsin-like mid-wavelength sensitive (Rh2), and long-wavelength sensitive (LWS) [61]. Cone opsin genes [91, 96, 132-134] and cone pigments [80, 91, 96, 135-142] were reported to be differentially expressed throughout the life history of several fish species. Additionally, spectral
sensitivity, which is the relative efficiency of detection of light as a function of wavelength, was also reported to change during the ontogeny of some fish species [272]. These alterations in spectral sensitivity were sometimes associated with a loss of certain cone classes and changes in the spatial arrangement of photoreceptors throughout the retina, i.e., cone photoreceptor mosaic [80-83].

By examining the visual system throughout ontogeny, the function of a given element/process in the visual system, and its effect on other elements/processes can be explored. For example, alterations in cone opsin gene expression (measured using reverse transcription-quantitative polymerase chain reaction - RT-qPCR) during ontogeny were qualitatively shown to accompany alterations in the frequency of cone photoreceptors (measured using microspectrophotometry – MSP) [80, 91, 96]. However, most previous studies have looked at only one or two elements/processes in the visual system, making it difficult to draw association with other elements and levels of processing. With this in mind, we studied the developmental trajectory of visual function in fry, juvenile and adult Nile tilapia through the examination of six elements: (i) lens transmission, (ii) sensitivity of photoreceptors, (iii) cone pigment expression – estimated based on photoreceptor sensitivity, (iv) geometry of the cone mosaic, (v) sensitivity of the outer plexiform layer (OPL), and (vi) retinal circuitry – estimated based on comparison of photoreceptor and OPL sensitivity. The Nile tilapia (Oreochromis niloticus; Cichlidae) is thought to be the riverine ancestor of the cichlid fishes of the East African Great Lakes, which represent the largest vertebrate radiation on Earth [173]. It is one of the worlds’ most heavily
cultured fish species and recently has gained significance as a vertebrate model for biological research [273].

The visual system of the Nile tilapia has been the subject of several past studies. Lens transmission in tilapia was shown to change throughout ontogeny, with the wavelength of half-maximum transmission ($T_{50}$) increasing with lens diameter [47]. However, it is unknown whether these changes in lens transmission are accompanied by changes in other elements of the visual system. Additionally, all four classes of cone opsin genes identified in vertebrates have been found in the Nile tilapia. However, in the tilapia, further gene duplications have resulted in numerous cone opsin genes that produce seven spectrally distinct cone pigments with peak sensitivities: SWS1 (360 nm), SWS2b (425 nm), SWS2a (456 nm), Rh2b (472 nm), Rh2αβ (518 nm), Rh2αα (528 nm) and LWS (560 nm) [134]. Nile tilapia has been shown to express different complements of opsin genes throughout ontogeny, with notable changes in SWS1 and LWS [96, 134]. These ontogenetic changes in opsin gene expression agree qualitatively with alterations in the frequency of cone photoreceptors [96]. Spectral sensitivity, on the other hand, was measured only for tilapia adults [167], thus, the relationships between ontogenetic changes in spectral sensitivity and those in other elements of the visual system are currently unknown. Further, in tilapia, short-wavelength sensitive pigments (SWS1, SWS2b, and SWS2a) reside in single cones, whereas long-wavelength sensitive pigments (Rh2b, Rh2αα, Rh2αβ, and LWS) reside in double cones [96]. Adult tilapia have cone photoreceptors arranged in a square mosaic, with a single cone surrounded by four double cones [274, 275]; but the pattern in fry and juveniles is yet to be studied. Lastly,
considering the persistent retinal neurogenesis in fish, it may be that the circuitry, i.e., the synaptic and electrical coupling between different retinal cells, also changes throughout ontogeny. Such an ontogenetic change in circuitry has not been reported previously.

Here we show that Nile tilapia undergo ontogenetic changes in lens transmission, and photoreceptor and OPL sensitivity. An ontogenetic decrease in ultraviolet (UV) photoreceptor sensitivity resulted from reduction in the expression of the SWS1 cone pigment, and was associated with reduction in lens transmission at UV wavelengths. We found that OPL sensitivity to UV and long wavelengths decreased with age, and these changes were the result of ontogenetic changes in both photoreceptor sensitivity in the UV range and retinal circuitry in the long wavelength part of the spectrum.

Methods

Fish care and holding conditions

Fry \((n = 20)\), juvenile \((n = 15)\), and adult \((n = 19)\) Nile tilapia \((O. niloticus,\) Redfish Ranch, Courtney, BC, Canada) were used. The age and body mass of fish were \(67\pm19\) (average\(\pm\)s.d.) days post fertilization \(\text{(d.p.f.)}\) and \(1.08\pm0.55\) g for fry, \(119\pm53\) d.p.f. and \(8.41\pm1.54\) g for juveniles, and \(255\pm20\) d.p.f. and \(76.61\pm20.72\) g for adults. Fish were held in our aquatic facility tanks under a 12h:12h light-dark photoperiod at 25\(\pm\)1 °C. Facility lighting comprised of full spectrum fluorescent lamps \((\text{UV-Blue actinic and BlueMax lamps; Full Spectrum Solutions, Jackson, MI, USA})\). All experimental and animal care procedures were approved by Queen’s University Animal Care Committee under the auspices of the Canadian Council for Animal Care.
Fish preparation

We recorded electroretinogram (ERG) to estimate photoreceptor and OPL sensitivity. Prior to ERG recordings, fish were immersed in a solution of 125 mg L\(^{-1}\) tricaine methanesulfonate (MS-222) until they reached stage III anaesthesia [29]. A general anaesthetic (metomidate hydrochloride; 0.3 mg g\(^{-1}\) body mass; Maranil; Syndel Laboratories, Qualicum Beach, B.C., Canada) and an immobilizing agent (pancuronium bromide; 0.05 mg g\(^{-1}\) body mass; Conier Chem and Pharma, Chongqing, China) were injected subcutaneously. Test fish were placed in a holding cradle in a Faraday cage and irrigated with aerated fresh water (temperature = 20±1°C, flow rate = 3-10 ml s\(^{-1}\)).

Electroretinogram (ERG) experimental apparatus

The optical system and recording apparatus have been described in detail elsewhere [95, 168]. Two background channels using 250 W halogen lamps (24V ELC, Eiko, Kansas City, KS, USA) provided constant background illumination to light adapt the eye. A bifurcated optical fiber (fused silica, numerical aperture, NA = 0.22; Fiberoptic Systems, Simi Valley, CA, USA) guided light from the two background channels to the electrophysiology rig. The intensity and spectral composition of background illumination was manipulated using interference cutoff filters and neutral density filters (Corion, Franklin, MA, USA). The stimulus channel used a 150 W xenon arc lamp and monochromator (Photon Technology International, London, ON, Canada; 150 W bulb, Ushio, Cypress, CA, USA). The wavelength, intensity, and duration of the stimulus were manipulated using a 0-2.7 optical density (OD) neutral density wedge (fused silica; Melles-Griot, Rochester, NY, USA), a filter wheel with 9 neutral density
filters of 0 to 4.0 OD at 0.5 OD increments, and an electronic shutter (UniBlitz D122 Shutter, Vincent Associates, Rochester, NY, USA). An optical fiber (fused silica; NA = 0.55; Fiberoptic Systems) guided light from the stimulus channel to the electrophysiology rig. The background and stimulus beams were superimposed one on another to produce a beam 1.0 cm in diameter at the plane of the fish eye.

**ERG recording and analysis**

ERG recordings commenced at least one hour after the onset of the light phase and completed before the onset of the dark phase to eliminate any circadian rhythm effects [191]. A glass electrode (1.5 mm outer diameter, 1 mm inner diameter, borosilicate glass; World Precision Instruments, Sarasota, FL, USA) pulled to a tip diameter of 80-125 μm (P-97 Flaming/Brown Micropipette puller; Sutter Instruments, Novato, CA, USA) was loaded with saline (0.684 M sodium chloride) and inserted into a saline-filled chlorided AgCl half-cell (A-M systems, Sequim, WA, USA). The electrode tip was placed on the dorsal-nasal corneal surface of the right eye. The ground electrode was placed on the caudal fin and a chloride-silver reference electrode was placed on the head of the fish. Fish were light adapted to the background light for 30 minutes prior to recordings. The duration of the light stimulus was 500 ms with an interstimulus interval of 5 s. The ERG signal was amplified and filtered using band-pass filter settings (10 Hz low pass, 100 Hz high pass) via an isolated bioamplifier (BMA-200, Bioamplifier, CWE Incorporated, Ardmore, PA, USA). This amplified signal was then analyzed with a 16-bit A/D data acquisition system (Micro 1401; Cambridge Electronic Design, Cambridge, England, UK) and Signal 4.0 software. Spectral sensitivity was measured in 10 nm
increments, from 340 to 700 nm, in a staggered wavelength presentation in order to prevent adaptation to specific spectral regions. At each wavelength, the ERG response to eleven stimulus intensities (irradiance levels) was determined. A third order polynomial was fit to the response versus irradiance (RI) curve and the threshold irradiance that corresponded to a response criterion of 30-40 μV was interpolated [197]. Sensitivity was estimated as the reciprocal of this threshold irradiance. Log relative sensitivity curves were created by normalizing the log absolute sensitivity values to the maximum sensitivity across the spectrum [222].

The response of the outer plexiform layer and photoreceptors

An electroretinogram accounts for the response of the outer plexiform layer (OPL) of the retina, representative of the response of cone photoreceptors, horizontal cells and bipolar cells. A typical ERG waveform consists of an initial hyperpolarization phase (a-wave) representing the response of photoreceptors to the onset of light, followed by a depolarization phase (b-wave) representing the response of ON bipolar cells to the onset of light (but may also be affected by other retinal neurons, e.g., amacrine cells) [193, 194]. Accordingly, the response of the OPL was measured as the potential change between the a-wave and b-wave, whereas, the response of photoreceptors alone was measured as the amplitude of the a-wave. However, due to the strong depolarization of ON bipolar cells, the response of photoreceptors (a-wave) was typically masked. To isolate the response of photoreceptors, sodium-L-aspartate (ASP) was injected into the ocular media of fish. ASP is a substrate of the glutamate transporter in photoreceptors that competitively inhibits glutamate uptake [276] and thereby increases glutamate at the
synapse, mimicking constant darkness. ASP is routinely used to isolate photoreceptor responses in the ERG [195, 277-279]. To account for variation in ocular media volume, different volumes and concentrations of ASP in Ringer’s solution [280] were used for different life stages (Fry, 5 μl 70 mM; Juvenile, 10 μl 70 mM; Adult, 10 μl 210 mM), resulting in an estimated final ocular concentration of 6.77 mM. To ensure constant effect of ASP throughout the experiments, ERG recordings commenced at least 20 minutes following the ASP injection and were completed within 250 minutes following the injection (S. Sabbah, F. E. Hauser, and C. W. Hawryshyn, unpublished).

