

# Diurnal rhythm of cone opsin expression in the teleost fish *Haplochromis burtoni*

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## Abstract

The biochemical and morphological specializations of rod and cone photoreceptors reflect their roles in sight. The apoprotein opsin, which converts photons into chemical signals, functions at one end of these highly polarized cells, in the outer segment. Previous work has shown that the mRNA of rod opsin, the opsin specific to rods, is renewed in the outer segment with a diurnal rhythm in the retina of the teleost fish *Haplochromis burtoni*. Here we show that in the same species, all three cone opsin mRNAs (blue, green, and red) also have a diurnal rhythm of expression. Quantitative real-time polymerase chain reaction (PCR) with primer pairs specific for the cone photoreceptor opsin subtypes was used to detect opsin mRNA abundance in animals sacrificed at 3-h intervals around the clock. All three cone opsins were expressed with diurnal rhythms similar to each other but out of phase with the rod opsin rhythm. Specifically, cone opsin expression occurs at a higher level near the onset of the dark period, when cones are not used for vision. Finally, we found that the rhythm of cone opsin expression in fish appears to be light dependent, as prolonged darkness changes normal diurnal expression patterns.

**Keywords:** Cone photoreceptor, Opsin, Diurnal rhythm, Temporal regulation, *Haplochromis (Astatotilapia) burtoni*

## Introduction

Vertebrate eyes are adapted for vision over a wide range of light intensities allowing animals to exploit nearly the full extent of the light/dark cycle (Land & Fernald, 1992). Adaptations for light detection in different species include the shape and size of the rod and cone photoreceptors, the relative abundance of photoreceptor types and their spatial distribution (Wikler & Rakic, 1990) as well as the regulation of key cellular and molecular processes, as reviewed by Puzzolo (1989). Among vertebrates, fish visual systems have been useful model systems for analysis because evolutionary adaptations for visual function are particularly evident. For example, the peak wavelengths of cone photoreceptor absorption in fish match key features of their optical environment (McFarland & Munz, 1975). Since absorption peak wavelength depends critically on key amino acids in opsin, fish visual pigment genes appear to be particularly sensitive to selective pressures (Fernald & Liebman, 1980). For the experiments reported here, we used the

African cichlid fish, *Haplochromis (Astatotilapia) burtoni* (*H. burtoni*), a species that displays elaborate social behavior mediated primarily by visual cues, making *H. burtoni* particularly appropriate for analysis of visual mechanisms (Fernald, 1990).

During evolution, regular light/dark changes have had a profound impact on visual systems including the evolution of regular rhythms in biological processes ranging from behavioral to biochemical. *Diurnal* rhythms are synchronized to the ambient light cycle but do not persist under constant lighting conditions, while *circadian* rhythms persist with a cycle of ~24 h even under constant lighting conditions (Jacklet, 1989). Examples of both diurnal and circadian rhythms are found in features of vertebrate eyes where they regulate many processes including cell addition (Chiu et al., 1995), photoreceptor position and function (Burnside, 1976), opsin synthesis (Korenbrodt & Fernald, 1989; Pierce et al., 1993; von Schantz et al., 1999), and disc shedding (Besharse et al., 1977; Young, 1978).

Among the most essential and robust rhythmic processes in the retina is the renewal of the photosensitive opsin proteins. Although all cells renew their molecular constituents through removal and new synthesis, photoreceptor cells have enhanced renewal processes that are typical of sensory systems responsive to physical signals from the environment (Fernald, 1991). Discs containing photoreceptor pigments in the rod outer segment undergo daily renewal, being assembled at the outer segment base, displaced outward by new discs, and eventually shed at the tip where they are phagocytized (Young & Bok, 1969). Net disc membrane assembly

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and shedding are balanced, resulting in the nearly constant length of the photoreceptor outer segments. In contrast, new cone opsin is not gradually displaced outward from the outer segment's base, but appears to be incorporated randomly in the cone outer segment (Young, 1969, 1976). Understanding how cone opsin renewal is controlled and balanced requires more information about the molecular basis of this process. Here we report that the abundance of mRNA associated with the daily renewal of the three cone opsins in *H. burtoni* retina varies as a function of time of day, and that this rhythm is out of phase with the rhythm of rod opsin mRNA. Furthermore, we show that the rhythm of cone opsin expression is under diurnal rather than circadian control.

