

Phosphodiesterase Inhibitors Sildenafil and Vardenafil Reduce Zebrafish Rod Photoreceptor Outer Segment Shedding

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Submitted: March 28, 2017

Accepted: September 12, 2017

Citation: Campbell LJ, Jensen AM. Phosphodiesterase inhibitors sildenafil and vardenafil reduce zebrafish rod photoreceptor outer segment shedding. *Invest Ophthalmol Vis Sci*. 2017;58:5604–5615. DOI:10.1167/iov.17-21958

PURPOSE. The vertebrate rod photoreceptor undergoes daily growth and shedding to renew the rod outer segment (ROS), a modified cilium that contains the phototransduction machinery. It has been demonstrated that ROS shedding is regulated by the light–dark cycle; however, we do not yet have a satisfactory understanding of the molecular mechanisms that underlie this regulation. Given that phototransduction relies on the hydrolysis of cGMP via phosphodiesterase 6 (PDE6), we examined ROS growth and shedding in zebrafish treated with cGMP-specific PDE inhibitors.

METHODS. We used transgenic zebrafish that express an inducible, transmembrane-bound mCherry protein, which forms a stripe in the ROS following a heat shock pulse and serves as a marker of ROS renewal. Zebrafish were reared in constant darkness or treated with PDE inhibitors following heat shock. Measurements of growth and shedding were analyzed in confocal z-stacks collected from treated retinas.

RESULTS. As in dark-reared zebrafish, shedding was reduced in larvae and adults treated with the PDE5/6 inhibitors sildenafil and vardenafil but not with the PDE5 inhibitor tadalafil. In addition, vardenafil noticeably affected rod inner segment morphology. The inhibitory effect of sildenafil on shedding was reversible with drug removal. Finally, cones were more sensitive than rods to the toxic effects of sildenafil and vardenafil.

CONCLUSIONS. We show that pharmacologic inhibition of PDE6 mimics the inhibition of shedding by prolonged constant darkness. The data show that the influence of the light–dark cycle on ROS renewal is regulated, in part, by initiating the shedding process through activation of the phototransduction machinery.

Keywords: cGMP, rods, outer segments, photoreceptor renewal

Vertebrate photoreceptors have a highly polarized and uniquely compartmentalized morphology that allows for sensitive detection and processing of light. A highly modified cilium called the outer segment (OS) contains the photopigments (opsins) and phototransduction machinery responsible for photon detection. The rod outer segment (ROS) is cylindrically shaped, and the photopigment Rhodopsin, which comprises approximately 95% of total protein, is embedded into discrete membranous discs that are tightly packed perpendicularly to the axoneme (reviewed in Ref. 1). The demand for OS disc membrane and protein turnover is so great that an estimated 9 to 10 billion opsin molecules are turned over every second in the human retina.² Rhodopsin is synthesized in the inner segment (IS) and transported to the ROS through the narrow transition zone, or connecting cilium, from which the ROS extends.³ This highly compartmentalized morphology and the tight, membranous packing require a unique pathway for disposal and recycling of ROS disc membranes and proteins.

Evidence for a unique ROS renewal mechanism was gained from experiments using pulse labeling with radioactive amino acids and autoradiography.^{4,5} These experiments demonstrated that newly synthesized proteins appear in the base of the OS and displace toward the distal tip of the OS over time until they

are shed by the photoreceptor and phagocytosed by the adjacent retinal pigment epithelium (RPE).^{6–8} As a consequence, the newest discs are at the base and the oldest are at the tip of the OS. In addition, to maintain constant OS length, the growth and shedding processes must be balanced. If the ROS shedding rate exceeds the growth rate, then the ROS will degenerate. ROS degeneration is the earliest histopathologic feature of retinitis pigmentosa, a heterogeneous collection of inherited blinding diseases in humans.⁹ Many animal models of retinitis pigmentosa and other blinding diseases present with progressive ROS shortening followed by photoreceptor cell death^{10–13}; however, we currently lack a satisfactory understanding of the molecular mechanisms that regulate ROS renewal.

The earliest identified regulator of ROS renewal was the diurnal light–dark cycle. In all vertebrate species studied, shedding at the distal tip of the ROS follows a cyclical burst of activity shortly after the daily onset of illumination.^{14–17} In addition, frogs that are exposed to prolonged darkness decrease the amount of ROS material that is shed.¹⁸ These observations prompted us to ask if the phototransduction machinery may autonomously regulate the shedding process.

In the dark, cGMP concentration is high and bound to a cyclic nucleotide-gated channel (CNG) that allows passage of



Na^+ and Ca^{2+} into the cell for a depolarized resting state. Light activates Rhodopsin, a G-protein-coupled receptor, which initiates a process that leads to activation of phosphodiesterase 6 (PDE6) and hydrolysis of cGMP. As a result, CNG channels close and the photoreceptor cells hyperpolarize (reviewed in Ref. 19). Hypothetically, inhibition of cGMP hydrolysis would mimic the dark state and prevent illumination-triggered shedding. Drugs such as sildenafil (Viagra; Pfizer, New York, NY, USA), vardenafil (Levitra; Bayer Pharmaceuticals, Leverkusen, Germany), and tadalafil (Cialis; Eli Lilly, Indianapolis, IN, USA) competitively, but reversibly, inhibit the cGMP-specific PDE5.²⁰ It has been shown that sildenafil and vardenafil are also potent inhibitors of purified PDE6, while tadalafil is PDE5 selective.²¹ We sought to examine ROS shedding and growth rates in zebrafish exposed to these PDE5/6 inhibitors.

This study describes the effects of prolonged darkness and PDE inhibition on zebrafish ROS renewal. We use the previously described, genetically encoded marker of ROS renewal in zebrafish²² to measure growth and shedding following exposure to prolonged darkness and the PDE5/6 inhibitors. Our analysis shows that prolonged darkness reduces the rate of ROS shedding in larval and adult zebrafish. Similarly, the shedding rate is also reduced in larval and adult zebrafish treated with the PDE5/6 inhibitors sildenafil and vardenafil, but not the PDE5-selective inhibitor tadalafil. These data provide a link between the long-accepted regulation of ROS renewal by the light-dark cycle and the molecular machinery of phototransduction.

MATERIALS AND METHODS

Zebrafish Care and Maintenance

All fish lines were maintained according to standard methods²³ at 28°C on a 14/10-hour light-dark cycle except where noted otherwise. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the University of Massachusetts Amherst Institutional Animal Care and Use Committee and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All fish lines were a mixed *AB/albino*^{b4/b4} background.^{24,25} The *Tg(Xla.rbo:EGFP)* line was obtained from James Fadool (Florida State University, Tallahassee, FL, USA).²⁶ The *Tg(bsp70:HA-mCherryTM)* line was described previously.²² The *Tg(opn1sw1:mCherry)* line was generated using the pTol system.²⁷ The *opn1sw1:mCherry* plasmid was constructed using the p5E-SWS1 plasmid²⁸ and pME-mCherry (KK386)²⁹ in a three-way recombination with pTolDestR4-R2pA (NL465).³⁰

The genotypes of individual transgenic carriers for all lines were determined by fluorescence microscopy. *Tg(Xla.rbo:EGFP)*-positive fish were identified by green fluorescent retinas, *Tg(bsp70:HA-mCherryTM)*-positive fish by red fluorescent lenses, and *Tg(opn1sw1:mCherry)*-positive fish by red fluorescent retinas.

Heat Shock Induction of HA-mCherryTM Expression

Transmembrane-associated mCherry (HA-mCherryTM) expression was induced in larval and adult fish with 45-minute incubation in 38°C water. After heat shock, the fish were returned to 28°C water, exposed to experimental conditions, and otherwise reared under standard methods in the fish facility. Dark-reared fish were maintained in total darkness within an independently controlled light box.