**Background light conditions**

Spectral sensitivity of each fish was evaluated under three background light conditions (BG1-BG3), designed to differentially activate the various cone pigment classes. Short-wavelength sensitivity was expected to be lowest under BG1, higher under BG2, and highest under BG3. The number of photons collected by the various cone pigment classes under the background conditions used was estimated using a quantum catch model:

\[
Q_i = \sum_{\lambda=300}^{800} A_i(\lambda)E(\lambda)
\]

where \(Q_i\) denotes the quantum catch of cone pigment \(i\), \(A_i(\lambda)\) denotes the absorbance of cone pigment \(i\) at a wavelength \(\lambda\), and \(E(\lambda)\) denotes the photon irradiance of the background light at a wavelength \(\lambda\). Absorbance spectra for cone pigments were
constructed for the seven cone pigments reported in the Nile tilapia using the absorbance templates of Govardovskii et al. [176] - note the $\lambda_{\text{max}}$ of A1-reconstituted visual pigments is provided in parentheses: SWS1 (360 nm), SWS2b (425 nm), SWS2a (456 nm), Rh2b (472 nm), Rh2aα (518 nm), Rh2aβ (528 nm), and LWS (561 nm) [134]. The spectral overlap of Rh2aα and Rh2aβ necessitated calculating an average $\lambda_{\text{max}}$ of 523 nm. The 523-nm $\lambda_{\text{max}}$ absorbance spectrum was used for subsequent analysis and is hereafter referred to as Rh2a.

The irradiance provided under the various background conditions was measured at the plane of the fish eye using a spectroradiometer (QE65000; Ocean Optics, Dunedin, FL, USA) connected to a 2 m optical fiber (QP600-2-UV/VIS; Ocean Optics) that was fitted with a cosine corrector (CC-3-UV; Ocean Optics). The spectroradiometer setup was calibrated for absolute irradiance using a NIST (National Institute of Standards and Technology, Gaithersberg, MD, USA) calibrated Halogen-Deuterium dual light source (200-1000 nm, DH-2000-CAL; Ocean Optics).

**Lens transmission measurement**

The spectral transmission of fish lens was measured following a protocol described elsewhere [49, 167]. Lenses were surgically removed from the eyes at the completion of ERG recordings and were mounted in a hole that was drilled in a black plastic block fitted inside a standard sample cuvette. Transmission measurements between 300 and 800 nm were carried out using a bench-top spectrophotometer (Cary 300; Varian, Palo Alto, CA, USA) and were normalized between 0 and 1. For each fish, 6-10 transmission measurements were acquired from both lenses and averaged.
**Retinal whole-mounts imaging**

To study the spatial arrangement of cone photoreceptors in the retina, we viewed DAPI (4',6-diamidino-2-phenylindole)-stained retinae of representative individuals from each age group under a laser scanning microscope. Whole-mount DAPI staining procedure was adopted from a protocol described elsewhere [281]. Fish were dark-adapted for one hour before dissection and eyes were enucleated under deep red light. Neural retina was separated from other ocular tissues and fixed in 4% paraformaldehyde (PFA) buffered in phosphate-buffered saline (PBS), pH 7.4, overnight at 4°C. Retinae were washed several times in PBS and dehydrated through a graded series into methanol and stored at -20°C. Retinae were rehydrated through a graded series into PBS containing 0.1% Tween-20 (PBST) and rinsed four times in 100% PBST for 5 minutes each. To enhance penetration of the stain, the tissue was partially digested with 10 µg/µl of proteinase K (diluted 1/1000 Tris-EDTA buffer) for 30 minutes at 37°C. The tissue was then rinsed in PBST, secondarily fixed in 4% PFA-PBS for 20 minutes at room temperature (RT), and rinsed with PBST (5 cycles of 5 minutes each) prior to staining. Retinae were incubated in 10 µl of 1:200 DAPI staining solution (DAPI [10 mg/ml solution] in PB) for 1-5 minutes at RT and rinsed in PB. Finally, retinae were placed on microscope slides and viewed under a laser scanning confocal microscope (LSM 710; Carl Zeiss).

**Statistical model of photoreceptor spectral sensitivity**

The sensitivity of retinal photoreceptors is the sum of the response of all cones present in the retina. Since it is not possible to measure the sensitivity of each cone
separately in a single retina, an alternative approach is to infer the cone-specific sensitivity using our knowledge of how cones respond and the cumulative sensitivity of photoreceptors. Cumulative sensitivity of retinal cone photoreceptors was modeled as the summed absorbance of all possible cone pigments:

\[
S(\lambda) = T(\lambda) \sum_{i=1}^{n} k_i A(i, \lambda) \\
= T(\lambda) \sum_{i=1}^{n} k_i [aA_2(i, \lambda) + (1-a)A_1(i, \lambda)]
\]

where \(T(\lambda)\) represents the lens transmission at a wavelength \(\lambda\), \(A(i, \lambda)\) represents the absorbance of cone pigment class \(i\) \([i = 1, 2, ..., n]\) (Nile tilapia, \(n = 6\)), \(k_i\) denotes the contribution of cone pigment \(i\) to spectral sensitivity (cone weight), and \(S(\lambda)\) is the spectral sensitivity adjusted for the measured lens transmission. To account for different proportions of \(A_1\) (retinal) and \(A_2\) (3,4-dehydroretinal) chromophores, the absorbance of cones with \(A_1\) and \(A_2\) chromophores, \(A_1(i, \lambda)\) and \(A_2(i, \lambda)\), was taken into account, where \(a\) \([0 \leq a \leq 1]\) represents the proportion of the \(A_2\) chromophore. Cone absorbance templates for the \(A_1\) and \(A_2\) chromophores were generated following Govardovskii et al. [176] while accounting for the \(\lambda_{\text{max}}\) shift associated with varying \(A_2\) proportion [105].

The statistical model was created by combining the deterministic model (eq. 2) with the appropriate error distribution. We denote the expected spectral sensitivity by \(y(\lambda)\) and the observed spectral sensitivity by \(Y(\lambda)\). Thus, spectral sensitivity at a particular
wavelength is distributed as $Q(y(\lambda), V(y))$, which has a quasi-likelihood distribution with an expectation $y(\lambda)$ and a mean-variance relationship of $V(y)$:

$$y(\lambda) = S(\lambda)$$

$$Y(\lambda) \sim Q(y(\lambda), V(y))$$

Note that the properties of the quasi-likelihood function are similar to those of the log-likelihood function, except that instead of specifying a probability distribution for the data, only a relationship between the mean and the variance is specified in the form of a variance function. Since the deterministic model is non-linear, we need to pay particular attention to the error distribution, which could have a large influence on the fits. To estimate the mean-variance relationship, we calculated the mean and variance of photoreceptor sensitivity across all individual fish that were used for the measurement of sensitivity under each of the three background conditions, for each of the life stages. This analysis suggested a power relationship between the mean ($y$) and variance ($V(y)$) of photoreceptor sensitivity. The function has the form

$$V(y) = dy^p$$

which is the general form for Tweedie distributions, where $d$ is the dispersion parameter and $p$ determines the specific class of distribution [282]. For all groups examined, $p$
ranged between 1.07 and 1.83, which falls within the range of compound Poisson
distributions. To accommodate model fitting in a statistical software environment, we
assumed $p = 1.5$ for all groups examined. To estimate the cone weights in each individual
fish, observed sensitivity was fit to the statistical model, while the lens transmission of
each respective fish was taken into account. The dispersion parameter $d$ was estimated
from the spectral sensitivity data during the fitting process. For simplicity, spectral
sensitivity values that typically ranged $10^{-13}$-$10^{-16}$ photons cm$^{-1}$ s$^{-1}$ were multiplied by
$10^{13}$ prior to analysis. Thereafter, spectral sensitivity curves were standardized.
Specifically, for each individual, spectral sensitivity was divided by the maximum
sensitivity across the spectrum. The resultant value was multiplied by the maximum
sensitivity across all fish in the group. Analyses were performed in R 2.11.1 (R
Development Core Team 2011).

Derived cone weights could be used to estimate the expression of cone pigments
in the retina. The absolute sensitivity, time-to-peak response, and slope of the response-
irradiance relationship in fish do not vary significantly between cone classes, i.e., the
various cone classes have the same properties except for their spectral sensitivity [221,
280]. Consequently, the expression of each cone pigment in the retina could be estimated
as the weight of each cone pigment divided by the sum of weights of all cone pigments.

**Statistical analysis**

Prior to performing statistical analyses, normality of all data was examined using
the Kolmogorov-Smirnov test, and homogeneity of variance was confirmed using the
Cochran’s $C$-test [209]. The requirement for normality was not met; therefore,
nonparametric statistical analysis was performed. To examine the variation in age and body mass across sensitivity types (photoreceptor vs. OPL), the nonparametric Mann-Whitney $U$-test (MW) was used. To examine the effect of background condition on cone pigment expression (estimated based on photoreceptor sensitivity), we compared cone expression under the three background conditions using the nonparametric ANOVA, Kruskal-Wallis (KW). To examine the effect of ontogeny on cone pigment expression, we compared cone expression across the three life stages using KW. Following KW analysis, post-hoc multiple comparisons were performed with p-values calculated while accounting for the number of comparisons made [283]. Our results for both KW and MW are presented and interpreted without the use of conservative corrections for multiple comparisons such as the Bonferroni correction ($\alpha = 0.05$). While the use of Bonferroni and related procedures may reduce Type I errors, they also reduce statistical power and increase the chance of Type II error, especially in cases of smaller sample sizes [284]. We therefore chose to report observed effect size (i.e., $\eta^2$ values) along with exact p-values to allow the reader evaluation of biological importance. The larger the effect size, the larger is the difference between treatment groups (e.g., photoreceptor vs. OPL sensitivity). The effect size for KW was calculated as $\eta^2 = H/(N-1)$, where $H$ denotes the $H$ statistic of KW and $N$ denotes the total number of samples in all groups examined [285]. The effect size for MW was calculated similarly, but with the $Z$ statistic of MW replacing the $H$ statistic. Statistical analysis was performed using the Statistica software (Statsoft, Tulsa, OK, USA).
Results

Lens transmission changes with ontogeny

Spectral lens transmission was measured in fry, juvenile, and adult Nile tilapia. Lens transmission at all life stages increased toward long wavelengths, showing a rapid increase between 350 and 400 nm (Figure 4.1A). The wavelength at half-maximum lens transmission $T_{50}$ significantly positively correlated to fish age (Figure 4.1B). Therefore, as the fish age, UV wavelengths were filtered out by the lens, precluding UV wavelengths from reaching the retina.

Photoreceptor sensitivity changes with ontogeny

Photoreceptor and OPL spectral sensitivity were measured under three background light conditions for fry, juvenile, and adult Nile tilapia. Photoreceptor sensitivity was estimated from the $a$-wave of ERG of ASP-treated retina, whereas, OPL sensitivity was estimated from the $b$-wave of ERG of untreated retina. See Figure 4.2 for ERG waveforms recorded from untreated and ASP-treated retina, the spectral irradiance of the background conditions used, and the quantum catches of cone pigments.