## Materials and methods

### Animals

*H. burtoni* derived from a wild-caught population were raised in aquaria under conditions similar to their native equatorial habitat in Africa's Lake Tanganyika (pH 8, 28°C; Fernald, 1977; Fernald & Hirata, 1977a,b). Aquarium lighting was provided by full spectrum lights which had an intensity at the surface of the water of ~600 lux (Li-Cor Quantum/Radiometer/Photometer, Li-Cor, NE). This is in the midrange of the intensities measured in the natural habitat (Fernald & Hirata, 1977a). Fish were kept in a 12-h light, 12-h dark cycle including 10 min of transitional twilight in the morning and evening. Light onset occurred at 9 AM and dark onset at 9 PM. Fish were fed every morning with cichlid pellets and flakes (AquaDine, Healdsburg, CA). Both male and female fish (3–4 cm, standard length) were used in these experiments. All experiments and animal handling were in compliance with the animal care and use guidelines at Stanford University and were approved by the Stanford University Administrative Panel on Laboratory Animal Care.

Fish were moved into experimental aquaria from colony tanks (three fish per aquarium) with identical water and lighting conditions as the rearing aquaria, and acclimated for 7 days before experiments. Separate experimental aquaria were used to allow collection of fish in complete darkness without disrupting other animals. To collect tissue at night, the aquarium room was accessed through a light-tight door, and all tissue processing steps were performed in complete darkness using an infrared viewer (FJW Industries, Mt. Prospect, IL) or under minimal illumination with adjustable safelight lamps (GBX-2 safelight filter, Eastman Kodak).

During two separate around-the-clock collections of normal retinal tissue, three fish each were sacrificed using rapid cervical transection at 3-h intervals over a 24-h period beginning at 9 AM. For each time point, the six retinas and adhering pigment epithelium were rapidly isolated, pooled in chilled TRIzol® reagent (Invitrogen, Carlsbad, CA), mechanically homogenized with a Tissue Tearor (Biospec Products, OK), and kept frozen in TRIzol® reagent at –80°C until RNA extraction.

To measure the effect of light deprivation on opsin expression at the time of maximum expression, 30 fish were kept under normal light–dark conditions until 15 of them were moved one night to complete darkness for the duration of 2 days. On the second day, three fish each of the 15 fish in darkness were removed at 2 PM, 4 PM, 6 PM, 8 PM, and 10 PM. The two retinas of each individual fish were combined and processed for RNA isolation as above. In parallel, pairs of retinas from three control fish main-

tained in normal light–dark conditions were collected at 2 PM, 4 PM, 6 PM, 8 PM, and 10 PM and processed identically.

### RNA extraction and PCR sample preparation

Total RNA was extracted from all retina samples following a standard protocol (TRIzol®, Invitrogen). RNA was DNase-treated to remove genomic DNA contamination (TURBO DNA-free, Ambion, TX). 1.0 µg total RNA was reverse transcribed (SuperScript II RNase H<sup>-</sup> reverse transcriptase; Invitrogen, Carlsbad, CA) using an anchored poly T primer, VdT18. A fraction of the cDNA from all samples of a given sample set was pooled and diluted serially from 1:10 to 1:100,000 to serve as concentration standards. All individual samples were diluted 1:100 in ultrapure, nuclease-free ddH<sub>2</sub>O.

