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Retinal development and photoreceptor synaptic ultrastructure are altered by abnormal rearing light regimes

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SUMMARY

The effects of light rearing regimen on distal retinal development and photoreceptor ultrastructure were investigated using light and electron microscopy. Zebrafish larvae were reared in constant light, control/cyclic light (14 hr light/10 hr dark), or constant dark conditions until 4 or 8 days post-fertilization (dpf). Gross retinal morphology was not altered by light rearing conditions; however, ultrastructural differences were noted both within and between age groups. Significant differences were seen in photoreceptor outer segments (OS) and synaptic ribbons, the size of cone photoreceptor mitochondria, and postsynaptic horizontal cell spinules. Larvae reared in constant dark displayed reduced pigment dispersion; OS development was delayed and cone mitochondria were smaller at 4dpf, two results that reversed by 8dpf.

Photoreceptor terminals of larvae reared in all treatment conditions displayed anchored synaptic ribbons with arciform densities and no significant differences in ribbon number. Ribbons were 30-40% longer in photoreceptor terminals within the constant light treatment. The number of horizontal cell spinules invaginating into cone terminals varied and the spinule-to-ribbon ratio was higher in control and constant light-reared tissue by more than 2x at 4dpf. By 8dpf, this ratio was significantly highest in retinas reared in control/cyclic light conditions. Taken together, these results show that abnormal light rearing conditions affect synaptic structure in distal retina.

These changes suggest a mechanism for the physiological and behavioral deficits reported in zebrafish larvae grown under constant light and/or dark conditions.

Key words: Synaptic ribbons – Spinules – Outer plexiform layer – Light

INTRODUCTION

The natural light-dark cycle leads to structural and physiological changes that modulate neuronal activity and change visual responsiveness in the vertebrate visual system. In fish, retinomotor movements, involving both photoreceptors and pigment epithelium, follow circadian rhythms which optimize visual processes by coordinating structural changes with the dark-light cycle (Blaxter and Jones, 1967; Dearry and Barlow, 1987; Burnside and Ackland, 1984). In addition, synaptic structures are subject to adaptive changes driven by environmental illumination levels. Specifically, the process of light adaptation involves assembly of photoreceptor synaptic ribbons and formation of postsynaptic horizontal cell spinules, while dark adaptation leads to ribbon disassembly (Allwardt et al., 2001; Emran and Dowling, 2010) and a reduction in spinule formation (Kroger and Wagner, 1996). These structural alterations affect visual function (Emran and Dowling, 2010) with changes

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(reductions) in ribbon length being associated with visual function deficits (Balkema et al., 2001; Emran and Dowling, 2010).

Studying the effects of either prolonged light exposure or prolonged light deprivation on the visual system during development is important for identifying specific neurons and circuits that are sensitive to anomalous light conditions, as well as for determining the cellular mechanisms underlying the observed physiological changes. The effects of continuous light and continuous darkness on the vertebrate retina have been investigated in several species, including mammals, amphibians, and fish (Kuwabara and Funahashi, 1976; Tucker and Hollyfield, 1977; Raymond et al., 1988; Ando and Noell, 1993). The onset, extent and severity of light damage vary by species, animal age, length of exposure, and light intensity. Research using zebrafish has established that abnormal lighting alters retinal physiology and visual behavior. Abnormal light rearing conditions change spectral sensitivity, as measured by ERGs (Saszik and Bilotta, 1999), and visual acuity, as measured by the optomotor response, in zebrafish larvae (Bilotta, 2000). Orientation-selective ganglion cell responses and tectal organization are altered following constant dark-rearing (Lowe et al., 2013), a condition that also reduces hatching success, growth and survival in zebrafish larvae (Villamizar et al., 2014).

To date, the reported physiological and behavioral changes identified using ERGs are not supported by published reports of changes in retinal anatomy. Some anatomical studies of zebrafish reared in constant light (Robinson and Dowling, 1994) or constant dark (Robinson and Dowling, 1994; Easter and Nicola, 1996) report no differences in retinal morphology compared to control animals. Previously, we showed that the fine structure of the optic nerve of zebrafish larvae reared in constant light and constant dark does not vary from that of control animals (Chapman et al., 2012). In contrast, Vihtelic and Hyde (2000) reported a loss of cells in the outer nuclear layer and a reduction in the thickness of the photoreceptor layer in adult albino zebrafish following continuous light exposure. Similarly, Lyday (2001) described alterations in retinal thickness in larval zebrafish reared in different lighting conditions up to 15 days post fertilization (dpf). Bilotta and Saszik (2001) proposed that the abnormal physiological responses seen in zebrafish larvae reared in altered light conditions might be caused by changes in synaptic structure, rather than retinal gross morphology, and no studies to date have examined that proposal.

The purpose of this study was to investigate, using light and electron microscopy, the effects of prolonged constant light, constant darkness, and cyclic (control) light (14 hour light/10 hour dark) on gross anatomy and ultrastructure of the distal retina in zebrafish larvae, focusing on photoreceptors and the synapses they make in the outer plexiform layer. Our data identify changes in presynaptic ribbon length and postsynaptic spinule density in cone pedicles, suggesting that light alters these components of photoreceptor synapses, in agreement with published physiological (Saszik and Bilotta, 1999) and behavioral (Bilotta, 2000) experiments with zebrafish larvae reared under the same conditions.