Phosphodiesterase Inhibitors

Sildenafil citrate (Pfizer), vardenafil HCl trihydrate (SelleckChem, Houston, TX, USA), and tadalafil (SelleckChem) were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich Corp., St. Louis, MO, USA) at 100 μM for stock concentration. Working concentrations were diluted further in DMSO prior to addition to fish water such that final DMSO concentration was 0.05% for all experiments except the sildenafil dilution series, which used 0.1%. Larval fish were treated with inhibitor for 3 days. Adult fish were treated with inhibitor for 6 days with a change of water and fresh inhibitor after 3 days.

Immunofluorescence

Larvae and adults were processed for immunofluorescence as previously described.^{28,51} Samples were collected and fixed 4 to 6 hours after the onset of illumination. Animals reared in the dark were maintained in a dark box for the duration of treatment and were not exposed to light except at the time of collection (same time of day as light-cycle control) when a red-filtered light was used for approximately 5 minutes to collect tissues and place in fixative in the dark. Antibodies used included rabbit anti-green fluorescent protein (GFP) primary antibody at 1:2000 (ThermoFisher Scientific, Waltham, MA, USA) and corresponding Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody at 1:2000 (ThermoFisher Scientific), mouse IgG₁ monoclonal anti-HA primary antibody at 1:1000 (Covance, Princeton, NJ, USA) and corresponding Rhodamine Red-conjugated goat anti-mouse IgG₁ secondary antibody at 1:100 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), R6-5 mouse IgG_{2A} monoclonal anti-Rhodopsin primary antibody³² at 1:200 and corresponding Alexa Fluor 647-conjugated goat anti-mouse IgG_{2A} secondary antibody at 1:100 (Jackson ImmunoResearch Laboratories), and K42-6 mouse IgG₃ monoclonal primary antibody³² at 1:100 and corresponding Alexa Fluor 488-conjugated goat anti-mouse secondary antibody at 1:200 (ThermoFisher Scientific). mCherry fluorescence was visualized directly from the ultraviolet cone-expressed transgene (*opn1sw1:mCherry*).

Imaging

Images were generated as z-stacks of optical sections using a Zeiss LSM 700 Confocal system with a 40 \times /1.4 NA oil objective and processed with Zen software (Carl Zeiss, Thornhill, NY, USA) and Adobe Photoshop (San Jose, CA, USA). Z-stacks for the *Tg(Xla.rbo:EGFP)*; *Tg(bsp70:HA-mCherryTM)*; *alb*^{b4/b4} images were collected with a thickness of 24 to 28 μm for larvae and 18 to 20 μm for adults, both with step size of 0.426 μm ; z-stacks for the *Tg(opn1sw1:mCherry)*; *alb*^{b4/b4} images were collected with a thickness of 20 to 24 μm with step size 0.386 μm . Representative images are projections of a subset of z-sections as described in the figure legends. Measurement analyses were performed within the three-dimensional z-stacks using the line function in Volocity 3D imaging software (PerkinElmer, Waltham, MA, USA).

Statistics and Quantification of Outer Segment Renewal

Linear mixed effects models were fit using R³³ and the lme4 package.³⁴ Data were graphed using the ggplot2 package.³⁵ Linear mixed effects models determine response means while considering terms of fixed and random error.³⁶ We used this modeling to determine treatment means and mean differences between treatment and control. We fit a random intercept model with treatment (standard 14-hour light/10-hour dark

[LD] or total darkness [DD] or inhibitor with concentration) assigned as a fixed effect and individual fish as random effect. Residual plots did not reveal any obvious deviations from homoscedasticity or normality. Results were considered significant if the 95% confidence interval (CI) for the mean difference did not span zero.

RESULTS

Constant Darkness Reduces ROS Shedding

Previous studies have shown that ROS shedding is initiated by illumination and suppressed by prolonged exposure to DD.^{16,18,37,38} We analyzed ROS renewal using *Tg(bsp70:HA-mCherryTM)* zebrafish, which, following a heat shock pulse, transiently express the HA-tagged mCherry fluorescent protein with a transmembrane domain (TM) that is incorporated into a small number of nascent ROS discs to form a stripe. ROS growth distance (D^G) was measured from the base of the ROS to the distally displaced HA-mCherryTM stripe (Fig. 1, red). ROS shedding distance (D^S) was measured from the HA-mCherryTM stripe to the ROS tip. Since D^S represents the growth that occurred prior to heat shock minus any shedding, comparison between control and treated fish reveals changes in the amount of ROS material shed during treatment. We measured D^G and D^S from individual rods within three-dimensional confocal z-stacks of retinal sections immunolabeled with anti-Rhodopsin (Fig. 1, blue). All experiments were performed using *alb^{b4/b4}* fish that lack most melanin pigment in the RPE cells,^{24,25} because the pigment in microvillar processes of pigmented RPE obscures ROS fluorescence and prohibits measurement analysis. We performed linear mixed effects analyses of the relationships between D^S or D^G and treatment conditions. The fixed effects were the treatment conditions. We report the mean treatment effects and mean differences compared to control along with 95% CIs and graphically represent the range of data with box plots. The random effects component included a random intercept for each fish per treatment.

We first examined the effects of prolonged darkness on ROS renewal in larval zebrafish. At 5 days post fertilization (dpf), *Tg(Xla.rbo:EGFP); Tg(bsp70:HA-mCherryTM)*; *alb^{b4/b4}* fish were heat shocked and reared in DD or LD until 8 dpf (Fig. 2A). Rod morphology was unchanged in the DD-reared larvae, except for an accumulation of phagosome-like bodies distal to the ROS (Fig. 2B). The mean D^S in LD-reared larvae was 5.7 μ m (95% CI: 4.4, 7.1 μ m), while D^S in DD-reared larvae was 7.5 μ m (95% CI: 6.2, 8.8 μ m) (Fig. 2C). This was a mean difference of 1.7 μ m (95% CI: -0.1, 3.6 μ m), indicating that ROS shedding was reduced by 0.58 μ m per day. To determine if ROS growth was affected by DD, we measured D^G and found no difference between LD-reared larval D^G of 4.8 μ m (95% CI: 4.0, 5.6 μ m) and DD-reared larval D^G of 4.9 μ m (95% CI: 4.2, 5.7 μ m) (Fig. 2D).

We next examined whether DD reduces shedding in adult rod photoreceptors where the ROS have reached mature steady-state length. We quantified D^G and D^S in adult *Tg(Xla.rbo:EGFP); Tg(bsp70:HA-mCherryTM)*; *alb^{b4/b4}* zebrafish that were heat shocked and reared in LD or DD for 6 days (Fig. 2E). We observed that a subset of rods from adults reared in DD had contracted IS such that the OS layer spanned a greater area than in the LD control (Fig. 2F). This is consistent with previous observations that teleost fish rod photoreceptors elongate in the light and shorten in the dark through actin-dependent mechanisms in the IS.³⁹ The rods with contracted IS were not measured for D^G and D^S so that the subset of measured rods in DD-reared adults was comparable to the subset of measured rods in LD-reared adults. D^S was 17.4 μ m