### Primers for quantitative real-time PCR

Primer design for the three cone opsins was based on cDNA sequences obtained from *H. burtoni* retinal mRNA using standard PCR cloning techniques (GenBank accession numbers AY660538 [blue-sensitive cone opsin], AY660539 [green-sensitive cone opsin], and AY660540 [red-sensitive cone opsin]). The identity of putative *H. burtoni* cone opsin cDNAs was confirmed by sequence alignments (nucleotide–nucleotide BLAST, National Center for Biotechnology Information, MD) and *in situ* hybridization (data not shown). In particular, the 1053 nucleotides of putative *H. burtoni* blue cone opsin cDNA are 99% (1045/1053) identical to closely related Malawi cichlid *Dimidiochromis compressiceps*'s (*D. compressiceps*) putative blue-sensitive opsin SWS-2A mRNA (Carleton & Kocher, 2001), and the alignment contained no gaps. The 1056 nucleotides of putative *H. burtoni* green cone opsin cDNA are 99% (1052/1056) identical to *D. compressiceps*'s putative green-sensitive opsin RH2 mRNA (Carleton & Kocher, 2001), and the alignment contained no gaps. The 1071 nucleotides of putative *H. burtoni* red cone opsin cDNA are 99% (1062/1071) identical to *D. compressiceps*'s putative red-sensitive opsin LWS mRNA (Carleton & Kocher, 2001), and the alignment contained no gaps.

Primers for quantitative real-time PCR (qPCR) were designed (Beacon Designer 2, Biosoft International, CA), synthesized (Invitrogen), and screened for PCR efficiency using standard curve analysis (see *PCR data analysis*), for yield of a single amplification product using gel electrophoresis, and for absence of primer dimer formation using melt curve analysis (MyiQ™ Single-Color Real-Time PCR Detection System; Bio-Rad Laboratories, Hercules, CA). Primers chosen for red cone opsin qPCR were: 5'-AGT CTC CAG GAT GGT CGT TGT C-3' and 5'-CTT CAG AGC CAT CGT CCA CTT G-3', generating a 245-bp amplicon; primers for green cone opsin: 5'-TCT CTG GTC ACT TGT TGT CCT G-3' and 5'-GGA GCC AGT GTG TAG TAG TCA G-3', generating a 216-bp amplicon; and primers for blue cone opsin: 5'-TGT TCC TGT TCT GCT TCT GC-3' and 5'-GGC ATC CAG CAC ACC AAG-3', generating a 187-bp amplicon. The "housekeeping gene" glyceraldehyde 3-phosphodehydrogenase (G3PDH; GenBank accession number AF123727) was used to control for sample differences with respect to total cDNA content as a result of loading inaccuracies or varying reverse transcription efficiency among samples. Primers for G3PDH generating a 78-bp amplicon were 5'-CAC ACA AGC CCAs'ACC CAT AGT CAT-3' and 5'-AAA CAC ACT GCT GCT GCC TAC ATA-3'.

### Quantitative real-time PCR

Polymerase chain reactions were performed (iCycler; Bio-Rad, Hercules, CA), and reaction progress in 30  $\mu$ l reaction volumes was monitored by fluorescence detection at 490 nm during each annealing step. Reactions contained 1x IQ SYBR<sup>®</sup> Green Super-Mix (Bio-Rad), 0.5  $\mu$ M of each primer, and 0.75 ng cDNA (RNA equivalent). Reaction conditions were 1 min at 95°C; then 40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C; followed by a melting curve analysis over the temperature range from 95°C to 4°C. All reactions were run in triplicate.

### PCR data analysis

Fluorescence readings for each sample were baseline subtracted, and suitable fluorescence thresholds were automatically determined by the MyiQ<sup>™</sup> software. To obtain comparable threshold cycle numbers, the threshold was imposed consistently on all samples of a given PCR run, and the average threshold cycle number ( $C_T$ ) was then calculated from the triplicates of each sample. The serially diluted concentration standards yielded standard curves with slopes that provided a measure of PCR efficiency based on the equation:  $E = 10^{-1/\text{slope}}$  (Pfaffl, 2001).  $E$  varied between runs with different primer pairs and runs with different sample sets, so the PCR efficiency was evaluated for each primer pair in each PCR run. Percent opsin expression relative to G3PDH expression was calculated for each sample with the equation:

$$X = R * E(\text{G3PDH})^{C_T(\text{G3PDH})} / E(\text{opsin})^{C_T(\text{opsin})} * 100$$

(percent relative expression, based on Pfaffl, 2001). In this equation  $R$  corrects for different amplicon lengths:  $R = \text{length (amplicon of G3PDH)} / \text{length(amplicon of opsin)}$ , where the amplicon length is measured in number of base pairs. Although G3PDH levels can vary significantly across different tissues (Pfaffl, 2001), rendering G3PDH a poor normalizing gene in many cases, this study used G3PDH expression strictly to control for total cDNA content in samples of the same tissue type, namely the teleost retina. For both sets of around-the-clock tissue, the percentage of opsin expression relative to expression at 6 PM (the maximum measured expression level) was calculated by dividing  $X$  of each time point of a given set by  $X$  at 6 PM ( $X_{6 \text{ PM}}$ ) of that set and then multiplying by 100. Using values from both independent sets of around-the-clock tissue, average opsin expression and standard error were then calculated for each time point with 6 PM defined as 100%.