MATERIALS AND METHODS

Animal maintenance

Adult wildtype zebrafish (*Danio rerio*) were allowed to spawn and the embryos collected. At 24 hours post-fertilization (hpf), the embryos were placed into one of three light conditions: constant light, constant dark, or cyclic (control) light (14 hour light/ 10 hour dark). Embryos were kept under these light conditions until aged 4 or 8 dpf. All were maintained in fresh water at 28.5°C and fed from a culture of mixed *Paramecium* species (*P. aurelia, P. caudatum,* and *P. micronucleatum*) starting at 4 dpf. Standard aquarium lights (spectral range: 400-600 nm, light intensity: ~6000-7000 cd/m^2) were used for both the constant and cyclic light treatments. Food density was maintained at a minimum of 10,000 *Paramecium* per liter, which is considered *ad libitum* (Westerfield, 2007). Larvae were checked at the same time each day.

Constant darkness was achieved by maintaining the larvae in a light-tight room for the duration of the experiments. Dim red light (≤ 15 minute) exposure (403 cd/m^2, >620 nm) was used to access the larvae in the constant dark conditions, similar to methods reported by others (Martin-Robles et al., 2012; Menger et al., 2005; Hodel et al., 2006). All protocols and procedures were approved by the Institutional Animal Care and Use Committee at American University.

Light and electron microscopy

All larvae were sacrificed at the same time of day, 2 hours into the light phase. Dark- reared larvae remained in dark conditions until the end of primary fixation. For transmission electron microscopy (TEM), two larvae per light rearing regimen were anesthetized using tricaine (MS-222) and then fixed in a Millonig's fixative for two hours, which consisted of 5% glutaraldehyde (Sabatini et al., 1963) in double strength phosphate buffer with $CaCl_2$ (Millonig, 1961). Larvae were then washed four times, for 15 minutes each, in phosphate buffer and then post- fixed in 1% osmium tetroxide in the same buffer for two hours. This was followed by dehydration through a graded (50%, 70%, 85%, 95%, 100%, 100%) ethanol-water series followed by two 15-minute washes in propylene oxide. The tissue was infiltrated over-

night at 20°C in a 1:1 mixture of epoxy resin and propylene oxide (Luft, 1961). A 3:1 mixture of epoxy resin and propylene oxide was used to further infiltrate the tissue at 20°C for another 9 hours. Finally, the specimens were embedded in epoxy resin and placed into a polymerizing oven at 45°C for 24 hours, followed by another 24 hours at 60°C.

Thin (0.25-1.0 µm) and ultrathin (50 nm) sections were cut with a Delaware diamond knife on a Sorvall Porter-Blum MT-2B ultramicrotome. The thin sections were mounted on glass slides and stained with an aqueous mixture of 1% methylene blue, 1% azure A and 1% sodium borate (Richardson et al., 1960). These sections were used for light microscopy (LM). The ultrathin sections were collected on copper grids (150-300 mesh), stained with uranyl acetate in 50% ethanol for 20 minutes (Gibbons and Grimstone, 1960) and 0.4% lead citrate for 10 minutes (Venable and Coggeshall, 1965), and viewed with a JEOL JEM 1010 transmission electron microscope. Electron micrographs were taken using Kodak Electron Microscope Film #4489 and developed in Kodak D-19. Prints from electron micrograph negatives were developed on Ilford multi-grade paper in a 1:2 Kodak Dektol-water developing solution, washed, fixed in Kodak acid fixer and rewashed.

Measurements

Digital measurements of the thickness (vitreal-scleral direction) of the neural retina, ganglion cell layer (GCL), inner plexiform layer (IPL), and inner nuclear layer (INL) were obtained from stained histological slides (Fig. 1) using an Olympus BX61 compound microscope and Metamorph (version 7.7.0.0; Olympus) imaging software. Forty sections were measured from two larvae for each age and light rearing condition. Each section was measured at five locations in central retina that were in the vicinity of the optic nerve and directly behind the lens. For consistency, measurements were obtained only from sections that had a visible cornea and lens. The sections were generated by cutting the larval head along the transverse plane, which cut the eyes in a plane parallel to the anterior-posterior axis (the lens being anterior) (Chapman et al., 2012). This procedure minimized any measurement error that could result from different sectioning angles.

The photoreceptor layer (PL) was measured from Bruch's membrane sclerad to the retinal pigment epithelium (RPE) to the vitread (proximal) edge of the outer nuclear layer (ONL) and, thus, it includes photoreceptor outer (OS) and inner segments (IS). Measurements of the PL, cone mitochondria, synaptic ribbons, and spinules in photoreceptor terminals were collected either from negatives or enlarged micrograph prints.

To quantify the numbers of ribbons per terminal, 60-80 photoreceptor terminals were assessed from two animals for each age and light treatment (total > 300 terminals per treatment). For calculations of spinules per terminal and the ratio of spinules to ribbons per terminal, ~ 20 cone terminals were surveyed for each age and light treatment. Only anchored ribbons were measured in the sections we surveyed which were collected from central retina as described above. Therefore, it is reasonable to assume that the sections studied from the 3 treatment conditions are equivalent and, as such, comparing ribbon measurements among the treatment groups is valid. The ratio of spinules-to-ribbons per terminal was calculated as previously described by Biehlmaier et al. (2003): the total number of spinules per terminal was divided by the number of ribbons in that terminal. Only receptor terminals that had visible ribbons and arciform densities were used for the calculations. The reported mitochondrial size was obtained by measuring mitochondria along the greatest dimension (i.e., Tarboush et al., 2012).

Statistical analyses of all measurements were performed using Microsoft Excel 2007. A one-way analysis of variance (ANOVA) was used to determine statistical significance of mean differences in retinal layer measurements and ribbon numbers. One-tailed t-tests were used to compare mean spinule numbers between different treatment conditions. Chi-square tests were used to compare spinule-to-ribbon ratios among the different treatment groups. For all statistical analyses used, P-value ≤ 0.05 was considered statistically significant.