Photoreceptor sensitivity between 400 and 700 nm did not vary appreciably across life stages (Figure 4.3), suggesting that the contribution of cone photoreceptor classes that are maximally sensitive in the 400-700 nm range (SWS2b, SWS2a, Rh2b, Rh2a, and LWS) did not vary with ontogenetic stage. In contrast, photoreceptor sensitivity in the UV spectral range (340-400 nm) varied with ontogenetic stage under all background conditions, with sensitivity in juveniles and adults lower than in fry. This
Figure 4.1 Lens transmission in the Nile tilapia throughout ontogeny

(A) Spectral lens transmission \( (n = 38) \) increased toward longer wavelengths. (B) The wavelength of half-maximum transmission \( T_{50} \) increased with the age of the fish. Age was measured in days post fertilization (d.p.f.).
Figure 4.2 ERG waveforms recorded from untreated and ASP-treated retina, the background light conditions used, and theoretical quantum catches of cone pigments

(A, B) An ERG waveform consists of an initial hyperpolarization phase (‘a.w.; \(a\)-wave) representing the response of photoreceptors to the onset of light, followed by a depolarization phase (‘b.w.; \(b\)-wave) representing the response of ON bipolar cells to the onset of light. (A) The response of the OPL was measured as the potential change between the \(a\)-wave and \(b\)-wave from an untreated retina. (B) To isolate the response of photoreceptors, retinae were treated with sodium-L-aspartate (ASP). The response of photoreceptors alone was measured as the amplitude of the \(a\)-wave from an ASP-treated retina. (C) Spectral sensitivity was measured under three background light conditions (BG1-BG3), each designed to emphasize the activity of different cone classes. (D) Quantum catches of cone pigments were estimated for each background condition while accounting for the lens transmission and the absorbance of visual pigments with A2 chromophore. For each background condition, bars represent the quantum catches of the SWS1, SWS2b, SWS2a, Rh2b, Rh2a, and LWS cone pigments (left to right). Quantum catches for fry, juvenile, and adult Nile tilapia were practically similar, thus quantum catches are presented for juveniles only.
Figure 4.3 Photoreceptor sensitivity across different background conditions (BG1-BG3; A-C) for fry, juvenile, and adult Nile tilapia

Photoreceptor sensitivity in the UV spectral range (340-400 nm) varied considerably with ontogeny for all background conditions tested, with sensitivity in juveniles and adults lower than in fry. In contrast, photoreceptor sensitivity at longer wavelengths (400-700 nm) varied only slightly with ontogeny. Error bars represent one standard error from the mean.
reduction in sensitivity during ontogeny could arise from two possibilities: (i) reduction in the proportion of UV wavelengths reaching the retina due to a reduction in lens transmission of UV light, and/or (ii) reduction in the expression of the SWS1 cone pigment that is maximally sensitive between 360 and 373 nm (dependent on chromophore composition).

To identify the source of reduction in photoreceptor sensitivity to UV wavelengths, we fit a statistical model to the observed spectral sensitivity to estimate the expression of cone pigments in the retina. Photoreceptor sensitivity was modeled while accounting for the lens transmission and the absorbance of all possible cone pigments. See Figure 4.4A for example fits. To examine ontogenetic changes in cone pigment expression, we compared cone expression across the three life stages. For each life stage, cone expression data for all fish under the three background conditions were pooled (expression of cone pigments did not vary significantly across background conditions [Table 4.1]). Apart from the expression of the SWS1 cone pigment, the expression of all other cone pigments did not differ significantly across life stages (Figure 4.4B; detailed statistics in figure caption). The expression of SWS1 in fry was significantly higher than in adults, but not in juveniles (cone expression of juveniles and adults did not differ significantly). Thus, the ontogenetic reduction of photoreceptor sensitivity to UV wavelengths was a result of reduction in the expression of SWS1 cone pigment in addition to the change in lens transmission; suggesting that fry versus juveniles and adults had a higher expression level of SWS1 cone pigment.
Figure 4.4 Variation in photoreceptor sensitivity and cone pigment expression with ontogeny

(A) Example fits (lines) to photoreceptor sensitivity (circles) measured in fry, juvenile, and adult Nile tilapia; spectral sensitivity curves are vertically displaced for clarity. (B) Variation in cone pigment expression with ontogeny (estimated from photoreceptor sensitivity). The expression of SWS1 differed significantly with ontogeny (Kruskal Wallis, $H = 9.08$, d.f. = 2, $N = 49$, $\eta^2 = 0.189$, $P = 0.011$). Post hoc analysis revealed that cone expression in fry was significantly higher than in adults ($P = 0.029$), but not in juveniles ($P = 0.077$). Cone expression in juveniles and adults did not differ significantly ($P = 1.000$). Expression of all other cone classes did not vary significantly with ontogeny (Kruskal Wallis, d.f. = 2, $N = 49$; SWS2b: $H = 3.48$, $\eta^2 = 0.073$, $P = 0.175$; SWS2a: $H = 1.16$, $\eta^2 = 0.024$, $P = 0.560$; Rh2b: $H = 0.56$, $\eta^2 = 0.012$, $P = 0.757$; Rh2a: $H = 1.10$, $\eta^2 = 0.023$, $P = 0.576$; LWS: $H = 1.16$, $\eta^2 = 0.024$, $P = 0.559$). The proportion of the A2 chromophore did not differ significantly across life stages (Kruskal Wallis, $H = 0.39$, d.f. = 2, $N = 49$, $\eta^2 = 0.008$, $P = 0.823$) and equalled 0.59±0.22 (average±s.d.), suggesting a mixed-chromophore retina in Nile tilapia.
Table 4.1 Variation in cone pigment expression across background light conditions

<table>
<thead>
<tr>
<th>Cone class</th>
<th>Fry</th>
<th></th>
<th></th>
<th>Juvenile</th>
<th></th>
<th></th>
<th></th>
<th>Adult</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$H(2,15)$</td>
<td>$\eta^2$</td>
<td>$P$</td>
<td>$H(2,19)$</td>
<td>$\eta^2$</td>
<td>$P$</td>
<td>$H(2,15)$</td>
<td>$\eta^2$</td>
<td>$P$</td>
<td></td>
</tr>
<tr>
<td>SWS1</td>
<td>3.29</td>
<td>0.235</td>
<td>0.192</td>
<td>1.16</td>
<td>0.064</td>
<td>0.559</td>
<td>4.57</td>
<td>0.326</td>
<td>0.101</td>
<td></td>
</tr>
<tr>
<td>SWS2b</td>
<td>0.85</td>
<td>0.061</td>
<td>0.653</td>
<td>6.84</td>
<td>0.380</td>
<td>0.032</td>
<td>1.04</td>
<td>0.074</td>
<td>0.594</td>
<td></td>
</tr>
<tr>
<td>SWS2a</td>
<td>1.14</td>
<td>0.081</td>
<td>0.565</td>
<td>2.57</td>
<td>0.143</td>
<td>0.276</td>
<td>2.47</td>
<td>0.176</td>
<td>0.291</td>
<td></td>
</tr>
<tr>
<td>Rh2b</td>
<td>0.01</td>
<td>0.001</td>
<td>0.993</td>
<td>0.63</td>
<td>0.035</td>
<td>0.728</td>
<td>1.83</td>
<td>0.131</td>
<td>0.399</td>
<td></td>
</tr>
<tr>
<td>Rh2a</td>
<td>5.77</td>
<td>0.412</td>
<td>0.056</td>
<td>0.46</td>
<td>0.026</td>
<td>0.794</td>
<td>3.45</td>
<td>0.246</td>
<td>0.178</td>
<td></td>
</tr>
<tr>
<td>LWS</td>
<td>1.44</td>
<td>0.103</td>
<td>0.484</td>
<td>1.64</td>
<td>0.091</td>
<td>0.44</td>
<td>4.82</td>
<td>0.344</td>
<td>0.089</td>
<td></td>
</tr>
</tbody>
</table>

To examine the variation in cone pigment expression across background light conditions, the nonparametric ANOVA, Kruskal-Wallis, was used. The $H$ statistic, degrees of freedom (d.f.), and number of samples (N) are presented as $H$(d.f.,N). The effect size ($\eta^2$) and p-value ($P$) are indicated. The larger the effect size - the larger is the difference in cone pigment expression between fish used for measuring photoreceptor sensitivity under the three background conditions. p-values for cone pigments that showed significantly different expression across the three background conditions ($P < 0.05$) are marked in bold. Apart from the expression of SWS2b in juveniles, the expression of all cone pigments, for all life stages, did not vary significantly across background conditions.
Square cone mosaic does not change with ontogeny

The geometry of the square cone mosaic did not change during ontogeny, showing a single cone surrounded by four double cones (Figure 4.5). Given that single cones in tilapia express short-wavelength sensitive opsin pigments, the single cone either shifted its pigment expression from SWS1 to SWS2a/b or coexpressed SWS1 along with SWS2a/b with changing ontogenetic stage.

OPL sensitivity changes with ontogeny

OPL sensitivity changed considerably in the UV range, with sensitivity in juveniles and adults lower than in fry (Figure 4.6). Additionally, OPL sensitivity changed to a greater extent between 400 and 700 nm, with long wavelength sensitivity (500-700 nm) in adults lower than in fry and juveniles (similar to a sensitivity increase in the 400-500 nm range). These differences in OPL sensitivity between the adult stage and the fry and juvenile stages could be explained by (i) comparable changes in photoreceptor sensitivity and (ii) change in the retinal circuitry. These two mechanisms are evaluated below.

Retinal circuitry changes with ontogeny

A means to examine the extent of ontogenetic change in sensitivity is to calculate the sensitivity difference between fry and adults (‘fry – adult difference’). Thus, no ontogenetic change in sensitivity would result in zero difference across the spectrum, whereas, an ontogenetic change in sensitivity would result in a non-zero difference (Figure 4.7A). Moreover, if ontogenetic changes in OPL sensitivity arise from changes in
Figure 4.5 Cone square mosaic in fry, juvenile, and adult Nile tilapia

Spacing between cone photoreceptors in the fry was smaller than in the juvenile and adult. However, cone square mosaic stayed fixed throughout ontogeny, exhibiting a single cone surrounded by four double cones. The age and body mass of fish were 96 days post fertilization (d.p.f.) and 1.8 g for the fry; 138 d.p.f. and 10.2 g for the juvenile; and 273 d.p.f. and 86 g for the adult. Scale bar applies to all panels.
Figure 4.6 Sensitivity of the outer plexiform layer (OPL) across different background conditions (BG1-BG3; A-C) for fry, juvenile, and adult Nile tilapia.