Similarly, for the light-deprivation study, percent opsin expression relative to 6 PM levels of normal fish was determined for light-deprived and normal fish.

### Statistical analysis

The basis for our one-way analysis of variance (ANOVA; SPSS 11.0, SPSS Inc., IL) of normal opsin expression over a 24-h period were two independent measurements of relative opsin expression at each of eight equally spaced time points (from 9 AM to 6 AM). Each measurement represents the average relative opsin expression of three different fish at that time point as measured in a pooled tissue sample.

Opsin expression in normal versus light deprived fish was analyzed by univariate analysis of variance. For each of five time

points between 2 PM and 10 PM, opsin expression in three individual normal fish was compared to three individual light-deprived fish.

## Results

### Diurnal rhythm of expression of blue, green, and red light-sensitive opsin

For the three cone opsins, mRNA concentrations were measured by quantitative real-time PCR at 3-h intervals over a complete diurnal cycle (12-h light/dark cycle). When normalized with respect to a "housekeeping gene" (G3PDH), mRNA levels of all three cone opsins exhibited robust and pronounced diurnal rhythms that were similar in their time course (Fig. 1). Individual mRNA concentrations of all three cone opsins varied significantly as a function of time of day ( $F_{\text{blue}}[7,15] = 12.07$ ,  $P_{\text{blue}} \leq 0.001$ , Fig. 1A;  $F_{\text{green}}[7,15] = 10.88$ ,  $P_{\text{green}} \leq 0.002$ , Fig. 1B; and  $F_{\text{red}}[7,15] = 4.96$ ,  $P_{\text{red}} \leq 0.019$ , Fig. 1C; from independent one-way ANOVAs). All three opsin mRNA concentrations exhibited a similar pattern of low expression immediately after light onset at 9 AM and a peak in transcript abundance at 6 PM. The increase in relative expression was 3.8-fold  $\pm 0.1$  for blue cone opsin, 24.8-fold  $\pm 0.4$  for green cone opsin, and 23.3-fold  $\pm 4.9$  for red cone opsin.