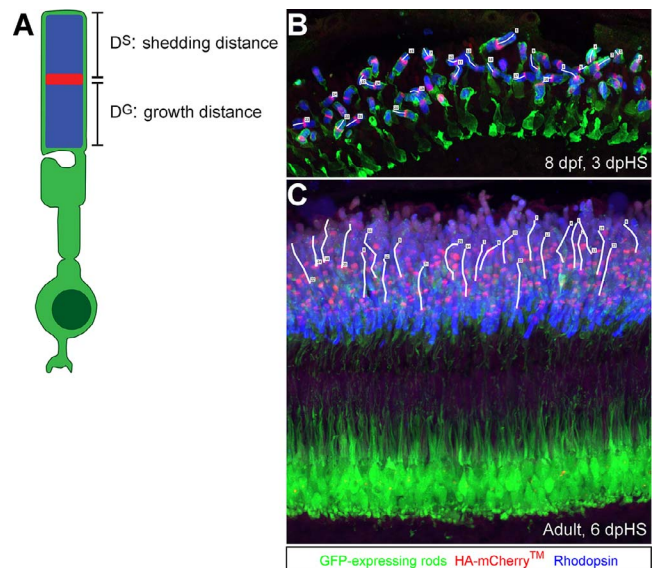


FIGURE 1. Transgenic fluorescent marker is a tool for measuring rod outer segment renewal. (A) Schematic representation of a *Tg(Xla.rbo:EGFP); Tg(bsp70:HA-mCherryTM)* rod photoreceptor with HA epitope- and transmembrane domain-tagged mCherry (red stripe) that has inserted into nascent rod discs and displaced toward the outer segment distal tip during the several days following a heat shock pulse. Growth distance (D^G) is measured from the base of the outer segment to the mCherry stripe; shedding distance (D^S) is measured from the mCherry stripe to the distal tip of the outer segment. (B, C) Representative z-projection images with D^S measurements from (B) 8 dpf, 3 days post heat shock (dpHS), and (C) adult, 6 dpHS. Measurements are made in the three-dimensional z-stack. Photoreceptors express GFP (green) and are labeled with anti-Rhodopsin antibody (blue).

(95% CI: 16.3, 18.4 μ m) in LD-reared adults, and D^S was 22.4 μ m (95% CI: 21.4, 23.5 μ m) in DD-reared adults (Fig. 2G). This was a mean difference of 5.1 μ m (95% CI: 3.6, 6.5 μ m), indicating that DD reduced the amount of material shed by 0.84 μ m per day. Although larval D^G remained unchanged in DD as compared to LD, adult D^G was decreased in DD. D^G in LD-reared adults was 7.9 μ m (95% CI: 7.3, 8.6 μ m), and D^G in DD-reared adults was 6.0 μ m (95% CI: 5.4, 6.7 μ m; Fig. 2H). This was a mean difference of 1.8 μ m (95% CI: -2.8, -0.96 μ m), indicating that exposure to DD reduced ROS growth by 0.32 μ m per day. Taken together, these observations show that constant dark suppresses ROS shedding in zebrafish similar to that previously observed in other species and that the *Tg(bsp70:HA-mCherryTM)* line is a powerful tool to measure ROS renewal kinetics.

D^G and D^S measurement analysis revealed inherent variability between ROS within a retina and between fish from the same treatment condition (Supplementary Fig. S1). The linear mixed effects model provides measure of this variability. The standard deviation for the random effect assigned to fish indicates the variability in D^G or D^S due to sampling individuals. The standard deviation for the fish random effect on LD/DD larval D^G was 0.78 μ m and on D^S was 1.3 μ m. On adult D^G , the standard deviation for fish random effect was 0.59 μ m and on D^S was 0.77 μ m. Standard deviation for the residual random effect indicates the variability in D^G and D^S that stems from factors outside the scope of this study. The residual standard deviation for larval D^G was 1.1 μ m and for D^S was 1.7 μ m. On adult D^G , the residual standard deviation was 1.1 μ m and on D^S was 3.5 μ m.