In the UV range (340-400 nm), OPL sensitivity in juveniles and adults was lower than in fry, whereas at long wavelengths (500-700 nm), sensitivity in adults was lower than in fry and juveniles. These trends were evident under all background light conditions. The ontogenetic sensitivity increase at short wavelengths observed under BG1 is equivalent to the ontogenetic sensitivity decrease at long wavelengths observed under BG2 and BG3 (the apparent opposite trends are merely a result of choice of normalization method). Error bars represent one standard error from the mean.
Figure 4.7 Mean Sensitivity difference between fry and adults

(A) A theoretical zero sensitivity difference across the spectrum that reflects no ontogenetic change in sensitivity, and a theoretical non-zero sensitivity difference that reflects an ontogenetic change in sensitivity at long wavelengths (sensitivity in fry is higher than in adults). (B) A theoretical correlation between the fry – adult difference in photoreceptor and OPL sensitivity, suggesting that ontogenetic changes in OPL sensitivity to short wavelengths arise from changes in photoreceptor sensitivity. (C, D) Sensitivity difference between fry and adults was calculated for photoreceptor sensitivity as well as for OPL sensitivity for background light conditions BG2 and BG3. Differences in photoreceptor and OPL sensitivity significantly correlated in the UV spectrum (340-400 nm; BG2 - Pearson $r = 0.9365$, $P = 0.0059$, average spectral difference = 0.78 and 0.51 log units for photoreceptor and OPL sensitivity; BG3 - Pearson $r = 0.8858$, $P = 0.0188$, average spectral difference = 1.05 and 0.44 log units for photoreceptor and OPL sensitivity), suggesting that ontogenetic changes in the UV range of OPL sensitivity arise from changes in photoreceptor sensitivity. In contrast, differences in photoreceptor and OPL sensitivity did not correlate at longer wavelengths (500-700 nm, BG2 - Pearson $r = 0.1646$, $P = 0.4759$, average spectral difference = 0.04 and 0.47 log units for photoreceptor and OPL; BG3 - Pearson $r = -0.3335$, $P = 0.1629$, average spectral difference = 0.03 and 0.59 log units for photoreceptor and OPL), suggesting ontogenetic changes in the retinal circuitry.
photoreceptor sensitivity, photoreceptor and OPL sensitivity are expected to correlate (Figure 4.7B). To examine whether ontogenetic changes in photoreceptor sensitivity correlate to changes in OPL sensitivity, we compared the fry - adult difference between photoreceptor sensitivity and OPL sensitivity (Figure 4.7C,D). This was possible only for the BG2 and BG3 background conditions while ensuring matching of fish age and body mass (see Table 4.2 for detailed statistics). In the UV spectrum (340-400 nm), differences in photoreceptor and OPL sensitivity significantly correlated, indicating that ontogenetic changes in the UV range of OPL sensitivity arise from changes in photoreceptor sensitivity. However, unexpectedly, at longer wavelengths (500-700 nm), differences in photoreceptor and OPL sensitivity did not correlate. Additionally, differences in photoreceptor sensitivity typically did not deviate from zero difference while those in OPL did. This suggests that at long wavelengths, ontogenetic changes in OPL sensitivity do not arise from changes in photoreceptor sensitivity, and likely arise from changes in the retinal circuitry. At long wavelengths, OPL sensitivity in fry was higher than in adults (positive difference), indicating reduced OPL sensitivity to long wavelengths in adults.

Discussion

This study systematically traced ontogenetic changes across several elements and processing levels in the visual system of fish, i.e. from the transmission of the lens that controls the wavelengths reaching the retina, to the retinal circuitry that processes the signals from photoreceptors prior to being conveyed to downstream elements in the eye and visual centers in the brain. This study delivers several significant findings for the understanding of the developmental trajectory of visual function in fish. Nile tilapia was
Table 4.2 Variation in fish age and body mass across sensitivity types (photoreceptor vs. OPL).

<table>
<thead>
<tr>
<th></th>
<th>Fry</th>
<th>Juvenile</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Age (d.p.f.)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BG1</td>
<td>0.00</td>
<td>13</td>
<td>0.000</td>
</tr>
<tr>
<td>BG2</td>
<td>0.48</td>
<td>12</td>
<td>0.044</td>
</tr>
<tr>
<td>BG3</td>
<td>0.68</td>
<td>15</td>
<td>0.049</td>
</tr>
<tr>
<td><strong>B. Body mass (g)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BG1</td>
<td>0.38</td>
<td>13</td>
<td>0.032</td>
</tr>
<tr>
<td>BG2</td>
<td>0.18</td>
<td>12</td>
<td>0.016</td>
</tr>
<tr>
<td>BG3</td>
<td>0.00</td>
<td>15</td>
<td>0.000</td>
</tr>
</tbody>
</table>

To examine the variation in age (A) and body mass (B) across sensitivity types (photoreceptor vs. OPL), the nonparametric Mann-Whitney U Test (d.f. = 1) was used. The Z statistic (Z), number of samples (N), effect size (η²), and p-value (P) are indicated. p-values for treatments that differed significantly (P < 0.05) in fish age or body mass (BM) are marked in bold. The larger the effect size - the larger is the difference in age or BM between fish used for measuring photoreceptor and OPL sensitivity. Age and BM of fry did not differ significantly between photoreceptor and OPL sensitivity for either background condition. Age, but not BM, of juveniles differed significantly between photoreceptor and OPL sensitivity for all background conditions. Age and BM of adults differed significantly between photoreceptor and OPL sensitivity for BG1 but not for BG2 or BG3. Therefore, photoreceptor sensitivity in fry could be compared to OPL sensitivity across all background conditions. Photoreceptor sensitivity in juveniles could not be compared to OPL sensitivity at any background condition. Photoreceptor sensitivity in adults could be compared to OPL sensitivity only for BG2 and BG3. Fish age was measured in days post fertilization (d.p.f.) and body mass was measured in grams (g).
shown to undergo ontogenetic changes in lens transmission, photoreceptor sensitivity, and sensitivity of the outer plexiform layer. The decrease in photoreceptor sensitivity observed during ontogeny resulted from reduction in the expression of the SWS1 cone pigment, and was associated with a reduction in lens transmission at UV wavelengths. Additionally, OPL sensitivity to UV and long wavelengths decreased with age. These changes in OPL sensitivity were a result of (i) ontogenetic changes in photoreceptor sensitivity, mediated through a reduction in the expression of the SWS1 pigment, in the UV spectral range, as well as (ii) ontogenetic changes in retinal circuitry leading to reduced sensitivity at longer wavelengths.

**Association between different elements of the visual process**

What elements/processes in the visual system can serve as predictors to the visual sensitivity of fish? The ability of elements/processes of the visual system to serve as predictors to visual sensitivity was wavelength-dependent. The reduced expression of the SWS1 cone pigment accompanied a reduction in lens transmission to UV wavelengths, and led to a reduction in photoreceptor sensitivity and OPL sensitivity to UV wavelength. Thus, reduced OPL sensitivity to UV wavelengths could be predicted based on the transmission of the lens, cone pigment expression, or photoreceptor sensitivity. In contrast, the observed reduction in OPL sensitivity to long wavelengths could not be predicted either by the lens transmission, cone pigment expression, or photoreceptor sensitivity. Therefore, any attempt to understand visual function or to relate it to the sensory world while using the lens transmission, expression of cone pigments, or photoreceptor sensitivity as predictors would likely fail. In this regard, the use of the
expression of cone opsin genes or the frequency of cone photoreceptors in predicting visual sensitivity is also likely to fail since these measures are strongly linked to the expression of cone pigments. Thus, our results emphasize the importance of considering the visual sensitivity of fish, rather than its proxies, to allow for an accurate and efficient investigation of visual functions and adaptations.

**Ontogenetic changes in lens transmission**

Our results show a correlation between fish age and the wavelength of half-maximum lens transmission $T_{50}$. That is, lens transmission of UV wavelengths declined with fish age. In tilapia, ontogenetic changes in lens transmission were reported to follow two phases [47]. In the first phase, lens diameter and rate of lenticular pigment accumulation increase, resulting in a steady increase in $T_{50}$. In the second phase, lenticular pigment accumulation slows down but lens diameter continues to increase, resulting in no further increase in $T_{50}$. We found no evidence for the levelling off of $T_{50}$. It is possible that the previously reported levelling off of $T_{50}$ was found in fish older than those represented in the current study. Unfortunately, details regarding the age of the fish studied previously were not provided, therefore precluding the confirmation of this possibility.

**Ontogenetic changes in cone pigment expression and photoreceptor sensitivity**

We report a reduced expression of the SWS1 cone pigment in adults as compared to fry. Lisney et al. [167] measured the spectral sensitivity of adult tilapia from an eye-cup (lens excluded) and found no evidence for contribution of the SWS1 pigment.
Additionally, gene expression studies have reported lower expression of the \textit{SWS1} opsin gene in adults as compared to larvae [96, 134]. Therefore, our results are in agreement with previous studies. Additionally, past gene expression studies have reported increased expression of the \textit{LWS} opsin gene with ontogeny [96, 134]. Our results show a slight, non-significant, ontogenetic increase in the expression of the LWS cone pigment. The disagreement between the current and past studies probably arises from differences in the classification of fish to the different life stages. For example, in the current study, fish whose age was 67±19 (average±s.d.) days post fertilization (d.p.f.) were classified as the youngest group (fry), whereas, in a previous study [134], fish whose age was 14-18 d.p.f. were classified as the youngest group (larva). It is possible that changes in the expression of the LWS pigment occurred at an earlier age, not represented in our sample. Thus, the discrepancy between the \textit{LWS} gene (past studies) and pigment (current study) expression levels might be a consequence of classification differences.

\textbf{Ontogenetic changes in cone mosaic}

The square cone mosaic in the Nile tilapia stayed fixed throughout ontogeny, with a single cone surrounded by four double cones, as reported for adults [274, 275]. Single cones in tilapia can express any of the short wavelength cone pigments, SWS1, SWS2b, and SWS2a [96]. Yet, single cones can also potentially co-express any combination of the three short wavelength pigments. Our results show that the relative expression of the SWS1 cone pigment was highest in fry and declined with ontogeny, suggesting that the SWS1 cone pigment was the primary pigment expressed in single cones in fry. With ontogeny, however, the relative expression of the SWS2b and/or SWS2a increased. This
raises the possibility of co-expression of the SWS2b and SWS2a cone pigments, or even co-expression of all three short wavelength cone pigments in juveniles and adults. This possibility of co-expression of cone pigments needs to be confirmed by measuring the absorbance of individual single cones using MSP, measuring the sensitivity of individual single cones using electrophysiological techniques, or by localizing the mRNA of cone opsin genes using *in situ* hybridization. Additionally, the eccentricity of the retinal samples used for examining the spatial arrangement of the cone mosaic could not be determined. Thus it is possible that sampling retina portions of different eccentricities would show cone mosaics of other configurations [82].

**Ontogenetic changes in retinal circuitry**

Our results suggest a change in the retinal circuitry when moving from fry to adults. This finding was based on low correlation of the fry – adult difference between photoreceptor and OPL sensitivity at long wavelengths. However, an alternative possibility could potentially explain the observed reduction in OPL sensitivity to long wavelengths during ontogeny. That is, since sensitivity of fry and adults was measured at different times, a change in the spectral distribution of the background illumination throughout the course of the experiment could have generated the differences observed in OPL sensitivity between fry and adults. In our study, however, the irradiance of the background channels was monitored and adjusted continuously throughout the experimentation period. Irradiance of background channels varied only slightly, with the standard deviation of relative irradiance ranging 0.005-0.116 and 0.006-0.151 log units (*n* = 3) across the spectrum, for the two background channels. Such small variation in
background irradiance cannot explain the sensitivity difference between fry and adults that exceeded 1 log unit in some cases (Figure 4.7C,D). In fact, to produce such large differences in spectral sensitivity of adults, spectral irradiance of background light needed to show differences of more than 4 log units (BG1-BG3; Figure 4.2C and Figure 4.6). In summary, this alternative explanation for the differences observed in OPL sensitivity between fry and adults seems remote; supporting our suggestion of an ontogenetic change in the retinal circuitry.

Remodelling of the retinal circuitry was previously reported during early development - prior to maturation of a functional visual system and robust visual behaviours. The sequence of retinal circuitry development is conserved across vertebrate species. Ganglion and amacrine cells are the first classes to differentiate, forming the earliest functional circuits in the inner plexiform layer (IPL) [286]. Subsequently, horizontal cells and photoreceptors differentiate and contact each other in the outer retina, forming the outer plexiform layer (OPL) [287]. The networks for the vertical flow of information in the inner and outer retina are later interconnected when bipolar cells differentiate and connect to the various cells in the IPL and OPL [288]. This sequence of development can reach completion in the prenatal stage, or typically no later than a few days or weeks after birth, e.g. prior to hatching in chicks, 70 hours post fertilization in zebrafish, 15 postnatal days in mice, and 35 postnatal days in the ferret [289, 290].