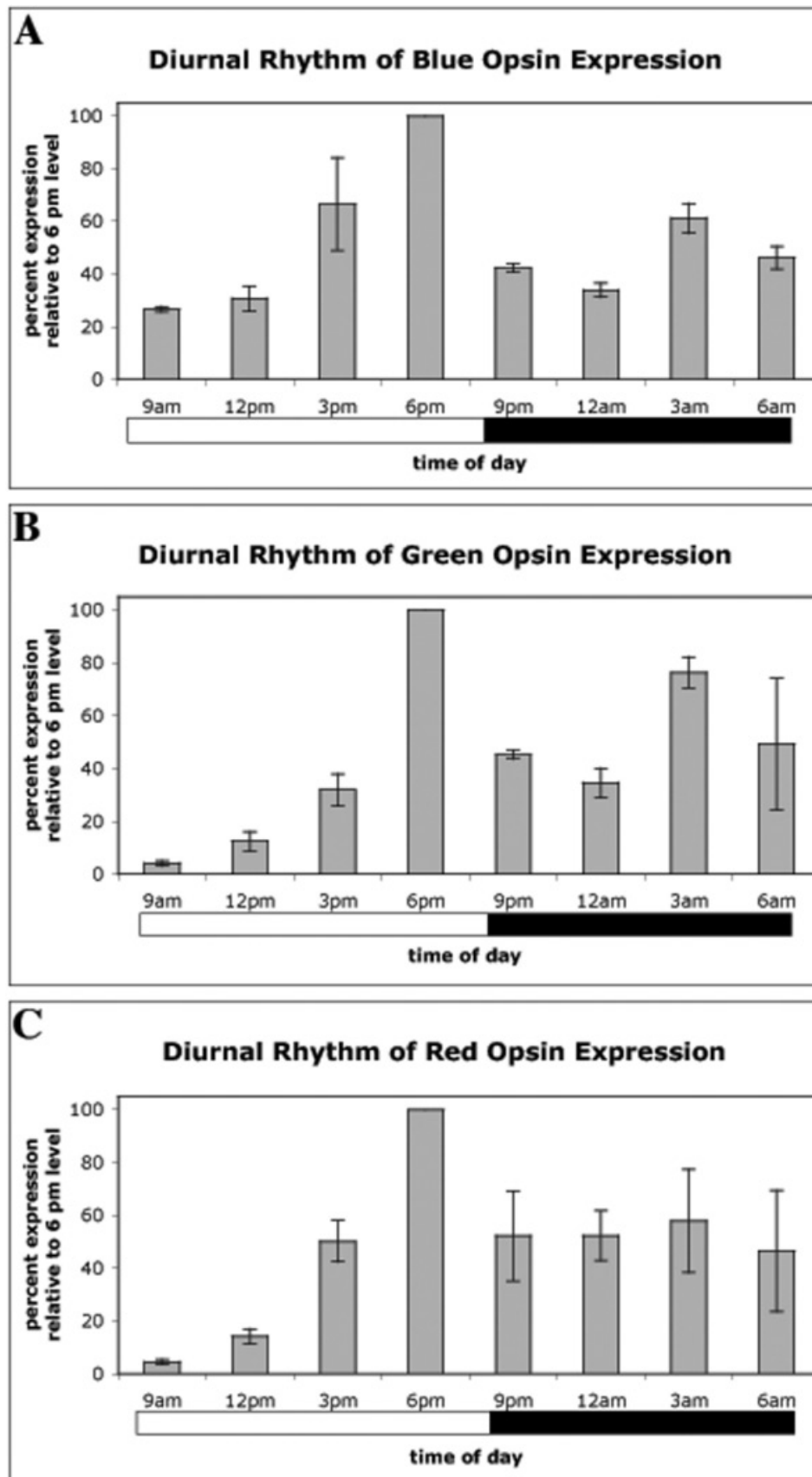
The diurnal rhythm of cone opsin expression had a distinctly different time phase from that previously reported for rod opsin mRNA in this species (Fig. 2, adapted from Korenbrot & Fernald, 1989). Rod opsin mRNA levels peaked 3 h after light onset in contrast to the 6 PM peak of all three cone opsin mRNA concentrations, 3 h before dark onset.