RESULTS

Retinal gross morphology

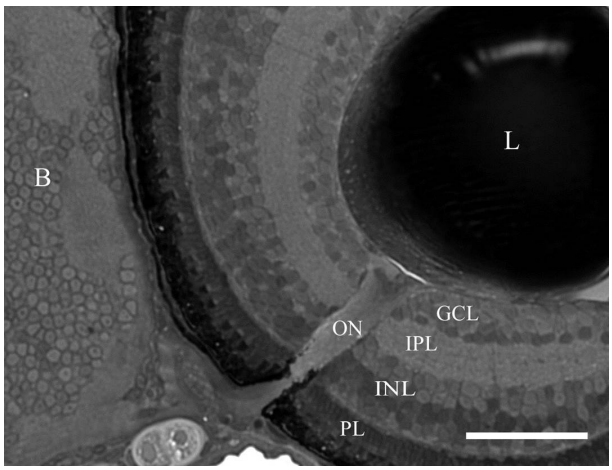

Fig. 1. Low magnification light micrograph of a retinal section. A transverse section of a 4 dpf zebrafish larval eye. The lens (L), optic nerve (ON), and brain (B) are visible. Retinal layers are labeled: ganglion cell layer (GCL), inner plexiform (IPL), inner nuclear layer (INL), outer nuclear layer (ONL) and photoreceptor layer

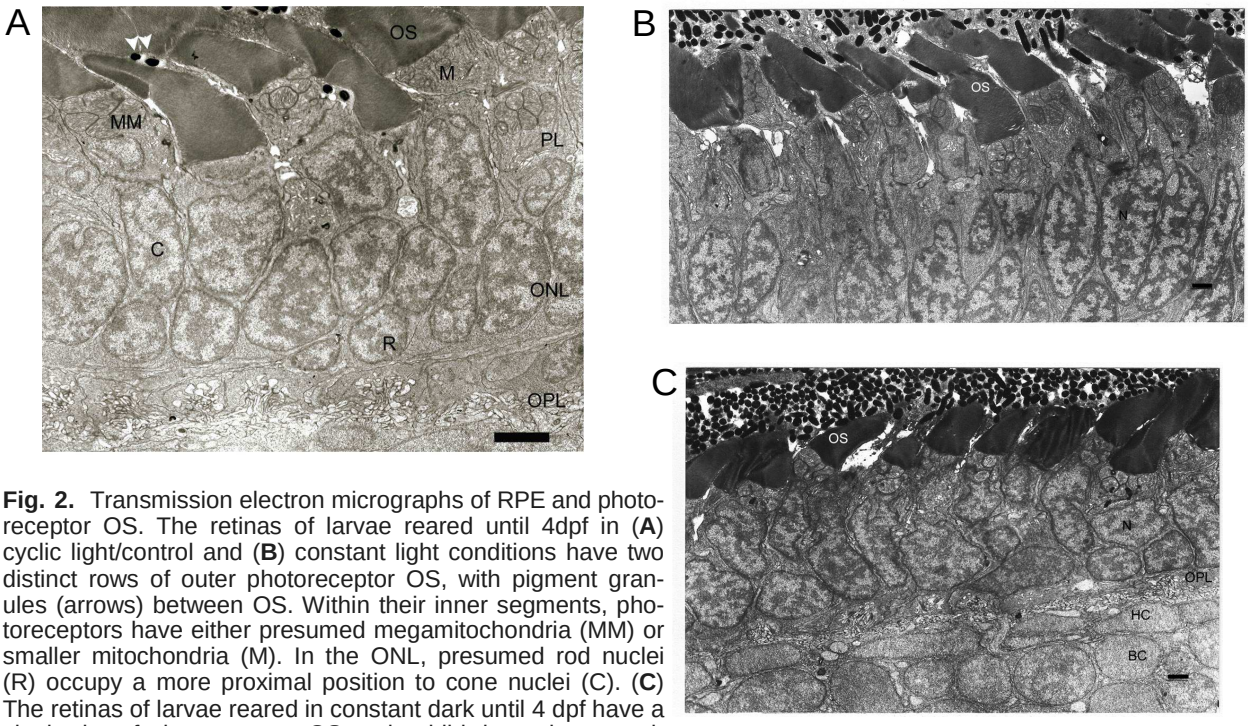

Fig. 2. Transmission electron micrographs of RPE and photoreceptor OS. The retinas of larvae reared until 4dpf in (**A**) cyclic light/control and (**B**) constant light conditions have two distinct rows of outer photoreceptor OS, with pigment granules (arrows) between OS. Within their inner segments, photoreceptors have either presumed megamitochondria (MM) or smaller mitochondria (M). In the ONL, presumed rod nuclei (R) occupy a more proximal position to cone nuclei (C). (**C**) The retinas of larvae reared in constant dark until 4 dpf have a single tier of photoreceptor OS and exhibit less pigment migration in the RPE. Similar differences in pigment granule migration in the RPE were seen in the 8dpf specimen reared in constant dark conditions (not shown). Scale bars in A, B and C = 1 µm. BC: bipolar cell nucleus; HC: horizontal cell nucleus; INL: inner nuclear layer; N: photoreceptor nucleus; ONL: outer nuclear layer; OPL: outer plexiform layer; RPE: retinal pigment epithelium; OS: photoreceptor outer segment.