Remodelling of the retinal circuitry was also reported to occur during aging - upon reaching adulthood. This often involves photoreceptor degeneration that leads to degeneration of neuronal processes and compensatory formation of new connections
For example, degeneration of rod photoreceptors in mice was associated with the loss of rod bipolar cells’ dendrites, re-distribution of glutamate receptors, reduced complexity of the horizontal cell network [292], and the transient contact of rod bipolar cells to cones [293]. Aging in mice was also associated with a reduction in the number of ganglion cells [294] and modification in the synaptic connections of rod bipolar cells and horizontal cells [295].

In sharp contrast to remodelling of the retinal circuitry during early development and aging, the remodelling of retinal circuitry reported in this study occurred between the fry and adult life stages, representing processes that take place following the maturation of the visual system but prior to reaching adulthood. This study is the first to suggest remodelling of the retinal circuitry in a time window that may allow for optimization of the visual system to the changing sensory environment. However, a detailed anatomical examination would be required in order to explore the mechanisms underlying the suggested change in circuitry.

**Potential adaptive significance of ontogenetic changes in OPL sensitivity**

*Reduction in UV sensitivity with ontogeny.* Tilapia adults are omnivorous, feeding mainly on macrophytes, but also on detritus, diatoms, and aquatic invertebrates that include copepods, cladocerans, and rotifers [296, 297]. In contrast, tilapia fry are largely planktivorous, feeding on diatoms, amphipods, insects, and copepods [128, 298, 299]. Zooplankton absorb UV light [9]. Consequently, UV sensitivity, mediated by the SWS1 cone class, may enhance the contrast of zooplankton against the water background, thereby aiding in their detection [52]. UV sensitivity is therefore expected to be
advantageous for the planktivorous tilapia fry, but not so for the omnivorous tilapia adults. Furthermore, UV sensitivity might be disadvantageous when not necessary, as it is presumed in tilapia adults. UV wavelengths are scattered strongly in the water media, and thus, sensitivity to, and the inclusion of UV wavelengths in image-forming light may blur and degrade the retinal image [300]. Thus, the ontogenetic reduction in UV sensitivity in tilapia likely has an adaptive value.

Reduction in long wavelength sensitivity with ontogeny. Mate-choice and male-male competition for territory are key processes in the life of Nile tilapia adults, and depend on visual stimulation and reliable visual assessment of the quality of conspecifics [301]. Nile tilapia evolved in rivers and pools in central and eastern Africa [302]. Like many freshwater habitats, these rivers likely exhibit turbid water that attenuates short wavelengths of light, resulting in a long-wavelength-shifted visual environment [253, 303]. Thus adult tilapia in their natural habitat are illuminated by the relatively broad light spectrum coming from above, and viewed against a long-wavelength-shifted water background. Under these conditions, a visual system with low sensitivity to long wavelengths in the adults would be largely insensitive to the long-wavelength light from the water background. Tilapia adults, which exhibit a brightly reflecting silvery trunk [302], would therefore appear brighter against a dark background. Thus, low sensitivity to long wavelengths may increase the radiance (luminance) contrast and conspicuousness of conspecific adults (‘contrast hypothesis’) [213, 304]. Unlike adult tilapia that occupy relatively deep water (down to 12 m), tilapia fry occupy very shallow water (<1 m) [298], and would be less subject to the influence of the long wavelength shift due to water
turbidity. Reduced sensitivity to long wavelengths is therefore expected to be more advantageous for tilapia adults than for tilapia fry. Thus, the reduction in long wavelength sensitivity with ontogeny in tilapia likely has an adaptive value. Yet, a more realistic situation would depend on chromatic rather than luminance contrast. Then, signals from different cone classes would form colour-opponent channels, through which the radiance arriving from the body pattern of conspecifics and from the background would be compared. Evaluation of such a possibility will need to wait until the configuration of colour-opponency in the Nile tilapia or related species is known.
Chapter 5

General Discussion

The overall objective of this thesis was to better understand the mechanisms that shape the diversity in colour vision of fish, and to explore the adaptive significance of this divergence. The cichlid model system illustrates that the visual system of fish may differ across species, sexes, individuals, and life stages of individuals. The large number of available cone opsin genes, which have resulted from multiple opsin gene duplications, facilitates this variation in the mechanisms of colour vision. My results suggest that the diversification of colour vision across species and across life stages of individuals contributes to sensory adaptations that enhance both the contrast of zooplanktonic prey, and the detection of optical signals of conspecifics. Therefore, both natural and sexual selection may work in concert to shape colour vision in fish. Additionally, my results suggest an important role for the complexity of environmental light in shaping the high-dimensional colour vision in fish.

In the second chapter of this thesis, I examined the variation in the number of cone classes that contribute to spectral sensitivity in females and males of three cichlid species from Lake Malawi. I found that ninety seven percent of fish examined employed cone complements of four to five pigments, with most *Melanochromis auratus* and *Protomelas taeniolatus* exhibiting five functional cone classes. The spectral sensitivity of all three species was shaped by both opponent and non-opponent cone interactions.
Therefore, these data indicate that the basic requirements for tetra- and pentachromatic colour vision are being satisfied in the cichlid species examined.

My results differ from the notion that Lake Malawi cichlids primarily exhibit three cone pigments [69, 97]. This discrepancy most likely arises from the tendency in recent gene expression studies to disregard cone opsin genes that are expressed at ‘low levels’ without any justification or discussion of potential implications [123, 305]. In fact, a recent large-scale survey of the expression of opsin genes in more than 50 Malawi cichlid species has shown that 43% of the examined species expressed four cone opsin genes, 13% of species expressed five cone opsin genes, and 4% of species expressed six cone opsin genes [73]. Furthermore, MSP studies have revealed up to seven cone pigment classes in the retina of various cichlids [69, 97]. My work suggests that even rare cone populations and opsin genes that are expressed at low levels contribute to the spectral sensitivity of fish. This emphasizes the need to standardize the interpretation of cone opsin gene expression levels and to seek an expression level threshold for the contribution of opsin genes to vision.

This study also provides evidence for sex differences in opsin gene expression, and possibly in spectral sensitivity, for cichlids. The most prominent sex differences occurred in *M. auratus*. Unlike the two other examined species, *M. auratus* males do not hold a permanent territory and both *M. auratus* females and males exhibit colourful, but very different, body patterns [178]. This information raises the possibility of an active role of males in mate-choice. Thus, I propose that the distribution of sex differences in the visual system across species might be associated with territorial behaviour and the
different roles of the sexes in mate-choice. However, the adaptive role of sexual
dimorphism in colour vision of Lake Malawi cichlids certainly requires further
examination. For example, it would be beneficial to behaviourally investigate the roles of
each sex in mate-choice, and whether not holding a permanent territory indeed allows the
males to choose between females.

Finally, the frequency of the SWS1 cone pigment across Malawi cichlid species
qualitatively correlated with planktivory. UV sensitivity mediated by the SWS1 cone
pigment enhances the contrast of zooplankton against the water background, and thus,
potentially aids in their detection [52, 116]. Thus, the presence of the SWS1 pigment in
the retina of cichlids likely has a significant adaptive value. Additionally, the frequency
of the LWS cone pigment across species quantitatively correlated with the proportion of
long wavelength reflectance in the colour pattern of conspecific males. Therefore, the
presence of the LWS pigment in the retina of cichlids likely also has a significant
adaptive value. Taken together, the variation in cone pigment frequency across species
seems to reflect visual adaptations that both enhance the detection of zooplanktonic prey
and signals important for mate-choice and male-male competition, suggesting that both
natural selection and sexual selection played a role in shaping colour vision in cichlids.

The tendency of expressing a large number of cone opsin genes or pigments is
general to teleost fish rather than restricted to the Cichlidae family. It is now clear that
opsin gene duplications resulted in an increase in the number of cone opsin genes in
teleost fish, an infraclass of ray-finned fish [68, 69, 71, 91, 134]. The numerous available
cone opsin genes facilitate the expression of diverse complements of large numbers of
cone opsins, and ultimately, pigments. For example, zebrafish express seven cone pigments [70, 74]; guppies express five cone pigments [87]; and salmonids [89, 90], black bream [91], goldfish [92, 93], and three-spine stickleback [94] express at least four cone pigments.

The unavoidable question is why do fish need to express so many cone pigment classes? Humans appear to adequately process the visual world through three cone photoreceptor classes. Why, then, do fish need to use a larger number of cone classes? What is the adaptive value of such high-dimensional colour vision systems? In the third chapter of this thesis, I aimed at exploring what has driven the increase in the number of cone classes in the high-dimensional colour vision systems of fish. I raised the possibility that the high complexity of underwater light has contributed to the high-dimensionality of fish colour vision. I evaluated the dimensionality of colour vision of fish in comparison to humans and old-world primates by examining the complexity of the colour-signals (radiance) reflected from aquatic and terrestrial objects, respectively. The underlying assumption was that to ensure recovery of an object’s reflectance, the visual system has to sample the colour-signal as accurately as possible.

I found that the number of cone classes required for reconstructing the colour-signal of aquatic objects was larger than that of terrestrial objects, regardless of whether the unfiltered colour-signal or the band-limited colour-signal passed by the cones was considered. These results show that fish would require four to six cone classes for reconstructing the colour-signal of aquatic objects at the accuracy level achieved by humans. The need for a larger number of cone classes for colour reconstruction was an
attribute of the aquatic environment, regardless of water type and ecosystem in concern. This suggests that the large diversity of cone opsin genes and high-dimensionality colour vision in fish have likely evolved to enhance the reconstruction of the complex colour-signals of aquatic objects.

It is important to note, however, that the presence of a large number of cone pigments in the retina may not necessarily suggest the ability of high-dimensional colour vision. First, cone pigments and the expression of cone opsin genes may be distributed differently across the retina. Such intraretinal variability in the proportions of cone pigments and the expression of opsin genes has been reported in many fishes, e.g., cichlids [306], salmonids [80, 81], archer fish (Toxotes chatareus) [307], zebrafish (Danio rerio) [308], and the rudd (Scardinius erythrophthalmus) and dragonet (Callionymus lyra) [309]. Second, multiple spectral cone classes, subserved by multiple cone pigments, may be used for tasks other than colour vision. For example, the presence of two spectral classes of photoreceptors and opponent interaction of their signals were suggested to allow for elimination of the flicker induced by surface waves from the retinal image, thereby aiding in the detection of moving prey and predators [310]. In such a hypothetical situation, the dimensionality of colour vision might be lower than it appears to be based solely on the number of spectral cone classes present.

Importantly, my results do not imply that all fish are expected to possess four to six cone classes. Fish might possess a smaller number of cone classes, but with this comes the inevitable loss in accuracy of signal reconstruction. Taken together, these findings significantly advance our understanding of the evolution of colour vision.
Additionally, the comparison of human and fish vision provides a rare and valuable window into how well fish detect the colours of their partners, competitors, and food, which in turn could improve our understanding of animal behaviour, ecology and evolution.