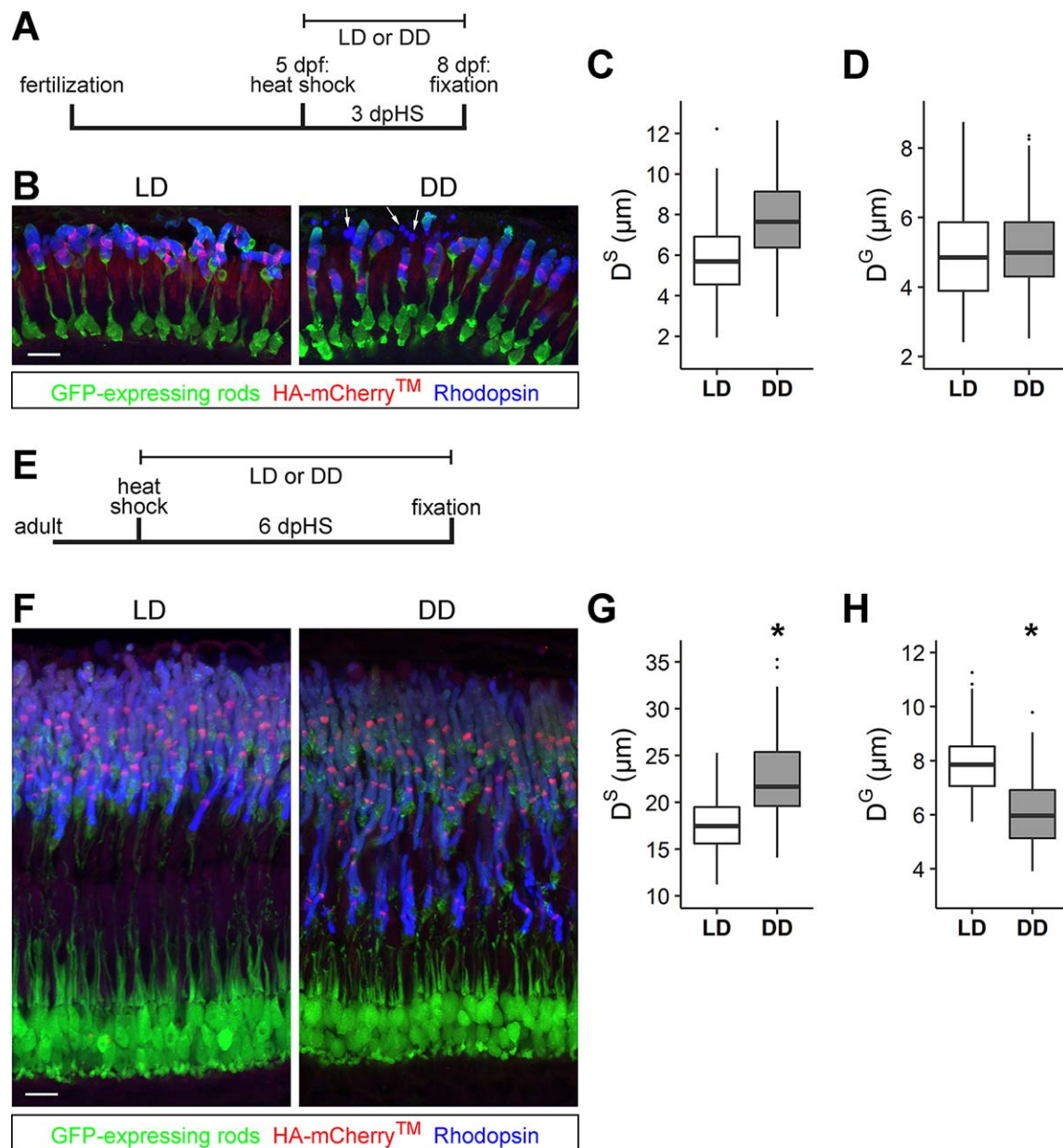


FIGURE 2. Constant darkness reduces rod outer segment shedding. (A) Schematic timeline for examining rod outer segment (ROS) renewal in 8 dpf, 3 days post heat shock (dpHS) *Tg(Xla.rbo:EGFP); Tg(bsp70:HA-mCherryTM)* fish after rearing in normal 14-hour light/10-hour dark cycle (LD) or constant dark (DD). (B) Representative images of the photoreceptor layer from LD- and DD-reared larvae immunolabeled with anti-GFP (green), anti-HA (red), and anti-Rhodopsin (blue) antibodies. Arrows in DD image indicate accumulation of phagosome-like bodies. Images are projections of a subset of z-sections totaling 11.09 μm . Scale bar: 10 μm . Quantification of (C) shedding distance (D^S) and (D) growth distance (D^G) in LD- and DD-reared larvae. Lower and upper hinges of box correspond to first and third quartiles; middle corresponds to median; whiskers extend 1.5 * interquartile range above and below the hinges; dots represent outliers. Graphs represent (C) $n = 4$ fish/treatment (96 LD, 157 DD outer segments) and (D) $n = 4$ fish/treatment (128 LD, 196 DD outer segments). (E) Schematic timeline for examining ROS renewal in adult *Tg(Xla.rbo:EGFP); Tg(bsp70:HA-mCherryTM)* fish heat-shocked and reared 6 days in LD or DD. (F) Representative images of the photoreceptor layer from LD- and DD-reared adults immunolabeled with anti-GFP (green), anti-HA (red), and anti-Rhodopsin (blue) antibodies. Images are projections of a subset of z-sections totaling 5.97 μm . Scale bar: 10 μm . Quantification of (G) D^S and (H) D^G in LD- and DD-reared adults. Graphs represent (G) $n = 3$ fish/treatment (129 LD, 142 DD outer segments) and (H) $n = 3$ fish/treatment (187 LD, 190 DD outer segments). *95% CI of difference compared to control does not span zero.

Phosphodiesterase Inhibitors Reduce Larval ROS Shedding

Since the onset of illumination triggers ROS shedding, we sought to determine if this is through the direct activation of the phototransduction cascade in rods. If so, then inhibition of the phototransduction pathway would mimic constant dark-

ness and reduce ROS shedding. Sildenafil, vardenafil, and tadalafil were developed as PDE5-specific inhibitors, but sildenafil and vardenafil are nearly equally effective inhibitors of rod PDE6,²¹ a critical component of the phototransduction cascade. At 5 dpf, we heat shocked *Tg(Xla.rbo:EGFP); Tg(bsp70:HA-mCherryTM)*; *alb^{b4/b4}* larvae and treated with sildenafil, vardenafil, or tadalafil for 3 days (Fig. 3A). Treatment

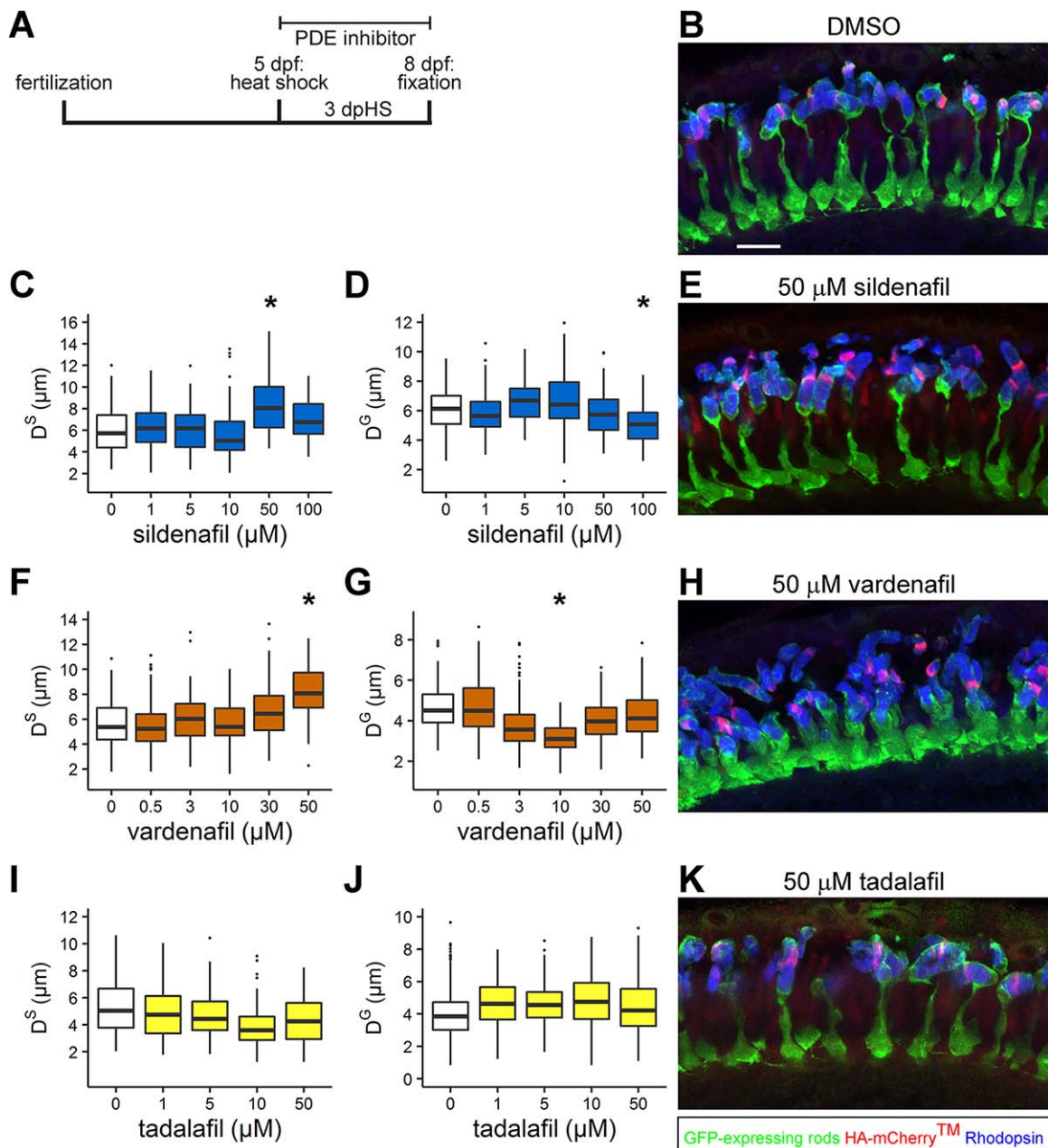


FIGURE 3. Sildenafil and vardenafil reduce larval rod outer segment shedding and vardenafil changes rod photoreceptor morphology. **(A)** Schematic timeline for examining rod outer segment renewal in 8 dpf, 3 days post heat shock (dpHS) *Tg(Xla.rbo:EGFP)*; *Tg(bsp70:HA-mCherryTM)*; *alb^{b4/b4}* fish after bathing in phosphodiesterase inhibitor. **(B, E, H, K)** Representative images of photoreceptor layers from 8 dpf, 3 dpHS larvae treated with **(B)** 0.05% DMSO, **(E)** 50 μ M sildenafil, **(H)** 50 μ M vardenafil, and **(K)** 50 μ M tadalafil. Retinal sections were immunolabeled with anti-GFP (green), anti-HA (red), and anti-Rhodopsin (blue) antibodies. Images are projections of a subset of z-sections totaling 5.97 μ m. Scale bar: 10 μ m. Quantification of **(C, F, I)** shedding distance (D^S) and **(D, G, J)** growth distance (D^G). Lower and upper hinges of box correspond to first and third quartiles; middle corresponds to median; whiskers extend 1.5 * interquartile range above and below the hinges; dots represent outliers. Graphs represent **(C, D)** sildenafil $n = 5$ fish/treatment (**[C]** ≥ 63 and **[D]** ≥ 93 outer segments); **(E, G)** vardenafil $n = 4$ fish/treatment (**[F]** ≥ 91 and **[G]** ≥ 122 outer segments); and **(I, J)** tadalafil $n = 4$ fish/treatment except for the 10 μ M tadalafil sample with $n = 3$ fish (**[I]** ≥ 100 and **[J]** ≥ 190 outer segments). *95% CI of difference compared to control does not span zero.