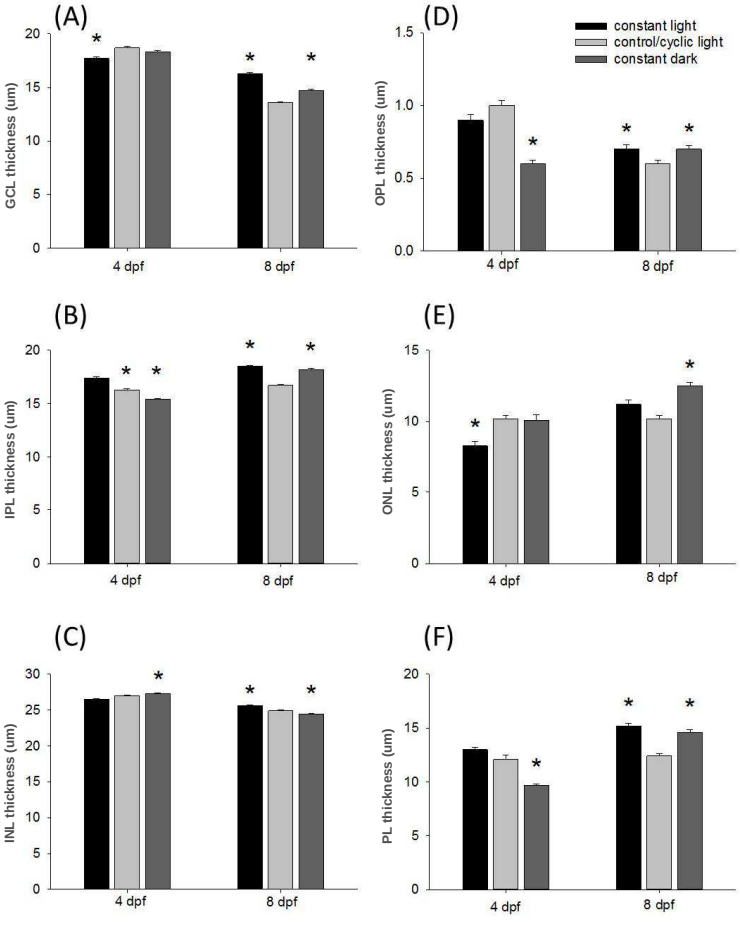

Fig. 3. Measurements of zebrafish retinal layers. Values (in µm) measured for 4 and 8 dpf larvae given as means ± SE. Asterisks indicate significant differences from control/cyclic light (ANOVA). (**A**) GCL = ganglion cell layer (4 dpf N = 593, $P = 0.032$; 8 dpf: N = 600, $P < 0.001$); (**B**) IPL = inner plexiform layer (4 dpf: N = 595, $P < 0.001$; 8 dpf: N = 600, $P < 0.001$); (**C**) INL = inner nuclear layer (4 dpf: N = 595, $P = 0.009$; 8 dpf: N = 599, $P < 0.001$); (**D**) OPL = outer plexiform layer (4 dpf: N = 91, $P = 0.027$; 8 dpf: N = 90, $P = 0.003$); (**E**) ONL = outer nuclear layer (4 dpf: N = 60, $p < 0.001$; 8 dpf: N = 60, $P = 0.059$); (**F**) PL = photoreceptor layer (4 dpf: N = 38, $P < 0.001$; 8 dpf: N

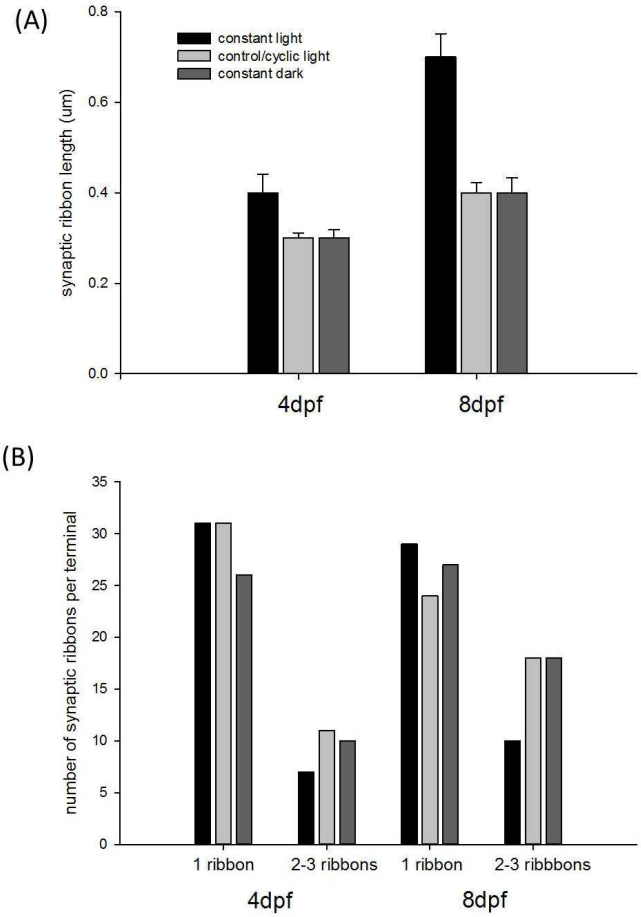

Fig. 4. Synaptic ribbon length and number. (**A**) Length of synaptic ribbons in larvae reared in cyclic light, constant light, and constant dark. Ribbons were longest in the constant light treatment. As animals grew from 4 to 8 dpf, ribbon length increased across the three light conditions, though they remained significantly larger in constant light-reared animals (N = 30; $P << 0.0001$)**.** Values are given as means ± SE in µm. (**B**) Number of synaptic ribbons per photoreceptor terminal at 4 and 8 dpf. There was no statistically significant difference in the number of ribbons per terminal in animals reared in the three lighting conditions at either 4dpf ($X^2 = 1.04$, df = 2, n = 116 $P = 0.595$) or 8dpf ($X^2 = 2.95$, df = 2, n = 126, $P = 0.229$). The graph shows the number of surveyed terminals with 1, 2, and 3 ribbons within each treatment.