### Alteration of relative cone opsin mRNA levels in light-deprived fish

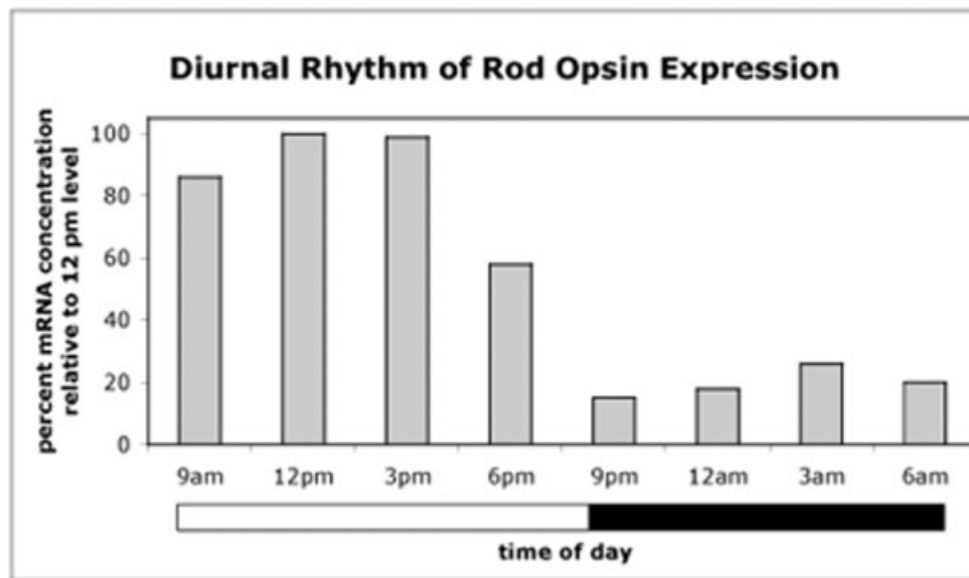
After 1 day of housing in complete darkness, light-deprived fish exhibited dramatically altered cone opsin mRNA expression compared to expression in control fish at the same times of day (Fig. 3). The significant difference in expression levels between light-deprived and normal fish was confirmed by three separate two-way mixed factor ANOVAs, which showed that all three two-way interactions between lighting condition and time of day were significant ( $F_{\text{blue}}[9,20] = 3.22$ ,  $P_{\text{blue}} = 0.014$ , Fig. 3A;  $F_{\text{green}}[9,20] = 4.25$ ,  $P_{\text{green}} = 0.003$ , Fig. 3B;  $F_{\text{red}}[9,20] = 4.10$ ,  $P_{\text{red}} = 0.004$ , Fig. 3C). Cone opsin mRNA levels during subjective night time in light-deprived fish did not coincide with levels of normally treated fish, but appeared to plateau at, or above, the peak concentrations of 6 PM. In particular, expression levels for all three opsins in light-deprived fish did not vary significantly between 8 PM and 10 PM ( $F_{\text{blue}} = 0.179$ ,  $P_{\text{blue}} = 0.694$ , Fig. 3A;  $F_{\text{green}} = 0.275$ ,  $P_{\text{green}} = 0.628$ , Fig. 3B;  $F_{\text{red}} = 0.128$ ,  $P_{\text{red}} = 0.739$ , Fig. 3C; pair-wise comparisons). Blue and red opsin expression at 10 PM in light-deprived versus normal fish was significantly different at the  $P \leq 0.05$  level, and in the case of green opsin nearly so ( $F_{\text{blue}} = 41.0$ ,  $P_{\text{blue}} = 0.003$ , Fig. 3A;  $F_{\text{green}} = 6.288$ ,  $P_{\text{green}} = 0.066$ , Fig. 3B;  $F_{\text{red}} = 79.5$ ,  $P_{\text{red}} = 0.001$ , Fig. 3C). Clearly the normal rhythm of cone opsin expression appeared to be disrupted due to the absence of light.

## Discussion

Opsin synthesis is a continuous process in both rod and cone photoreceptors because opsin constantly needs to be replenished.



**Fig. 1.** Diurnal rhythm of cone opsin expression. (A) Blue-sensitive cone opsin, (B) green-sensitive cone opsin, and (C) red-sensitive cone opsin. Average cone opsin expression is shown as a percentage of maximum expression, found to occur at 6 PM for each opsin. The bar below the time of day illustrates the light/dark cycle. Error bars represent standard error.



**Fig. 2.** Diurnal rhythm of rod opsin expression. Percent rod opsin mRNA is shown relative to the 12 PM level in samples of 10  $\mu$ g total RNA at different time points, as determined by Northern blotting (Korenbrodt & Fernald, 1989). The bar below the time of day illustrates the light/dark cycle.