with sildenafil or tadalafil did not cause larval lethality. Vardenafil concentrations above 50 μ M were larval lethal, so these concentrations were omitted from analysis. Gross morphology of treated larvae was assessed by measuring the standard length (SL, snout to peduncle; Supplementary Table S1).⁴⁰ Larvae treated with 50 and 100 μ M sildenafil were slightly smaller (4 mm) as compared to control (4–4.5 mm; 0.1% DMSO). SL was also smaller in larvae treated with vardenafil at 10 μ M and above: 10 μ M had SL of 3.75 to 4 mm,

30 and 50 μ M had SL of 3.5 mm, and 0.05% DMSO control had SL of 4 mm. Larvae treated with all concentrations of tadalafil had SL similar to control (3.5 mm; 0.05% DMSO). Rod photoreceptors from larvae treated with sildenafil and tadalafil were similar in morphology to DMSO-treated controls (Figs. 3B, 3E, 3K). In contrast, the morphology of rod photoreceptors treated with 50 μ M vardenafil was abnormal with dramatically shortened IS (Fig. 3H).

We quantified D^S and D^G from the PDE inhibitor-treated larvae. D^S in 50 μM sildenafil-treated larvae was 8.4 μm (95% CI: 7.4, 9.3 μm), while D^S was 6.0 μm (95% CI: 5.0, 7.0 μm) in vehicle control-treated larvae (Fig. 3C). This was a mean difference of 2.4 μm (95% CI: 1.0, 3.8 μm), indicating that the amount of shed material was reduced by 0.79 μm per day with 50 μM sildenafil treatment. No difference was seen with lower concentrations; however, D^S was 7.1 μm (95% CI: 6.1, 8.0 μm) in 100 μM sildenafil-treated larvae, and although this was a D^S increase of 1.1 μm , or 0.36 μm per day compared to control, the 95% CI of this difference (−0.28, 2.4 μm) does not indicate a significant increase. D^G was unaffected by sildenafil except at 100 μM , which was slightly reduced at 5.1 μm (95% CI: 4.4, 5.7 μm) compared to vehicle control of 6.1 μm (95% CI: 5.4, 6.7 μm ; Fig. 3D). This was a mean difference of 1.0 μm (95% CI: −1.9, −0.10 μm), or a decrease in growth of 0.34 μm day.

ROS shedding was reduced with 50 μM vardenafil as compared to vehicle control-treated larvae (Fig. 3F). D^S was 7.9 μm (95% CI: 7.1, 8.7 μm) with 50 μM vardenafil, while control D^S was 5.7 μm (95% CI: 4.9, 6.4 μm). This was a mean difference of 2.2 μm (95% CI: 1.2, 3.3 μm), indicating that ROS shedding was reduced by 0.74 μm per day. D^G was not significantly different in vardenafil-treated larvae except for a slight decrease in the 10 μM vardenafil-treated larvae (Fig. 3G), which had D^G of 3.3 μm (95% CI: 2.7, 3.8 μm) versus control D^G of 4.7 μm (95% CI: 4.1, 5.3 μm). This was a mean difference of 1.4 μm (95% CI: −2.3, −0.59 μm), indicating a decrease in growth of 0.47 μm per day; however, SL was also reduced.

In order to test whether the suppression of ROS shedding by sildenafil and vardenafil is due to inhibition of PDE6 and not PDE5, we treated larvae with tadalafil, a PDE5-selective inhibitor.²¹ Tadalafil had no effect on D^S and D^G as compared to control (Figs. 3I, 3J; Supplementary Fig. S2), indicating that sildenafil and vardenafil suppress shedding by inhibiting PDE6.

Sildenafil and Vardenafil Reduce Adult ROS Shedding

We next examined whether sildenafil and vardenafil reduce shedding in mature rod photoreceptors. We bathed adult zebrafish in inhibitor for 6 days following heat shock induction of the HA-mCherryTM stripe (Fig. 4A). We chose 50 μM concentration for both sildenafil and vardenafil because of its efficacy in larval fish. Adult zebrafish appeared normal with inhibitor treatment, although changes in rod morphology were observed with both sildenafil and vardenafil (Fig. 4B). Gaps in GFP-expressing rods appeared in the outer nuclear layer of sildenafil-treated adults, suggesting toxicity. However, we detected no increased apoptosis as measured by activated-caspase 3 antibody labeling (data not shown). The photoreceptor layer of vardenafil-treated adults was disorganized and inner segments appeared contracted (Fig. 4B), suggesting that vardenafil had a similar effect on larval and adult rod morphology. To quantify OS renewal, we measured D^S and D^G in the ROS of inhibitor-treated adults. D^S was 19.7 μm (95% CI: 18.1, 21.2 μm) with sildenafil, 18.6 μm (95% CI: 17.1, 20.2 μm) with vardenafil, and 15.8 μm (95% CI: 14.2, 17.4 μm) with vehicle control (Fig. 4C). The mean difference was 3.9 μm (95% CI: 1.6, 6.1 μm) between sildenafil-treated adults and vehicle control, indicating a reduction in ROS shedding of 0.64 μm per day. In vardenafil-treated adults, the mean difference in D^S as compared to vehicle control was 2.8 μm (95% CI: 0.62, 5.0 μm), indicating a reduction in ROS shedding of 0.47 μm per day. D^G was not different with sildenafil (7.3 μm ; 95% CI: 6.8, 7.8 μm) or vardenafil (7.1 μm ; 95% CI: 6.5, 7.6 μm) as compared to vehicle control (7.3 μm ; 95% CI: 6.7, 7.8 μm ; Fig. 4D). Taken together, these results suggest that PDE6 inhibition

has a similar effect on ROS shedding as dark rearing in adults but does not have the same inhibitory effect on growth.

ROS Shedding Resumes After Removal of Sildenafil

We next wanted to know if ROS from sildenafil-treated larvae would maintain their increased D^S after inhibitor removal or whether shedding would accelerate such that D^S would return to that of control ROS. We heat shocked at 5 dpf and treated fish with 50 μM sildenafil for 3 days, after which larvae were transferred to inhibitor-free water (wash). Larvae were fixed at 0, 1, 2, and 3 days of wash (dWash; Fig. 5A). Rod photoreceptors from sildenafil-treated larvae were not morphologically different than vehicle control (Fig. 5B). ROS shedding recovered following removal of inhibitor. D^S from sildenafil-treated, 0 dWash larvae was 1.5 μm longer (95% CI: 0.80, 2.2 μm) than the D^S of vehicle control. After 1 dWash the D^S was still increased by 1.6 μm (95% CI: 0.46, 2.8 μm) in sildenafil-treated larvae. By 2 dWash there was no difference in D^S . Both the sildenafil-treated and vehicle control larvae had D^S of 4.1 μm (95% CI: 3.7, 4.5 μm), suggesting that the shedding process resumed and accelerated to compensate for the reduced amount of material shed during sildenafil treatment (Fig. 5C).