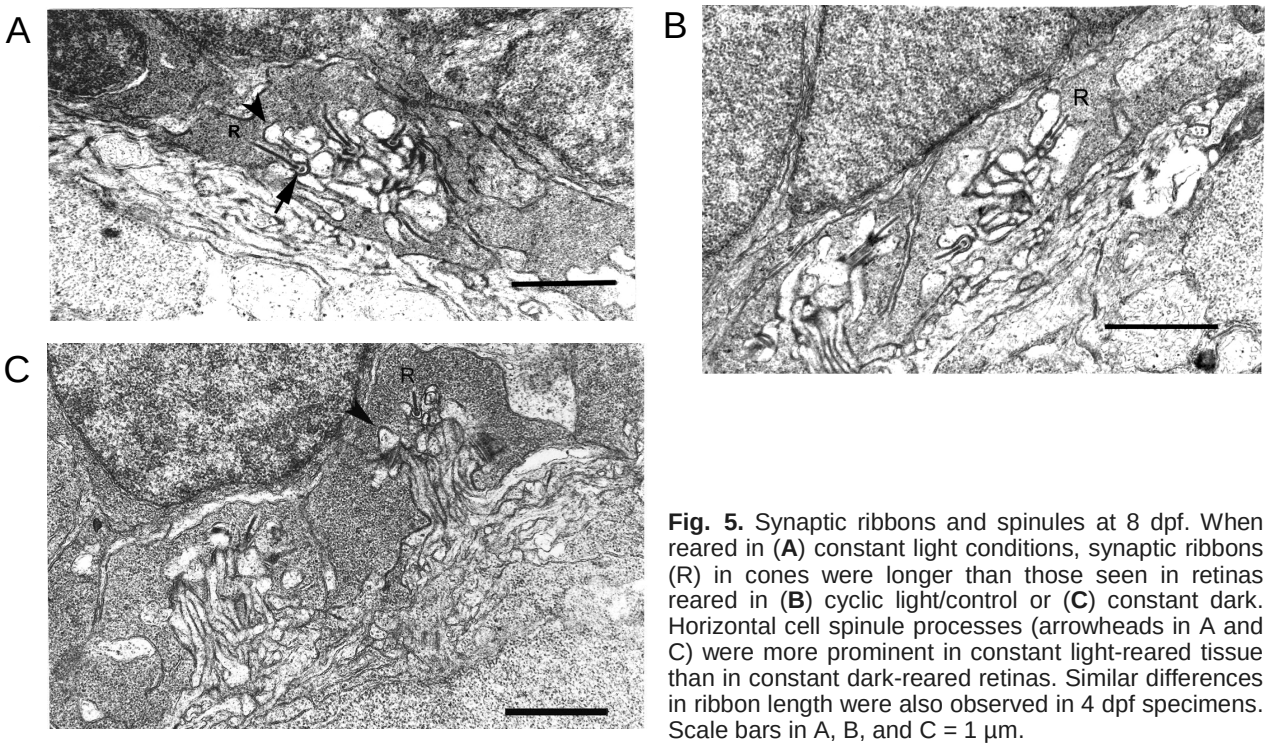

Fig. 5. Synaptic ribbons and spinules at 8 dpf. When reared in (**A**) constant light conditions, synaptic ribbons (R) in cones were longer than those seen in retinas reared in (**B**) cyclic light/control or (**C**) constant dark. Horizontal cell spinule processes (arrowheads in A and C) were more prominent in constant light-reared tissue than in constant dark-reared retinas. Similar differences in ribbon length were also observed in 4 dpf specimens. Scale bars in A, B, and C = 1 µm.