While “older” opsin is disposed of in rod photoreceptors *via* outward displacement of discs and ultimately disc shedding, opsin disposal in cone photoreceptors is complicated by the ubiquitous addition of new opsin within the entire outer segment (Young, 1969, 1976). Our data show that there is significant temporal regulation of cone opsin mRNA synthesis during the light/dark cycle in the African cichlid fish *H. burtoni*. Transcript abundance demonstrates that mRNA levels of all three cone opsins in *H. burtoni* reach their maximum in the subjective late afternoon. This is in agreement with a previous report from Pierce et al. (1993), who showed semiquantitatively that chick iodopsin mRNA levels peaked in the late afternoon and evening. Pierce et al. observed this rhythm both *in vivo* and in primary cultures of dispersed embryonic chick retina. Furthermore, the rhythm in chick retina persisted in the absence of external time cues suggesting circadian control. In contrast, our data from the *H. burtoni* retina indicate that continuous exposure to the light/dark cycle is necessary to maintain the rhythm of cone opsin expression in these fish. Species and developmental differences between chick embryos and mature *H. burtoni* might account for the difference between chicks’ circadian rhythm and *H. burtoni*’s rhythm of cone opsin expression, which is diurnal. In particular, chick photoreceptors appear to act as circadian pacemakers themselves, similar to cone photoreceptors in *Xenopus* as suggested by work in eyecup cultures (Cahill & Besharse, 1993). By the lack of intrinsic pacemaking cues in cone photoreceptors, fish such as *H. burtoni* reserve the capability to respond more flexibly to changes in light intensity and coloration than amphibians (*Xenopus*), higher vertebrates (chicks), and even mammals (mice; Korenbrot & Fernald, 1989; Pierce et al., 1993; von Schantz et al., 1999).

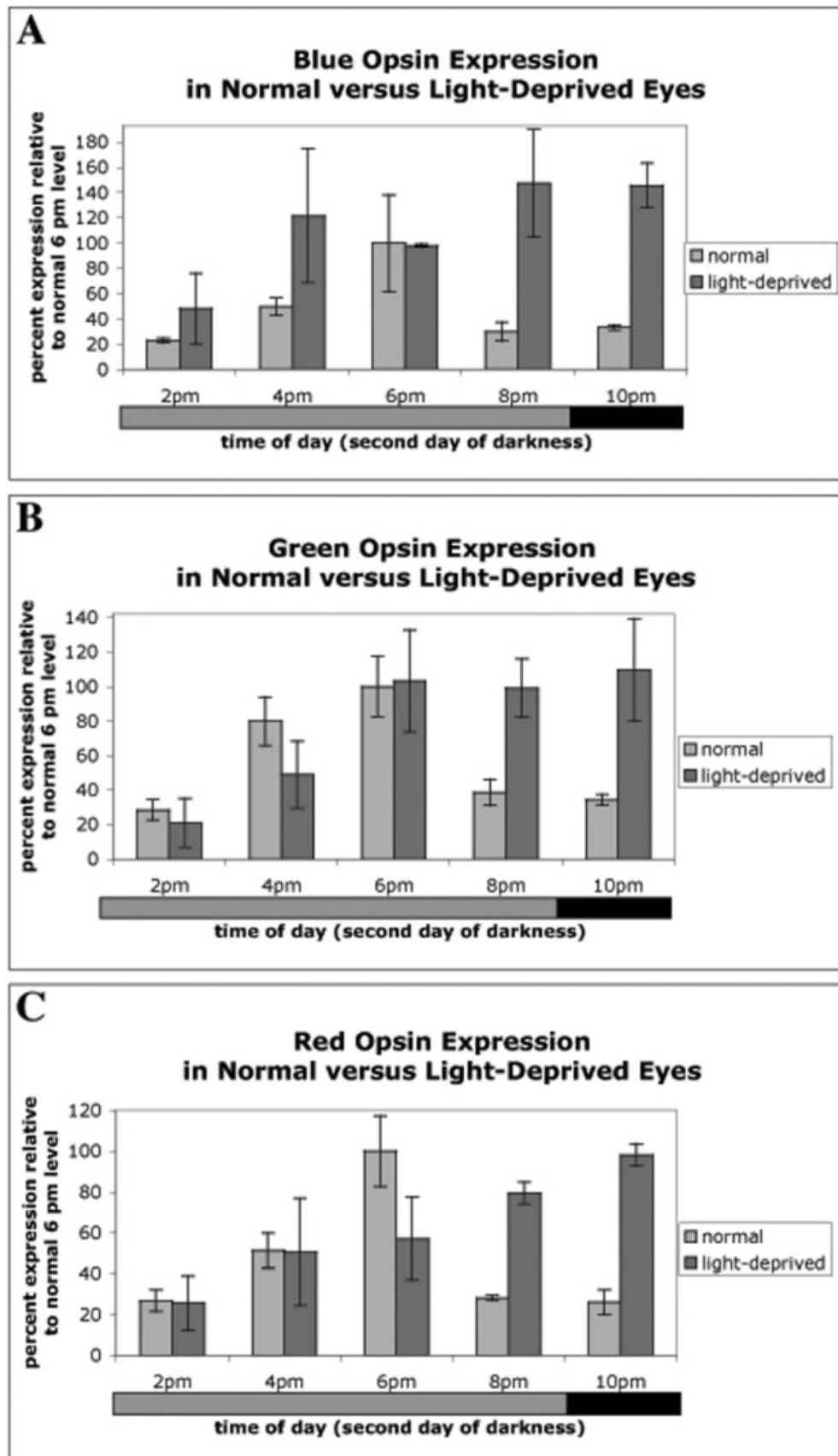
A second finding of our study is that cone opsin expression in *H. burtoni* is not in phase with rod opsin expression. Previous studies have shown that rod opsin transcript abundance peaks in the early morning, decreases as the day progresses, and finally tapers off to lower levels at night (Korenbrodt & Fernald, 1989). Thus, cone opsin and rod opsin expression are approximately 6 h