Cone Photoreceptors Degenerate With Exposure to PDE Inhibitors

While ROS shedding is triggered by the onset of illumination,³⁸ cone outer segment (COS) shedding has been shown to occur after the onset of darkness.^{17,41,42} Given these different shedding triggers, we sought to examine whether inhibition of the phototransduction pathway affects COS shedding. Unfortunately, because COS discs consist of a continuous membrane,⁴⁵ the HA-mCherryTM stripe does not form as in the discrete ROS discs. Therefore, we cannot measure COS growth and shedding rates independently.^{22,44} Instead, we examined COS with a transgenic zebrafish line, *Tg(opn1sw1:mCherry)*, that expresses mCherry in ultraviolet cones and labeled green COS with the monoclonal antibody, K42-6 (Supplementary Fig. S3). Larvae were bathed in 10, 25, or 50 μM inhibitor for 3 days starting at 5 dpf (Fig. 6A). Massive loss of ultraviolet cones and green COS was observed in larvae treated with 25 μM sildenafil (Fig. 6B). In vardenafil-treated larvae, ultraviolet cones were morphologically similar to vehicle control at 10 and 25 μM , but at 50 μM nearly all ultraviolet cones and many green COS were lost. Total length of the green COS (D^{total}) was quantified by measuring from the base to the distal tip of the K42-6-labeled COS (Fig. 6C). D^{total} was 10.8 μm (95% CI: 9.8, 11.8 μm) in control larvae and 12.2 μm (95% CI: 11.1, 13.2 μm) with 10 μM sildenafil. This was a slight, but not significant, increase of 1.4 μm (95% CI: −0.071, 2.8 μm). Due to the degree of cone cell loss in the larvae treated with 25 μM sildenafil, D^{total} was not measured. Larvae treated with 10 and 25 μM vardenafil also had increased D^{total} as compared to vehicle control. In 10 μM vardenafil-treated larvae, D^{total} was 12.2 μm (95% CI: 11.1, 13.2 μm); this was a slight, but not significant, increase of 1.4 μm (95% CI: −0.076, 2.8 μm). In 25 μM vardenafil-treated larvae, D^{total} was 12.4 μm (95% CI: 11.4, 13.4 μm); this was an increase of 1.6 μm (95% CI: 0.15, 3.0 μm). Despite the cone cell loss, the green COS from larvae treated with 50 μM vardenafil were abundant enough to measure and had D^{total} of 7.4 μm (95% CI: 6.4, 8.5 μm). As compared to vehicle control, this was a significant decrease of 3.4 μm (95% CI: −4.8, −1.92 μm). Altogether, these data suggest that ultraviolet and green cones are more sensitive to degenerative effects of sildenafil and vardenafil, and that lower, tolerable concentrations do not greatly affect green COS total length.

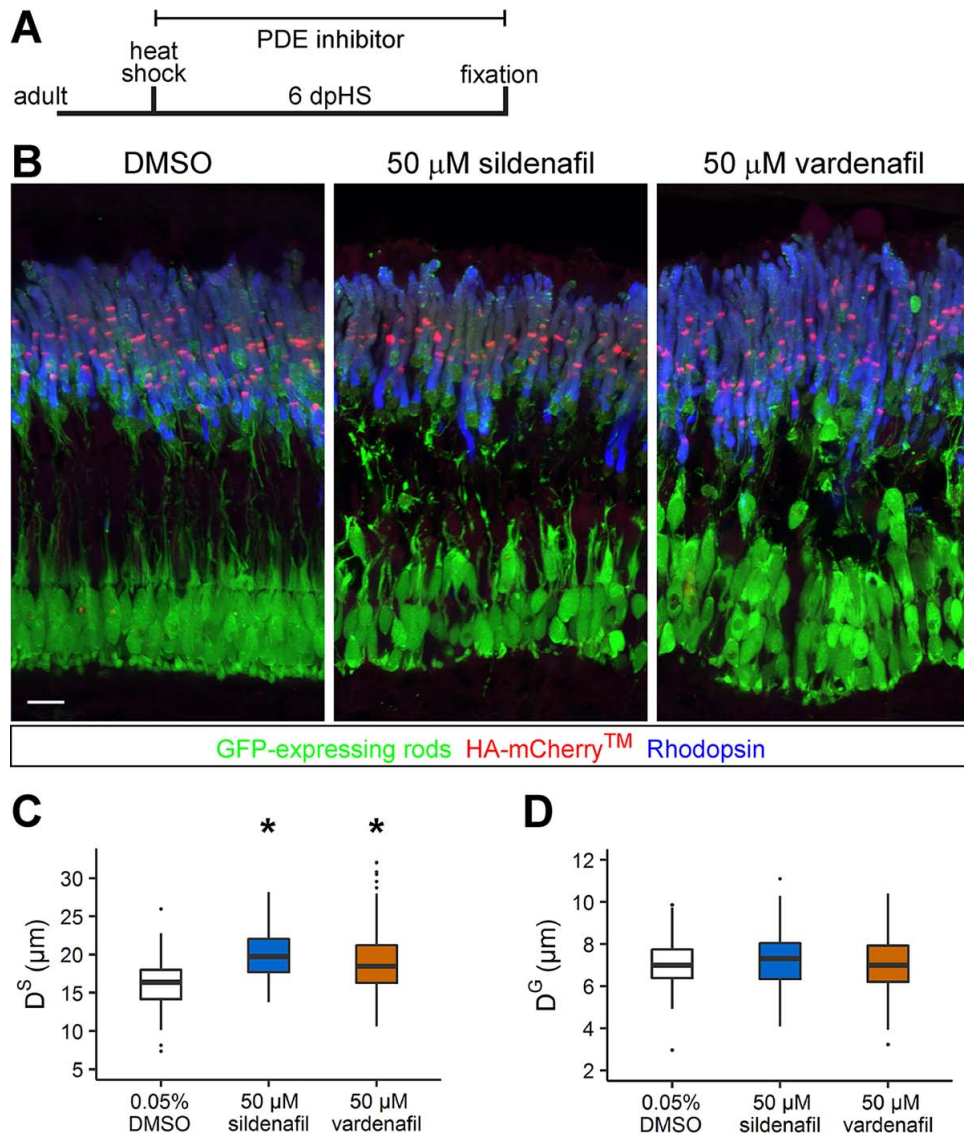


FIGURE 4. Sildenafil and vardenafil reduce adult rod outer segment shedding. **(A)** Schematic timeline for examining rod outer segment renewal in adult, 6 days post heat shock (dpHS) *Tg(Xla.rbo:EGFP); Tg(bsp70:HA-mCherryTM); alb^{b³/b⁴}* fish after bathing in phosphodiesterase inhibitor. **(B)** Representative images of photoreceptor layer from inhibitor-bathed adults immunolabeled with anti-GFP (green), anti-HA (red), and anti-Rhodopsin (blue) antibodies. Images are projections of a subset of z-sections totaling 5.97 μm. Scale bar: 10 μm. Quantification of **(C)** distance to shed (D^S) and **(D)** growth distance (D^G) in inhibitor-bathed adults. Lower and upper binges of box correspond to first and third quartiles; middle corresponds to median; whiskers extend 1.5 * interquartile range above and below the hinges; dots represent outliers. Graphs represent **(C)** $n = 3$ fish/treatment (123 DMSO, 102 sildenafil, 133 vardenafil outer segments) and **(D)** $n = 3$ fish/treatment (176 DMSO, 192 sildenafil, 171 vardenafil outer segments). *95% CI of difference compared to control does not pass zero.