In the fourth chapter of this thesis, I examined changes in the mechanisms of colour vision throughout the ontogeny of the Nile tilapia. I systematically traced ontogenetic changes across six elements and processing levels in the visual system that may potentially affect colour vision in fish. I found that the ontogenetic reduction observed in the sensitivity of the outer plexiform layer (OPL) to long wavelengths could not be predicted either by the cone pigment expression or photoreceptor sensitivity. This suggests that any attempt to understand visual function while using the expression of cone opsin genes and pigments, or photoreceptor sensitivity as predictors, would likely fail. Knowledge of the expression levels of cone opsin genes and pigments is valuable and may serve as the basis for determining the cone classes that contribute to spectral sensitivity. However, cone photoreceptors merely sample the light incident on the retina. Considering the extensive neural processing at post-receptoral sites, the use of the spectral location and expression levels of cone pigments to understand the sensory world of fish is all but optimal. This emphasizes the importance of considering the spectral sensitivity as recorded from post-receptoral sites in fish. In this study, I examined the sensitivity of the outer plexiform layer, which represents the signals of photoreceptors after relayed to horizontal and bipolar cells. However, to more fully understand the visual sensitivity in fish, psychophysical measures should be employed.
I have also found that the retinal circuitry in the Nile tilapia changed throughout ontogeny. Unlike the remodelling of the retinal circuitry during early development and aging, the remodelling of retinal circuitry reported in this study occurred between the fry and adult life stages, representing processes that take place following the maturation of the visual system but prior to reaching adulthood. This study is the first to suggest remodelling of the retinal circuitry in a time window that may allow for optimization of the visual system to the changing sensory environment. However, a detailed anatomical examination would be required in order to explore the mechanisms underlying the suggested change in circuitry.

Finally, my analysis suggests that the ontogenetic reduction in UV and long wavelength sensitivity likely has an adaptive value. Specifically, UV sensitivity, mediated by the SWS1 cone class, may enhance the contrast of zooplankton against the water background, thereby aiding in their detection [52]. UV sensitivity is therefore expected to be advantageous for the planktivorous tilapia fry, but not so for the omnivorous tilapia adults. Additionally, adult Nile tilapia occupy relatively deep water that exhibit a long-wavelength-shifted spectrum. Consequently, low sensitivity to long wavelengths in adults may increase the conspicuousness of conspecifics. On the other hand, tilapia fry occupy very shallow water and would be less subject to the influence of this depth-dependent long wavelength shift. In conclusion, the decrease in sensitivity to UV and long wavelengths with ontogeny might reflect changes in the foraging style and depth distribution of the different life stages of Nile tilapia. This study advances our understanding of ontogeny in visual systems and demonstrates that the association
between different elements of the visual process can be explored effectively by examining visual function throughout ontogeny.

The data presented in this thesis may significantly improve our understanding of the variable nature of fish colour vision across various divergence levels, the mechanisms that shape the diversity in colour vision, and in some cases, also the adaptive significance of the divergence observed. In spite of these advances in our understanding of the visual function in fish, many important challenges remain. A central challenge is to bridge the gap between our knowledge of the characteristics of cone pigments to that of the characteristics of colour channels. The number and type of these colour channels and the interactions between the different channels are the basis of colour vision [4]. Some possible approaches that can be employed to study the characteristics of cone pigments and the colour channels in fish are discussed below.

Several techniques are routinely used to estimate the location and expression levels of cone pigments in the retina. These techniques include quantitative polymerase chain reaction (qPCR), *in situ* hybridization, immunohistochemistry, microspectrophotometry (MSP), and electrophysiology. qPCR can provide information about the level of mRNA of opsin genes, and thus, provides only a proxy for the expression of cone pigments. *In situ* hybridization can provide information about the location of mRNA of opsin genes, but not about the expression levels of opsin genes or the expression and location of cone pigments. Immunohistochemistry can provide information about the location of cone pigments, but not about their expression level. MSP can provide information about the absorbance of cone photoreceptors. However, it
cannot provide conclusive information about the expression levels of cone pigments. This arises from two main reasons: (i) the absorbance of cone photoreceptors might reflect the absorbance of several cone pigments that are coexpressed in the same photoreceptor, and (ii) the dependence of the absorbance of cone photoreceptors on the chromophore composition in the retina introduces further complexity. Lastly, the expression levels of cone pigments can be approximated by fitting cone absorbance templates to sensitivity spectra that were determined electrophysiologically (e.g. [85, 167]). However, due to the contribution of post-receptoral processing to spectral sensitivity, this technique often yields fits of low quality that prevents cone pigments from being identified at high certainty.

The number and expression level of cone pigments and cone photoreceptors can be estimated based on some of the techniques mentioned above. However, the number and characteristics of colour channels, which can potentially be formed by any possible combination of cone signals, cannot be determined by any of those techniques. The characteristics of colour channels can be studied by psychophysically measuring the colour discrimination capabilities of fish (e.g. [259]). Additionally, the characteristics of colour channels can also be studied by determining the connectivity in the retina by recording the signals of complete populations of ganglion cells that represent the output signal of the retina [311]. For both approaches, considering the large number of cone pigments that might be involved in colour vision as well as the potential variation in the expression of cone pigments across populations, individuals, sexes, and life stages, the design of light stimuli and the interpretation of results would be certainly challenging. In
summary, both these approaches for studying the characteristics of the colour channels in fish are difficult, but fortunately, possible. Pursuing these approaches, I believe, will take us one step further in the long way toward answering the question that has fascinated people for centuries: How do animals see the world?
Summary

1. Functional diversity in the colour vision of cichlid fishes

1.1. Individual Lake Malawi cichlids possess complements of four to five cone pigments, with different cone classes likely interacting with one another. Thus, Lake Malawi cichlids satisfy the requirements for tetra- and pentachromatic colour vision.

1.2. Cone complements differ across Lake Malawi species, likely contributing to sensory adaptations that enhance the contrast of transparent prey and the detection of optical signals from conspecifics, suggesting a role for both natural and sexual selection in tuning colour vision.

1.3. Opsin gene expression, and possibly also spectral sensitivity, may be sex-dependent. This suggests that females and males may sample their visual environment differently, providing a neural basis for sexually dimorphic visual behaviour.

2. Colour vision requires more cone classes in aquatic than in terrestrial environments

2.1. The correspondence between the band-limits of cone photoreceptors and those of the colour-signal of objects suggests that the filtering properties of photoreceptors evolved to allow for recovery of high frequencies from the colour-signal of relevant objects.
2.2. Four to six cone classes are required for reconstructing the colour-signal of aquatic objects at the accuracy level achieved by humans. This holds regardless of whether the unfiltered colour-signal or the band-limited colour-signal passed by the cones is considered.

2.3. The need for a larger number of cone classes for colour-signal reconstruction is an attribute of the aquatic environment, and is true for diverse marine and freshwater ecosystems. This suggests that the large diversity of cone opsin genes and high-dimensional colour vision in fish are of adaptive significance, and have likely evolved to enhance the reconstruction of the complex colour-signal of aquatic objects.

3. **Ontogeny in the visual system of Nile tilapia**

3.1. Nile tilapia undergo ontogenetic changes in lens transmission, photoreceptor sensitivity, and post-receptoral sensitivity.

3.2. The decrease in photoreceptor sensitivity observed during ontogeny is a result of reduction in the expression of the SWS1 cone pigment, and is associated with a reduction in lens transmission at UV wavelengths.

3.3. Post-receptoral sensitivity to UV and long wavelengths decreases with age. These changes in post-receptoral sensitivity are a result of (i) ontogenetic changes in photoreceptor sensitivity, mediated through a reduction in the expression of the SWS1 pigment, in the UV spectral range, as well as (ii) ontogenetic changes in retinal circuitry leading to reduced sensitivity at longer wavelengths.
3.4. In addition to remodelling of the retinal circuitry during early development and aging, in the Nile tilapia, remodelling of retinal circuitry occurs also following maturation of the visual system but prior to reaching adulthood, and thus may facilitate optimization of the visual system to the changing sensory demands.

3.5. Observed reduction in post-receptoral sensitivity to long wavelengths can not be predicted either by the lens transmission, cone pigment expression, or photoreceptor sensitivity. This emphasizes the importance of considering the visual sensitivity of fish, rather than its proxies, to allow for an accurate and efficient investigation of visual functions and adaptations.
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Figure A1.1 Background isolation conditions for spectral sensitivity measurements

(A) The spectral irradiance provided under the various background isolation conditions. Background conditions: Long wavelength isolation (LW, red), Control (green), Dim short wavelength isolation (Dim SW, cyan), and Short wavelength isolation (SW, blue).

(B) The quantum catches of the six possible cone pigments under each condition. Cone pigment: SWS1 (black), SWS2b (violet), SWS2a (blue), Rh2b (green), Rh2a (orange), and LWS (red). Cone quantum catches were calculated while setting the A2 proportion of cones to 0.5 for the design of the background conditions.
Table A1.1 Summary of *t*-test results for the examination of the effect of sex on opsin gene expression

<table>
<thead>
<tr>
<th></th>
<th><em>M. auratus</em></th>
<th></th>
<th></th>
<th><em>P. taeniolatus</em></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>t</em></td>
<td>d.f.</td>
<td><em>P</em></td>
<td><em>t</em></td>
<td>d.f.</td>
</tr>
<tr>
<td><strong>SWS1</strong></td>
<td>1.536</td>
<td>8</td>
<td>0.1630</td>
<td>0.549</td>
<td>8</td>
</tr>
<tr>
<td><strong>SWS2b</strong></td>
<td>1.929</td>
<td>8</td>
<td>0.0898</td>
<td>0.316</td>
<td>8</td>
</tr>
<tr>
<td><strong>SWS2a</strong></td>
<td>1.132</td>
<td>8</td>
<td>0.2904</td>
<td>-1.306</td>
<td>8</td>
</tr>
<tr>
<td><strong>Rh2a</strong></td>
<td>-3.845</td>
<td>8</td>
<td><strong>0.0049</strong></td>
<td>-0.646</td>
<td>8</td>
</tr>
<tr>
<td><strong>Rh2b</strong></td>
<td>6.756</td>
<td>8</td>
<td><strong>0.0001</strong></td>
<td>1.404</td>
<td>8</td>
</tr>
<tr>
<td><strong>LWS</strong></td>
<td>0.850</td>
<td>8</td>
<td>0.4200</td>
<td>0.819</td>
<td>8</td>
</tr>
</tbody>
</table>

Values in bold indicate significant sex differences in expression. An experiment-wise error rate of 5% was corrected to 0.83% (*α* = 0.05/6 = 0.0083) following Bonferroni correction for 6 hypothesis tests that correspond to 6 opsin genes (Rh2αα and Rh2αβ were pooled because of genetic and functional similarity).
Table A1.2 Primer specifications for cone opsin genes