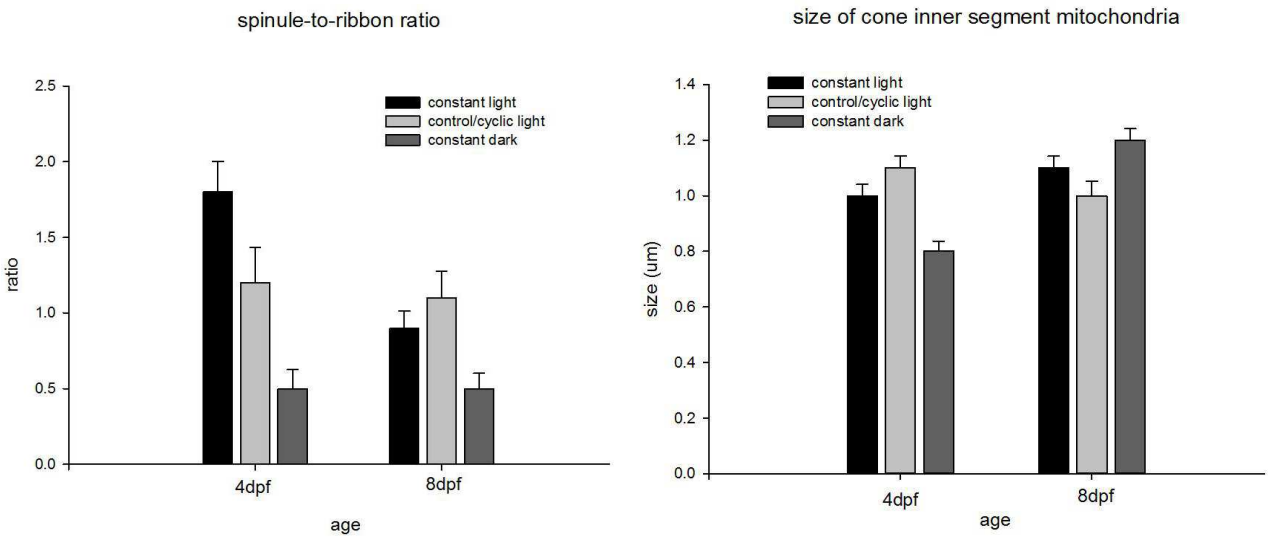

Fig. 6 (left). Ratio of spinules-to-ribbons per pedicle in 4 dpf and 8 dpf larvae. Dividing the number of spinules that invaginate into a cone pedicle by the total number of ribbons in that pedicle gives a measure of cone functionality (Biehlmaier et al., 2003). At 4 dpf, larvae reared in constant dark had the lowest ratio and larvae reared in constant light had the highest (ANOVA, $P = 0.00012$). By 8 dpf, the ratio was still significantly less in constant dark- reared retinas compared to the other two treatments (ANOVA, $P = 0.016$).

Fig. 7 (right). Size of cone inner segment mitochondria from retinas of larvae reared in the three lighting conditions. Mitochondria were significantly smallest in tissue from constant dark conditions at 4 dpf (ANOVA, $P < 0.001$), but this effect reversed at 8 dpf resulting in no significant differences in mitochondrial size at this age (ANOVA, $P = 0.096$). Values (in µm) are means ± SE calculated from measurements of all mitochondria present (both large and small).

At 4 days after fertilization (4 dpf, Fig. 1), all components of the zebrafish eye, including the retina, were clearly visible at low magnification in all animals, regardless of treatment (Chapman et al., 2012). Eyes from fish reared in control/cyclic light, constant light, and constant dark appeared similar, with no obvious gross morphological differences. Likewise, eyes from fish reared to 8dpf under the three different light conditions appeared similar (as in Chapman et al., 2012).

There were two consistent differences in retinal structure observed at both the LM and TEM levels in constant dark-reared retinas compared to constant light-reared retinas (Figs. 2A-C): (1) delayed development of a second tier of photoreceptor OS at 4 dpf and (2) reduced migration of pigment granules into projections that extend between photoreceptor OS (Fig. 2C).

Rearing in constant light (Fig. 2B), in contrast, did not cause any differences in retinal structure compared to controls (Fig. 2A). By 8 dpf, two tiers of photoreceptor OS were observed in tissue collected from larvae reared in all three treatments, although pigment dispersion remained reduced in constant dark-reared specimens.

To quantify observed changes in anatomy, retinal layer thicknesses were measured. At 4 dpf, thicker nuclear layers, but thinner plexiform layers (OPL and IPL), were found in the retinas of larvae reared in constant dark compared to either cyclic or constant light-treated larvae (Fig. 3A-F). These values were significantly different at this age, though overall retinal thickness was not significantly different across groups (average: 78-79 µm; $P = 0.779$). At 8 dpf, significantly thicker retinal layers were observed in both constant light and constant dark treatments compared to controls (Fig. 3) and the overall thickness of the neural retina was significantly increased in these two treatment groups ($P < 0.001$). Though significant differences in layer measurements were observed at both ages, the trends in retinal layer size over time (i.e., with age) were the same for animals reared under all three light conditions.

Synaptic ribbons

In general, animals reared in constant light conditions had significantly longer synaptic ribbons than their age-matched counterparts reared in either constant dark or cyclic light (Fig. 4A). Compared to 4 dpf larvae, synaptic ribbons (Figs. 5A-C) were longer in animals aged 8 dpf regardless of light rearing condition (Fig. 4A $P < 0.05$).

Synaptic functionality: spinules and ribbons

To determine possible differences in synaptic functionality within the three light-rearing conditions, two values were collected from each cone photoreceptor pedicle: (1) the number of synaptic ribbons with arciform densities and (2) the number of invaginating horizontal cell spinules. From these values, the per-terminal-ratio of spinules-to-ribbons was calculated (Biehlmaier et al., 2003). Only rib-

bons with arciform densities (Figs. 5A-C) were included based on the assumption that these ribbons were anchored to the membrane and, hence, functional. The numbers of synaptic ribbons with arciform densities did not vary significantly with light rearing condition at either 4 or 8 dpf (Fig. 4B).

The average number of spinules per pedicle counted in larvae reared in constant light (2.26 spinules) at 4 dpf was significantly higher than the number of spinules counted in both constant dark (0.71 spinules) and control (1.6 spinules) tissue (one-tailed t-test, $P < 0.05$). At 8 dpf, the average number of spinules per pedicle in larvae reared in constant light (1.5 spinules) was not significantly different from that seen in larvae reared in control conditions (1.46 spinules); however, the average number in larvae reared in constant dark (0.95 spinules) was significantly lower ($P <0.05$).

Similarly, the spinule-to-ribbon ratio within each cone pedicle significantly varied among the three light treatments (Fig. 6, ANOVA: $P <0.001$). Due to the reduced number of spinules observed in constant dark tissue, the average spinule-to-ribbon ratio was lowest for animals reared in this regimen at both 4 and 8 dpf (Fig. 6). This ratio was highest in retinas from animals reared under constant light conditions at 4 dpf, but then decreased at 8 dpf leaving the highest spinule-to-ribbon ratio in control animals at this age.

Cone mitochondria

Cones identified in this study contained the previously described mega-mitochondria (Kim et al., 2005, Tarboush et al., 2014) as well as small mitochondria, and both types of mitochondria were tightly packed within their IS. At 4dpf, the average size of cone mitochondria (large and small) in larvae reared in the constant dark treatment was significantly (~20%) smaller than the average size of mitochondria in larvae reared in either control/ cyclic light or constant light ($P < 0.01$; Fig. 7). Under constant light conditions, cone mitochondria were slightly smaller than those in control larvae, but the difference was not significant.