DISCUSSION

In this study, we present a molecular mechanism for the light-cycle regulation of ROS renewal. The diurnal light cycle, specifically the onset of light, is recognized as a key regulator of ROS shedding. Studies in several species^{14–18,45} demonstrate an increase in ROS shedding early in the light period during maintenance on a light-dark cycle. The nature of this regulation, whether circadian or illumination driven, is unclear and may be species specific. Rats maintained in continuous darkness for 3 days continue to follow a circadian burst of shedding.⁴⁵ In contrast, ROS shedding is initiated by illumination in frogs.³⁸ The molecular mechanisms underlying light regulation of shedding are unknown since progress in this area has been hampered by the lack of a tool for quantification. With the established *Tg(bsp70:HA-mCherryTM)* zebrafish,²² we

can induce expression of a fluorescent ROS marker and measure growth and shedding rates separately.

In the present study, we report that prolonged exposure to DD reduces ROS shedding in zebrafish. ROS in dark-reared larvae shed approximately 0.58 μm less per day as compared to diurnal light cycle-reared larvae, while the growth rate is unaffected at approximately 1.6 μm per day. If constant darkness prevents most, or all, ROS shedding, then we can calculate from these data that the developing, elongating ROS in larvae has a net growth rate of approximately 1 μm per day. It has been shown that rods contribute little to no spectral sensitivity function, as measured by ERG and optokinetic response, until around 20 dpf,⁴⁶ raising the question of the involvement of phototransduction as a mechanism for shedding in developing rods. However, the ROS that are present during early rod development are filled with light-sensing

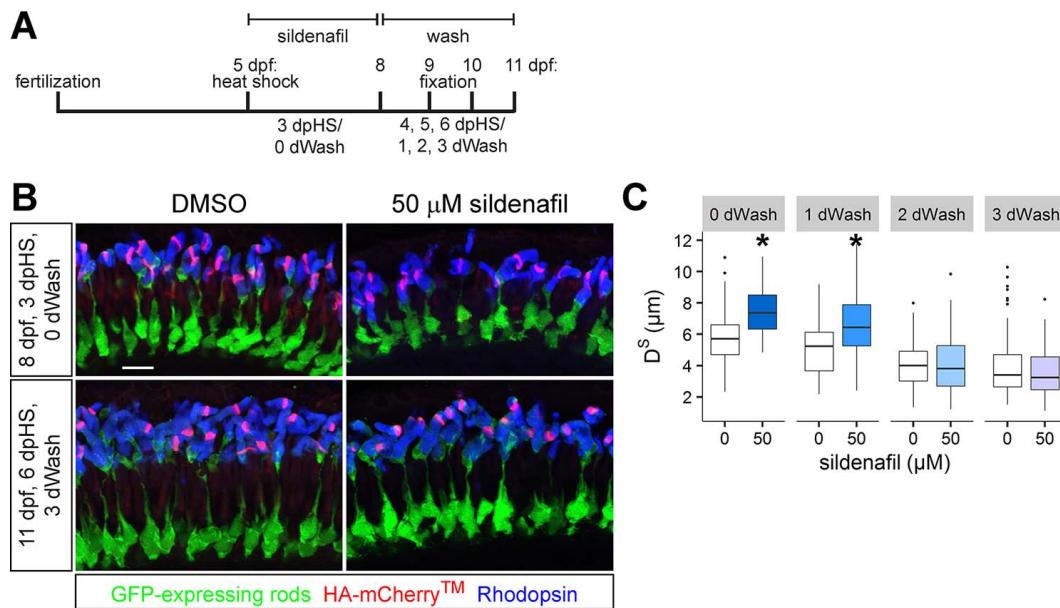


FIGURE 5. Rod outer segment shedding resumes following removal of sildenafil. **(A)** Schematic timeline for examining rod outer segment renewal in larval *Tg(Xla.rho:EGFP); Tg(bsp70:HA-mCherryTM); alb^{b4/b4}* fish following heat shock at 5 dpf, sildenafil treatment for 3 days post heat shock (dpHS), and 1 to 3 days sildenafil removal (dWash). **(B)** Representative images of photoreceptor layers from 0 and 3 dWash sildenafil-treated larvae immunolabeled with anti-GFP (green), anti-HA (red), and anti-Rhodopsin (blue) antibodies. Images are projections of a subset of z-sections totaling 8.1 μ m. Scale bar: 10 μ m. Quantification of shedding distance (D^S) following removal of **(C)** sildenafil for 0 to 3 days (dWash). Lower and upper hinges of box correspond to first and third quartiles; middle corresponds to median; whiskers extend 1.5 * interquartile range above and below the hinges; dots represent outliers. Graphs represent $n = 5$ retinas/condition (≥ 74 outer segments). *95% CI of difference compared to control does not span zero.

Rhodopsin,^{47,48} and they first shed at around 6 dpf (Campbell LJ, Jensen AM, unpublished observation, 2012). It is possible that while the entire phototransduction cascade may be nonfunctional during early rod development, individual components do have function. In this study, we report a single component of the phototransduction cascade in connection to shedding. It is still unclear whether just this component (PDE6 activity) or the entire phototransduction pathway triggers the shedding event.

Previous studies assessed ROS shedding by counting the number of phagosomes (shed ROS material) in light or electron micrographs.^{18,38} The accumulation of phagosome-like debris in the dark-reared larvae of our study would, on its own, suggest that shedding increases in dark-reared larvae. However, with our more direct method of measuring D^S , we find that the length from HA-mCherryTM stripe to distal ROS tip is increased compared to that in fish on a diurnal light cycle. The accumulation of phagosomes in dark-reared larvae (Fig. 2B, arrows) may indicate that some shedding is still occurring, albeit at a reduced amount, and that phagocytosis by the larval RPE may be inhibited. We did not observe phagosome accumulation in dark-reared adults (Fig. 2F), suggesting that an accumulation of phagosomes is a response of the developing, but not the adult, retina to constant darkness. However, a small decrease in ROS growth rate was observed in dark-reared adults. Since zebrafish rely heavily on vision,⁴⁸ it is possible that the fish consume less food during the 6-day dark rearing. A decrease in ROS growth due to decreased food consumption would be consistent with our observation that dietary restriction slows ROS growth (Campbell LJ, Jensen AM, unpublished observation, 2012). Alternatively, decreased ROS growth in the dark-reared adults may be due to a compensatory or homeostatic mechanism present in mature rods that reduces growth when shedding is reduced to maintain a constant steady-state ROS length.

Activation of the vertebrate phototransduction cascade results in the activation of the cGMP-specific PDE6, reduced cGMP concentration, closure of cGMP-gated cation ion channels, membrane repolarization, and ultimately, reduced neurotransmitter release (reviewed in Ref. 19).¹⁹ To test if the phototransduction pathway regulates ROS shedding, we pharmacologically inhibited PDE6. Sildenafil, vardenafil, and tadalafil were originally characterized as PDE5 inhibitors. It has also been demonstrated that sildenafil and vardenafil inhibit purified, activated (i.e., inhibitory γ subunits are absent) PDE6 about equally well as PDE5 and partially inhibit PDE6 in isolated, intact frog ROS.^{20,21} Therefore, we expected milder effects when exposing zebrafish to sildenafil and vardenafil than the photoreceptor degeneration and cell death that result from total loss of PDE6 in the *rd1* mouse model.⁴⁹ Exposure to the PDE5/6 inhibitors sildenafil and vardenafil reduces ROS shedding in larval and adult zebrafish. The peak reduction in larval ROS shedding with sildenafil and vardenafil (0.79 and 0.74 μ m/day, respectively) is comparable to that with dark rearing (0.58 μ m/day; Table). In contrast, treatment with the PDE5-specific inhibitor, tadalafil, does not affect larval ROS shedding, indicating that the reduction in ROS shedding with sildenafil and vardenafil is due entirely to inhibition of PDE6. Sildenafil and vardenafil also reduce adult ROS shedding (0.64 and 0.47 μ m/day, respectively) to levels approaching the reduction observed in dark-reared adults (0.84 μ m/day, Table).