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer Sequence a</th>
<th>Primer b (bp)</th>
<th>GC (%)</th>
<th>Tm c (°C)</th>
<th>Annealing site</th>
<th>Product b (bp)</th>
<th>Location</th>
<th>Input PCR template a</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWS1</td>
<td>UV F3 ACATCCCTGAAAG TCTGGGC</td>
<td>20</td>
<td>60</td>
<td>55.59</td>
<td>655-674</td>
<td>148</td>
<td>Exon III</td>
<td>AF191222</td>
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<tr>
<td></td>
<td>UV R2 AGCAAGCTGAGG TGGACAA TGGTGTGGTCCTCT TGCTGG</td>
<td>20</td>
<td>55</td>
<td>54.96</td>
<td>802-783</td>
<td>148</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SWS2b</td>
<td>Blue2 F2b GTCTTGAGG TCTGGG</td>
<td>21</td>
<td>57</td>
<td>55.96</td>
<td>437-457</td>
<td>151</td>
<td>Exon I</td>
<td>AF317674</td>
</tr>
<tr>
<td></td>
<td>Blue2 R2b GGAAGTGG GGAAGTGG</td>
<td>20</td>
<td>55</td>
<td>53.29</td>
<td>587-568</td>
<td>151</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SWS2a</td>
<td>Blue1 F1a GAGAGAGAGGAA GTGACCAG GCCTTTTGAGAAGA GAGCACG</td>
<td>20</td>
<td>60</td>
<td>53.94</td>
<td>1267-1286</td>
<td>156</td>
<td>Exon IV</td>
<td>AF247114</td>
</tr>
<tr>
<td></td>
<td>Blue1 R1a AGCAAGAACCACA AGAGACC GCTCTTGTGACCATC TGGATTC</td>
<td>20</td>
<td>45</td>
<td>48.37</td>
<td>165-184</td>
<td>357</td>
<td>Exon I-II</td>
<td>DQ088645</td>
</tr>
<tr>
<td>Rh2b</td>
<td>PaG-Rh2b-F12 GAGAGAGAGGAA GTGACCAG GCCTTTTGAGAAGA GAGCACG</td>
<td>20</td>
<td>50</td>
<td>51.93</td>
<td>521-502</td>
<td>357</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PaG-Rh2b-R2 GCTCTTGTGACCATC TGGATTC</td>
<td>20</td>
<td>50</td>
<td>51.93</td>
<td>521-502</td>
<td>357</td>
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<tr>
<td>Rh2a</td>
<td>Green1 F4 GAGAGAGAGGAA GTGACCAG GCCTTTTGAGAAGA GAGCACG</td>
<td>20</td>
<td>55</td>
<td>53.2</td>
<td>1160-1179</td>
<td>187</td>
<td>Exon IV</td>
<td>AF247122</td>
</tr>
<tr>
<td></td>
<td>Green1 R3 AGCAAGAACCACA AGAGACC GCTCTTGTGACCATC TGGATTC</td>
<td>22</td>
<td>45</td>
<td>53.51</td>
<td>1346-1325</td>
<td>187</td>
<td></td>
<td></td>
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<tr>
<td>LWS</td>
<td>Red F1a GCCCTCAGGATA CACATGG</td>
<td>18</td>
<td>50</td>
<td>48.57</td>
<td>853-870</td>
<td>271</td>
<td>Exon I</td>
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</tr>
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<td></td>
<td>Red R0 GCCCTCAGGATA CACATGG</td>
<td>21</td>
<td>48</td>
<td>51.15</td>
<td>1123-1103</td>
<td>271</td>
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<td></td>
</tr>
</tbody>
</table>

a Tails associated with restriction sites were removed from the original primer sequences [76, 134] with an exception of the Rh2b primer sequence that was designed in our lab.

b Length in base pairs.

c Melting temperature.

d Accession numbers of gene sequences used to assess the specificity of primers.
Table A1.3 Statistics of cone interactions for the most frequent cone pigment subset of each species

<table>
<thead>
<tr>
<th>Background</th>
<th>Spectral range (nm)</th>
<th>Cone weight coefficients</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$K_{SW1}$</td>
<td>$K_{SW2a}$</td>
</tr>
<tr>
<td><strong>M. zebra</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>340-400</td>
<td>0.919</td>
<td>-1.038</td>
</tr>
<tr>
<td>Subset #2</td>
<td>400-480</td>
<td>-0.173</td>
<td>0.848</td>
</tr>
<tr>
<td>LW</td>
<td>480-540</td>
<td>-</td>
<td>-0.979</td>
</tr>
<tr>
<td></td>
<td>540-620</td>
<td>-</td>
<td>-</td>
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<td>SW</td>
<td>340-400</td>
<td>0.371</td>
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<td></td>
<td>420-460</td>
<td>-0.856</td>
<td>1.313</td>
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<tr>
<td></td>
<td>460-520</td>
<td>-</td>
<td>-0.387</td>
</tr>
<tr>
<td></td>
<td>540-620</td>
<td>-</td>
<td>-</td>
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<td><strong>M. auratus</strong></td>
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<td></td>
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<tr>
<td>Control</td>
<td>340-380</td>
<td>1.064</td>
<td>-1.036</td>
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<td>Subset #3</td>
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<td>0.587</td>
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<tr>
<td></td>
<td>440-480</td>
<td>-</td>
<td>0.075</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td>540-640</td>
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<td>-</td>
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<td>LW</td>
<td>340-400</td>
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<td>420-480</td>
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<td></td>
<td>480-540</td>
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<td>-0.052</td>
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<td></td>
<td>540-620</td>
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<td>SW</td>
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<tr>
<td></td>
<td>520-560</td>
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<td>-</td>
</tr>
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<td></td>
<td>560-640</td>
<td>-</td>
<td>-1.894</td>
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<td><strong>P. taeniolatus</strong></td>
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<td>Control</td>
<td>380-420</td>
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<td>1.321</td>
</tr>
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<td>Subset #7</td>
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<td>520-580</td>
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</tr>
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<td></td>
<td>580-660</td>
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<td>-</td>
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<tr>
<td>Dim-SW</td>
<td>380-420</td>
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<td>460-520</td>
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<td>-0.068</td>
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<td></td>
<td>520-560</td>
<td>-</td>
<td>-</td>
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<td>-0.459</td>
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<td></td>
<td>540-580</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>560-660</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Background conditions used for spectral sensitivity measurements. Artificial conditions: LW - Long wavelength isolation, Control, SW - Short wavelength isolation, Dim-SW - Dim short wavelength isolation.

b Modeled spectral range.

c Total amount of variance accounted for by the model across the spectrum.
Appendix 2

Figure A2.1 Irradiance and surface reflectance spectra analyzed

Celestial (A) and aquatic (D) irradiance spectra as well as reflectance spectra of terrestrial (B-C) and aquatic (E-F) objects were analyzed. Spectra were spline interpolated at a 1 nm interval and normalized to the length of one.

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Figure A2.2 Example procedure of fitting a spectrum to linear models

An aquatic irradiance spectrum fitted to a linear model of one through six principal components (PCs). The goodness of fit, $R^2$, increases with the number of PCs included in the model, reaching an excellent fit with 6 PCs. That is, the given signal can be reconstructed with 6 independent parameters. This procedure was repeated for all spectra in a collection.
Figure A2.3 Irradiance and object reflectance expressed as band-limited functions

Celestial (A) and aquatic (D) irradiance as well as reflectance spectra of terrestrial (B-C) and aquatic (E-F) objects were expressed as band-limited functions.
Figure A2.4 Absorbance and sensitivity of cone photoreceptors in humans

Absorbance spectra for the S-, M-, and L-cone photoreceptors in humans (blue, green, and red solid lines, respectively) were generated based on visual pigment absorbance templates [176] and were corrected for lens [262, 263] and macula [264] transmission. Corrected absorbance spectra were quantitatively similar to the commonly used sensitivity spectra (dashed lines) for humans [261] in the wavelength (A) and frequency (B) domains. The band-limits of cone photoreceptors based on corrected absorbance spectra equalled 14.45, 9.57, and 8.39 cycles/μm, whereas, the band-limits of cone photoreceptors based on sensitivity spectra equalled 13.87, 10.55, and 8.79 cycles/μm for the S-, M-, and L-cone photoreceptors.
Table A2.1 $R^2$ coefficients for celestial and aquatic irradiance (A), and reflectance (B) and colour-signal (C) of terrestrial and aquatic objects (see details below table).

<table>
<thead>
<tr>
<th>A</th>
<th>Irradiance</th>
<th>Median</th>
<th>5th percentile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Celestial</td>
<td>Aquatic (Human VS)</td>
<td>Aquatic (Human VS)</td>
</tr>
<tr>
<td>PC</td>
<td>(Human VS)</td>
<td>(Fish VS)</td>
<td>(Human VS)</td>
</tr>
<tr>
<td>1</td>
<td>0.9956</td>
<td>0.9833</td>
<td>0.9835</td>
</tr>
<tr>
<td>2</td>
<td>0.9996</td>
<td>0.9966</td>
<td>0.9966</td>
</tr>
<tr>
<td>3</td>
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<td>0.9994</td>
<td>0.9995</td>
</tr>
<tr>
<td>4</td>
<td>0.9999</td>
<td>0.9999</td>
<td>0.9999</td>
</tr>
<tr>
<td>5</td>
<td>1.0000</td>
<td>1.0000</td>
<td>1.0000</td>
</tr>
<tr>
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<td>1.0000</td>
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</tr>
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<td>1.0000</td>
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</tr>
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</table>

<table>
<thead>
<tr>
<th>B</th>
<th>Reflectance of objects</th>
<th>Median</th>
<th>5th percentile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Terrestrial (Human VS)</td>
<td>Aquatic (Fish VS)</td>
<td>Aquatic (Human VS)</td>
</tr>
<tr>
<td>PC</td>
<td>(Human VS)</td>
<td>(Fish VS)</td>
<td>(Human VS)</td>
</tr>
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<td>0.9223</td>
<td>0.9078</td>
<td>0.9312</td>
</tr>
<tr>
<td>2</td>
<td>0.9845</td>
<td>0.9713</td>
<td>0.9855</td>
</tr>
<tr>
<td>3</td>
<td>0.9959</td>
<td>0.9862</td>
<td>0.9955</td>
</tr>
<tr>
<td>4</td>
<td>0.9984</td>
<td>0.9939</td>
<td>0.9985</td>
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<td>0.9971</td>
<td>0.9992</td>
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<td>0.9995</td>
</tr>
<tr>
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<td>0.9997</td>
</tr>
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<td>0.9995</td>
<td>0.9998</td>
</tr>
<tr>
<td>10</td>
<td>1.0000</td>
<td>0.9996</td>
<td>0.9999</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>C</th>
<th>Colour-signal of objects</th>
<th>Median</th>
<th>5th percentile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Terrestrial (Human VS)</td>
<td>Aquatic (Fish VS)</td>
<td>Aquatic (Human VS)</td>
</tr>
<tr>
<td>PC</td>
<td>(Human VS)</td>
<td>(Fish VS)</td>
<td>(Human VS)</td>
</tr>
<tr>
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<td>0.8438</td>
<td>0.8457</td>
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<td>0.9776</td>
<td>0.9568</td>
<td>0.9587</td>
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<td>0.9855</td>
<td>0.9865</td>
</tr>
<tr>
<td>4</td>
<td>0.9979</td>
<td>0.9943</td>
<td>0.9952</td>
</tr>
<tr>
<td>5</td>
<td>0.9990</td>
<td>0.9963</td>
<td>0.9970</td>
</tr>
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<td>6</td>
<td>0.9995</td>
<td>0.9976</td>
<td>0.9982</td>
</tr>
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<td>7</td>
<td>0.9996</td>
<td>0.9983</td>
<td>0.9989</td>
</tr>
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<td>0.9997</td>
<td>0.9990</td>
<td>0.9991</td>
</tr>
<tr>
<td>9</td>
<td>0.9998</td>
<td>0.9992</td>
<td>0.9994</td>
</tr>
<tr>
<td>10</td>
<td>0.9999</td>
<td>0.9994</td>
<td>0.9995</td>
</tr>
</tbody>
</table>
$R^2$ coefficients for linear models of one through ten principal components (PCs), for irradiance, reflectance, and colour-signal spectra. The median $R^2$ indicates the value above which the $R^2$ values corresponding to 50% of spectra in a collection fell. The 5th percentile of $R^2$ indicates the value above which the $R^2$ values corresponding to 95% of spectra in a collection fell. The median and 5th percentile of the $R^2$ coefficient are given for celestial ($n = 400$) and aquatic ($n = 80$) irradiance (A), reflectance of terrestrial ($n = 676$) and aquatic ($n = 351$) objects (B), and colour-signal of terrestrial ($n = 270,400$) and aquatic ($n = 28,080$) objects (C). For aquatic irradiance, and the reflectance and color signal of aquatic objects, spectra were fitted to linear models while accounting for the visible spectrum of fish (Fish VS), and again while accounting for the visible spectrum of humans (Human VS). $R^2$ values just exceeding the $R^2 = 0.99$ criterion are marked in bold.
Table A2.2 Band-limits of fish cone photoreceptors for different combinations of chromophore and lens transmission.