out of phase with each other. Assuming a lag time of a few hours between DNA transcription and photopigment synthesis and positioning within the cell, it appears that each photoreceptor replenishes its photopigment at a time when the photoreceptor is least active, suggesting a possible evolutionary selective pressure for this timing. Since rods are the primary cell type involved in night vision, allocating energy to rod opsin synthesis during the day would be advantageous for energy and metabolic considerations, and *vice-versa* for the cones. In particular, cone photoreceptors appear to conform to this hypothesis by preparing for protein synthesis—that is, by producing cone opsin mRNA—before dark onset (Fig. 1) so that photopigment synthesis and intracellular distribution can occur during the night.

When comparing the amplitude of the diurnal expression among the three cone opsins, there is clearly a smaller increase in blue opsin mRNA expression from 9 AM to 6 AM as compared to green and red opsin mRNA expression (Fig. 1). Assuming that each opsin mRNA molecule gives rise to the same number of protein molecules for all three cone opsins, it thus appears that there is comparatively less blue opsin renewal than green and red opsin renewal. This may be accounted for by the fact that blue cones are half as abundant in the retina and are substantially smaller than green and red cones (Fernald & Liebman, 1980). It could also be that blue cone opsin turnover is lower due to greater protein stability or less blue opsin usage, although the relationship between use and renewal is tenuous.

Interestingly, the duration of increased cone opsin mRNA expression in normal fish is substantially shorter than that of rod opsin expression. Clearly this could be due to the much larger number of rods than cones in the retina. In addition, the shorter duration of increased cone opsin expression perhaps reflects that there is less cone than rod disc shedding, with rods shedding about 10% of outer segment length every 4 to 5 days (Young & Bok, 1969).

How might the rhythms of opsin synthesis in rods and cones be controlled? Recent evidence suggests several possibilities. The



**Fig. 3.** Cone opsin expression in normal *versus* light-deprived eyes. (A) Blue-sensitive cone opsin, (B) green-sensitive cone opsin, and (C) red-sensitive cone opsin. Average cone opsin expression is shown as a percentage of maximum expression in normal fish, found to occur at 6 PM for each opsin. The bar below the time of day illustrates the light/dark cycle. Error bars represent standard error.

existence of non-rod, non-cone ocular photoreceptors has led to the identification of a new gene family of opsins in teleost fish with expression in different cell types, including horizontal cells as reviewed by Foster and Bellingham (2004). Clearly there are now many possible ways in which cone opsin mRNA levels described here could be regulated. Interestingly, in *H. burtoni*, earlier work showed that the growth of the lens is regulated by light in a restricted range of wavelengths (Kroger et al., 2001), suggesting that developmental and well as physiological processes could be regulated *via* these new pathways of photic detection.

Whether pacemaker function is in photoreceptor cells or other cell types, and whether they act separately or in concert, remains to be discovered. The discovery that mRNA synthesis in rods and cones of *H. burtoni* is out of phase under normal conditions contributes to the understanding of opsin renewal and provides a standard against which molecular and lighting factors can be tested to understand their role in controlling diurnal oscillations in retinal photoreceptors.

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