At 8 dpf, the size of cone mitochondria in retinas reared in constant dark was larger while the smallest mitochondria were observed in retinas from control animals (Fig. 7). These differences in mitochondrial size at 8 dpf were either not significant (constant light vs. control/cyclic light and constant light vs. constant dark) or marginally significant ($P = 0.05$: control/cyclic light vs. constant dark). As larvae grew from 4 to 8 dpf, the greatest increase in mitochondrial size (presumably due to growth) was observed in the photoreceptors of larvae reared in constant dark conditions ($P <0.0001$). Cone mitochondria from larvae reared in constant light also showed a significant increase in size ($P = 0.020$), while mitochondria in control tissue did not ($P =0.50$).

DISCUSSION

Altered lighting conditions are reported to change visual behavior (Bilotta, 2000) and retinal physiology in zebrafish (Saszik and Bilotta, 1999; Dixon et al., 2004). Our results suggest that altered light rearing conditions also cause changes in the ultrastructure of the distal retina, which could contribute to the altered functionality reported in the literature. Constant dark rearing results in a developmental delay and a constant state of dark (or semi-dark) adaptation; whereas, constant light conditions result in significantly longer synaptic ribbons and a reduced spinule number in photoreceptor terminals suggesting changes in neurotransmitter release and/or synaptic efficacy.

Constant dark rearing

In 4 and 8 dpf constant dark reared animals, pigment granules around photoreceptor OS were restricted to the body of the pigment epithelial cells, with few or no pigment granules migrating into the apical projections. Larvae reared in constant dark conditions had a thicker GCL and INL, but a thinner IPL and OPL, compared to larvae reared in constant and cyclic light, suggesting delayed retinal development caused by a delay in the normal apoptotic pruning of excess cells. This hypothesis is supported by four other observations: (1) at 4 dpf, larvae reared in the constant dark treatment have only one tier of OS, while larvae reared in constant or cyclic light have two distinct tiers, (2) cone mitochondria are smaller, (3) ribbons are shorter, and (4) larvae hatched later. These observations are consistent with published reports showing that constant dark rearing differentially affects visual responses and tectal innervation of ganglion cell types in zebrafish (Lowe et al., 2013) and significantly alters hatching time and reduces growth (Villamizar et al., 2014). We also hypothesize that these zebrafish larvae are in semi-dark adapted state characterized by a cessation of retinomotor movements (Wagner and Ali, 1977) resulting in the prolonged elongation of cone myoids in the dark and a thicker PL at 8 dpf.

Reports documenting the effects of dark rearing are mixed. In amphibians, total darkness leads to retinal cell loss (Hollyfield et al., 1980), but increases synaptic density in the IPL (Tucker and Hollyfield, 1977). Raymond et al. (1988) showed that exposure to constant light and dark for over a year led to a reduction in rod density in goldfish. In contrast, other studies, using zebrafish, failed to find gross anatomical changes in retinal layers, in spite of physiological differences (Bilotta, 2000; Bilotta and Saszik, 2001); though the spatial organization of ganglion cell inputs to the optic tectum is altered (Lowe et al., 2013). These inconsistencies may be attributable to differences in the length of light deprivation as well as species-specific differences. As we have shown in this study, the anatomical

changes that underlie the visual deficits are very subtle, as in tectum (Lowe et al., 2013), and pertain to retinal ultrastructure. Adult zebrafish maintained in constant dark conditions for 10 days have decreased BDNF/TrkB (two factors that promote neuronal development) mRNA levels (Sanchez-Ramos et al., 2013) suggesting light levels also impact gene expression.

The zebrafish PL accounts for 25-27% of total retinal thickness at 4 dpf and 30-33% at 8 dpf, compared to 52% of adult retinal thickness (Tarboush et al., 2012). Fish raised in constant and cyclic light have two distinct rows of photoreceptor OS at 4 dpf versus one row in animals reared in constant dark, resulting in a thinner PL in these larvae. Interestingly, the decreased PL thickness in dark-reared retinas did not correlate with a similar reduction in ONL thickness. In fact, larvae reared in constant light have the thinnest ONL. In zebrafish, the first photoreceptor OS appear at 60 hpf under normal conditions, ~ 10 hr after photoreceptor nuclei become discernible in the ONL (Schmitt and Dowling, 1999). The thinner PL, but only slightly thinner ONL, in 4 dpf animals reared in constant darkness again suggests a developmental delay in OS development. By 8 dpf, PL thickness has increased and a second tier of OS is present.

Mitochondria were some of the most conspicuous organelles in photoreceptor inner segments at both 4 and 8 dpf. Cone mitochondria were smallest in 4 dpf larvae reared in constant darkness. By 8 dpf, mitochondrial size was larger, though not statistically different, from those in controls, further suggesting an initial delay in development. Regardless of rearing light regimen, however, mitochondria in presumptive cones at 4 and 8 dpf are not adult-like.

Cone inner segments in adults contain multiple (2) mitochondria types (Tarboush et al., 2014). Larvae, however, lack these morphologically distinct types within a single inner segment (Tarboush et al., 2012; 2014), indicating the photoreceptors are still anatomically immature. In support of this interpretation, the mitochondria of 8 dpf control larvae averaged 1.03 µm in diameter, which is 40% smaller than the average size of the large mega-mitochondria in adult zebrafish (1.40 µm; Tarboush et al., 2012).