<table>
<thead>
<tr>
<th>Chromophore</th>
<th>Lens $T_{50}$ (nm)</th>
<th>SWS1</th>
<th>SWS2b</th>
<th>SWS2a</th>
<th>Rh2b</th>
<th>Rh2α</th>
<th>Rh2β</th>
<th>LWS</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₁</td>
<td>350</td>
<td>16.02</td>
<td>10.35</td>
<td>8.98</td>
<td>8.20</td>
<td>7.62</td>
<td>7.42</td>
<td>7.23</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>n.a.</td>
<td>11.72</td>
<td>9.38</td>
<td>8.59</td>
<td>7.81</td>
<td>7.62</td>
<td>7.42</td>
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<tr>
<td>A₂</td>
<td>350</td>
<td>10.55</td>
<td>8.40</td>
<td>7.62</td>
<td>7.03</td>
<td>6.25</td>
<td>6.05</td>
<td>5.66</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>n.a.</td>
<td>9.38</td>
<td>8.01</td>
<td>7.23</td>
<td>6.45</td>
<td>6.25</td>
<td>5.86</td>
</tr>
</tbody>
</table>

The band-limits (cycles/μm) of cone photoreceptors in fish depended on chromophore content and lens transmission. The band-limits of cone photoreceptors in fish that exhibited A₁ retina were higher than in fish that exhibited A₂ retina. Additionally, the band-limits of cone photoreceptors in fish with a non-UV-transmissive lens ($T_{50} = 400$ nm) were slightly higher than in fish with a UV-transmissive lens ($T_{50} = 350$ nm). However, fish that exhibit non-UV-transmissive lenses typically do not possess the SWS1 (UV) cone photoreceptor [44, 84], and thus, in these cases, the band-limits of the SWS1 photoreceptor are not shown. Consequently, fish with A₁ retina and a UV-transmissive lens would recover higher frequencies from the colour-signal of objects viewed.
Supplementary Methods

Description of irradiance and surface reflectance data sets

Aquatic spectral irradiance

Aquatic irradiance ($n = 80$) was measured in July 2008 at three near shore sites in Lake Malawi, near Cape Maclear on the northwestern part of the Nankumba Peninsula, Malawi (Otter Point - 14º 02’ 21.02” S 34º 49’ 20.33” E, Mawlamba - 14º 01’ 05.92” S 34º 48’ 59.91” E, and Mitande - 14º 00’ 58.02” S 34º 48’ 33.29” E) [214]. Lake Malawi is a clear, nutrient-low (e.g., chlorophyll=1-2 μg L$^{-1}$), oligotrophic lake [232]. To represent the irradiance spectra encountered at different positions in a given habitat, spectral irradiance was measured at two orientations (downwelling and sidewelling) and down to a depth of 15 m, a depth range that Lake Malawi cichlids typically occupy [162, 178]. Irradiance was measured using a thermoelectrically cooled spectroradiometer (QE65000, Ocean Optics, Dunedin, FL, USA) connected to a 30 m optical fiber (ZPK600-30-UV/VIS, Ocean Optics) that was fitted with a cosine corrector (CC-3-UV, Ocean Optics). Irradiance was measured at 1 nm intervals between 200 and 950 nm, with an effective spectral resolution of 1.9 nm (FWHM). Sampling time exceeded 2 seconds to average irradiance fluctuations induced by surface waves. The spectroradiometer setup was calibrated for absolute irradiance using a calibrated Halogen–Deuterium dual light source (200-1000 nm, DH-2000-CAL, Ocean Optics). All measurements were made at approximately noon under clear blue sky.
**Celestial spectral irradiance**

Celestial (zenith) irradiance \((n = 18)\) was measured just above the water surface at each of the sampling sites in Lake Malawi using the same spectroradiometer configuration described above. Additionally, three large data sets of celestial irradiance spectra were adopted. The first data set \((n = 66)\) was obtained at the Nouragues field station in French Guiana \((4° 05' \text{ N}, 52° 40' \text{ W})\) [229, 256]. The second data set \((n = 64)\) was collected at the Makerere University Biological Field Station (MUBFS) in Kibale Forest, Western Uganda [257]. Irradiance spectra included in these two data sets were measured at 4 nm intervals from 380 to 780 nm and under diverse meteorological conditions. Spectra in these two data sets were obtained from the Cambridge database of natural spectra (http://vision.psychol.cam.ac.uk/spectra/spectra.html). The third data set \((n = 252)\) was collected in Granada, Spain \((37° 118’ \text{ N} 3° 358’ \text{ W})\) under a full range of atmospheric conditions [254]. Irradiance was measured from sunrise to sunset at intervals of 1 hour and at 1 nm intervals from 300 to 1100 nm. The Granada data set was obtained from http://www.cns.nyu.edu/pub/ltm/Illuminants/Granada.

**Spectral reflectance of algae**

Spectral reflectance of algae \((n = 47)\) was measured in July 2008 at three near-shore sites around Cape Maclear, Lake Malawi (see the Aquatic spectral irradiance section for description of the study sites). Spectral reflectance of algae was measured using a custom built reflectance probe that included a diving flashlight (mini Q40, Underwater Kinetics, Poway, CA, USA) and a fiber-coupled spectroscopic system (Jaz, Ocean Optics). The tip of the flashlight was fitted with an adaptor that held the optical
fiber (QP600-2-UV-VIS, Ocean Optics) oriented at an angle of 45º to the examined surface. To reduce stray light in the measurement, the far side of the adaptor included a ring of black foam that used to seal the reflectance probe against the surface examined. A SCUBA diver held the reflectance probe against different underwater substrates covered with algae while readings were acquired and saved on a laptop computer in a boat. The irradiance spectrum of the flashlight allowed for a reliable reflectance measurement between 370 and 800 nm, and the spectroradiometer configuration resulted in an effective spectral resolution of 2.06 nm (FWHM) across this range. A measurement of a Spectralon diffuse reflectance standard (WS-1-SL, Ocean Optics) was taken as 100% reflectance, and a dark measurement was taken as zero reflectance.

**Spectral reflectance of the body pattern of fish**

Spectral reflectance of the body pattern of fish \(n = 304\) was measured using a spectroradiometer (USB2000, Ocean Optics) connected to one arm of a 2 m bifurcated optical fiber (BIF600-2-UV/VIS, Ocean Optics). The other arm of the fiber was connected to a light source (DH-2000-BAL, Ocean Optics) that provided a high output between 200 and 1000 nm. The common end of the bifurcated fiber was fitted with a flat black reflectance probe that showed a 3 mm diameter tip, cut at an angle of 45º. A measurement of a Spectralon diffuse reflectance standard was taken as 100% reflectance, and a dark measurement was taken as zero reflectance. The spectroradiometer configuration resulted in an effective spectral resolution of 2.06 nm (FWHM) between 200 and 950 nm. Fish were immersed in 500 ml water containing 2.0 ml of 1:10 clove oil:ethanol solution immediately after capture until the fish reached stage III anesthesia.
Fish were held submerged in water and spectral reflectance was measured at 16-23 different points across the body of the fish. Care was given to sealing the reflectance probe against the fish skin to reduce stray light. Spectral reflectance data were acquired from three individuals of each of five cichlid species: *Melanochromis auratus*, *Metriaclima zebra*, *Metriaclima aurora*, *Metriaclima callainos*, and *Protomelas taeniolatus*. All experimental and animal care procedures were approved by Queen’s University Animal Care Committee under the auspices of the Canadian Council for Animal Care.

**Spectral reflectance of fruits**

A large data set of fruit reflectance was adopted. This data set (n = 482 from 51 plant species) was obtained from fruits collected at the Makerere University Biological Field Station (MUBFS) in Kibale Forest, Western Uganda. Data were also collected at other sites within Kibale Forest (which also lie within the same reserve: 0° 13’ to 0° 41’ N and 30° 19’ to 30° 32’ E), at Budongo Forest and in Queen Elizabeth National Park [257]. The reflectance of fruits was measured at 4 nm intervals from 380 to 780 nm. Fruit reflectance spectra were obtained from the Cambridge database of natural spectra (http://vision.psychol.cam.ac.uk/spectra/spectra.html).

**Spectral reflectance of integumentary tissues of primates**

A data set of reflectance of fur, skin, and pelts of old-world primates (Catarrhine) was adopted. This data set (n = 194 from 13 species) was measured at zoos in Uganda and U.K. and at the Natural History Museum, London. Reflectance was measured at 4 nm intervals from 380 to 780 nm. Reflectance spectra of integumentary tissues were obtained
from the Cambridge database of natural spectra
(http://vision.psychol.cam.ac.uk/spectra/spectra.html).

**Aquatic spectral irradiance for eutrophic freshwater and marine systems**

The first data set \( (n = 131) \) included spectral irradiance measurements taken at marine systems off the coast of the North Pacific during July-August, 1991 (Saanich inlet - 48° 37' N, 123° 29' W and Trevor Channel - 48° 50' N, 125° 10' W) [253]. These sampling sites represent high-nutrient (e.g., chlorophyll = 2-19 \( \mu \)g L\(^{-1} \); depth-dependent), eutrophic marine systems. The second data set \( (n = 240) \) included spectral irradiance measurements taken at Lake Cowichan, Vancouver Island, British Columbia (48° 49' N, 124° 03' W) [255]. This sampling site represents a high-nutrient (e.g., chlorophyll = 1-13 \( \mu \)g L\(^{-1} \); depth-dependent), eutrophic freshwater system. For both data sets, downwelling and sidewelling irradiance were measured at 4 nm intervals from 300 and 850 nm and down to a depth of 15 m.

**Choice of spectral irradiance data and biological relevance**

To reliably represent the irradiance incident on terrestrial objects, downwelling celestial irradiance was analyzed (sidewelling celestial irradiance is typically lacking in the literature, and challenging to measure due to the inclusion of obstacles in the light path). However, light attenuation is considerably larger in water than in air [5]. Thus, the effect of the path length over which light is transmitted through the medium, and consequently also the effect of viewing orientation and water depth, on aquatic irradiance is relatively large. Therefore, to reliably represent the irradiance incident on aquatic objects, downwelling and sidewelling aquatic irradiance measured at different depths was
analyzed. The number of PCs required for reconstructing the downwelling irradiance was similar to that of sideward irradiance, with two and three PCs required for reconstructing 50% and 95% of spectra in the collection, respectively. Additionally, the band-limits of downwelling and sideward irradiance were comparable, with median band-limits of 5.66 and 5.85 cycles/μm for downwelling and sideward irradiance, respectively.