Animals reared in constant dark had fewer spinules invaginating into cone terminals than animals reared in either control or constant light conditions, in agreement with studies showing rarely any spinules in dark-adapted conditions. The observed reduction in spinule number in constant dark-reared retinas also suggests a semi-dark adapted state. Calculating the spinule-to-ribbon ratio at cone terminals as an index for functional changes (Biehlmaier et al., 2003) identified differences due to rearing light: the smallest ratio in larvae reared in constant dark.

Constant light rearing

Larvae reared in constant light displayed many characteristics that were similar to control animals. Pigment granules migrated between OS, consistent with light-dependent changes in RPE reported in several species (Bruenner and Burnside, 1986; Hodel et al., 2006; Blaxter and Jones, 1967; Collin et al., 1996; Dearry et al., 1990; Futter et al., 2004), including zebrafish (Menger et al., 2005; Hodel et al., 2006) and full dispersion of the RPE was observed. Two distinct rows of photoreceptor OS were evident at 4 dpf due to the presence of UV and/or blue cones (vitreal row) and rods and green and red cones (sclerad row).

However, larvae reared in constant light had the longest synaptic ribbons and the highest spinule-to-ribbon ratio at 4 dpf , though by 8 dpf this ratio was significantly smaller than controls due to a decrease in the number of spinules. This change in ratio suggests that the retina initially develops normally, but then degenerates due to phototoxicity as a result of continued light exposure. Interestingly, in another teleost (blue acara), cyclic exposure to monochromatic (not white) light also changes the spinule-to-ribbon ratio (Kroger and Wagner, 1996), and, in adult zebrafish, differences in BDNF and TrkB mRNA levels (Sanchez-Ramos et al., 2013) occurred after exposure to white vs. blue light, suggesting it is not just the presence of light but also the chromatic properties that can affect spinule dynamics and gene expression.

Zebrafish, and other fish species, reared in constant conditions do not display circadian rhythms (Hurd and Cahill, 2002; Vallone et al., 2007; Cuesta et al., 2014; Martin-Robles et al., 2012; Vuilleumier et al., 2006; Dekens et al., 2003). Importantly, circadian rhythms are not necessary for normal development (Lahiri et al., 2014), though light rearing conditions can affect this process (Villamizar et al., 2014). In our protocol, zebrafish larvae were placed into constant light or dark conditions at 1 hpf and maintained in these conditions until 4 or 8 dpf. Larvae in the constant light/dark treatments did not encounter cyclic light conditions and were maintained at a constant temperature, suggesting an absence of circadian rhythms (Cuesta et al., 2014; Martin- Robles et al., 2012; Vuilleumier et al., 2006; Dekens et al., 2003), particularly in the constant light-reared group. Dim red light was used to access (feed) larvae in the constant dark-reared group at the same time each day. Daily dim red light exposure did cause rhythmic locomotor activity in a minority of zebrafish larvae reared in constant darkness from 5-9 dpf (Hurd and Cahill, 2002) because the larvae became ‘phase locked’ to the time of access (Hurd and Cahill, 2002). If a similar ‘phase lock’ occurred in our conditions, the shorter ribbon length observed in the constant dark-reared retinas may reflect partial reassembly of the ribbons, again

suggesting a semi-dark adapted state. Clearly, control/cyclic light conditions are crucial for maintaining normal synaptic structure and functionality of photoreceptors during development.

Relating ultrastructural data with physiology

The adverse effect of rearing light level on retinal function was initially reported by Saszik and Bilotta (1999). They reared zebrafish larvae in constant light, constant dark, or cyclic light conditions until 6 dpf then transferred the larvae to cyclic light conditions and recorded ERG responses. Their results identified deficits in spectral sensitivities of the ERG b-wave: 6-8 dpf larvae reared in constant dark displayed deficits across all stimulating wavelengths used, while constant light-reared animals showed the largest changes in response to UV and short wavelength stimuli (Saszik and Bilotta, 1999; Dixon et al., 2004). No changes in a-wave spectral sensitivities were noted (Saszik and Bilotta, 1999) though a later study by the same investigators described differential effects of constant dark rearing on a- and b-wave threshold responses (Saszik and Bilotta, 2001). These studies suggested that light-induced deficits in ERG sensitivities are due to changes in synaptic efficacy between photoreceptor terminals and bipolar cell dendrites even though no obvious anatomical changes in photoreceptor-to-bipolar cell connections have been reported (Robinson and Dowling, 1994).

Given the known light-dependent changes in photoreceptor synaptic ribbons and spinules in outer retina, our results suggest that light induced changes in ERG components may be due to ultrastructural changes at this retinal synapse. Our data indicate that the formation and numbers of synapses and ribbons are independent of normal activity (Verhage et al., 2000; Hiesinger et al., 2006), while synaptic ultrastructure is clearly altered. Ribbon length varied based on light exposure, with ribbons either longer (constant light-reared) or shorter (constant dark-reared) than controls. Rearing in constant dark caused a delay in photoreceptor development and reduced synaptic functionality, while rearing in constant light only reduced synaptic functionality. These effects are evident early, 4 dpf, soon after hatching. Delayed OS development affects the receptors' ability to detect light, while reduced synaptic functionality suggests deficits in signal transmission. Synaptic alterations due to abnormal light levels do not appear to be permanent, however: (1) we observed recovery of retinal layer measurements at 8 dpf, (2) ERG responses of exposed larvae recover after 2 weeks in cyclic light (Saszik and Bilotta, 1999), and (3) spinule dynamics can be restored (Kroger and Wagner, 1996).

In conclusion, light deprivation and extended exposure to light during development affect the structure of ribbons and spinule synaptic elements associated with cone photoreceptor synapses in zebrafish outer retina. These synaptic alterations, subtle as they may be, correlate with reported functional deficits in animals exposed to similar abnormal lighting environments during development.

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