In addition to inhibiting ROS shedding, vardenafil also induces morphologic changes by shortening the IS in both larvae and adults, an effect that is also seen with dark rearing. Retinomotor movements in teleosts position the photoreceptors and RPE pigments for optimal function in different lighting conditions. The diurnal movements are thought to be regulated in a circadian manner and follow the oscillations of cAMP levels.⁵⁰⁻⁵² However, retinomotor movements have also been shown to be influenced by light as rod inner segment elongate in the light and contract in the dark.⁵⁹ This may be regulated by

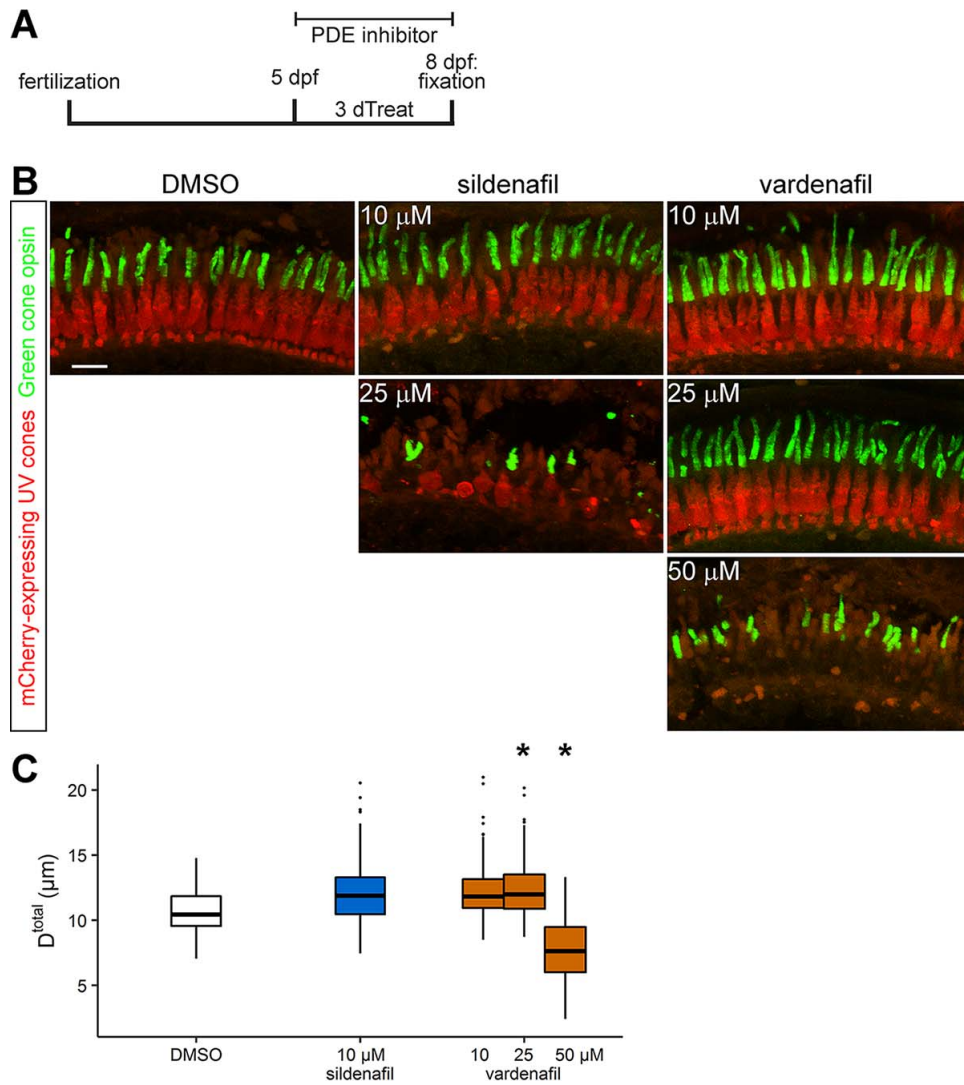


FIGURE 6. Cone photoreceptors are sensitive to sildenafil and vardenafil. (A) Schematic timeline for examining green cone outer segment length in 8 dpf *Tg(SWS1:mCherry); alb^{b4/b4}* larvae after bathing in phosphodiesterase inhibitor for 3 days (dTreat). (B) Representative images of photoreceptor layers from larvae treated with 0.05% DMSO, sildenafil, and vardenafil at indicated concentrations. Retinal sections were immunolabeled with green cone opsin antibody (green); ultraviolet cones express mCherry (red). Images are projections of a subset of z-sections totaling 7.33 μm. Scale bar: 10 μm. (C) Quantification of green cone outer segment full length (D^{total}). Lower and upper hinges of box correspond to first and third quartiles; middle corresponds to median; whiskers extend 1.5 * interquartile range above and below the hinges; dots represent outliers. Graph represents $n = 5$ fish/condition (DMSO = 162 outer segments, sildenafil = 169 outer segments, and vardenafil ≥ 175 outer segments). *95% CI of difference compared to control does not span zero.

cGMP levels since application of cGMP analogues inhibits RIS elongation in the light.⁵³ The vardenafil-treated retinas that have contracted RIS (Figs. 3H, 4B) presumably mimic the dark state with increased cGMP levels and inhibition of RIS elongation. Intriguing questions remain as to how cAMP and cGMP influence retinomotor movements as well as how circadian regulation is mechanistically independent from regulation by illumination.

We also observed that the reduction in shedding by the PDE5/6 inhibitor sildenafil is reversible. This is consistent with the mechanism of action of sildenafil as a competitive inhibitor. Sildenafil is structurally similar to cGMP and effectively blocks cGMP hydrolysis.⁵⁴ By removing sildenafil, we sought to examine the mechanistic nature of shedding: Would shedding accelerate so that D^S would rapidly return to control levels, or would shedding commence at the control rate? Our data show that D^S in sildenafil-treated larvae returns quickly to control values after 2 days of drug removal,

TABLE. Decrease in ROS Material Shed Per Day Treatment

Treatment	Age	D^S Increase Over Control, μm (95% CI)*	Per Day Decrease in Material Shed, μm†
DD	8 dpf	1.7 (-0.1, 3.6)	0.58
	Adult	5.1 (3.6, 6.5)	0.84
Sildenafil, 50 μM	8 dpf	2.4 (1.0, 3.8)	0.79
	Adult	3.9 (1.6, 6.1)	0.64
Vardenafil, 50 μM	8 dpf	2.2 (1.2, 3.3)	0.74
	Adult	2.8 (0.62, 5.0)	0.47

* Control for DD was LD; control for sildenafil was 0.1% DMSO; control for vardenafil was 0.05% DMSO.

† Per day decrease in material shed was determined by dividing the D^S increase over control by the number of days of treatment. Larvae were treated for 3 days; adults were treated for 6 days.

indicating that ROS shedding accelerates following removal of the inhibitor. This suggests that, rather than shedding a daily fixed amount or at a fixed rate, the mechanisms regulating ROS renewal are instead set to shed down to a level that is premarked for disposal.

A premarked level of disposal may be necessary given the daily bombardment of photons on the OS. Photoreceptors must endure considerable oxidative stress, yet immunocytochemical studies report that glutathione, a primary component of intracellular antioxidant defense systems, is present in all parts of the retina except the ROS and COS.⁵⁵⁻⁵⁹ It has been hypothesized that, in the absence of antioxidant systems, the fail-safe method to replace damaged molecules of the phototransduction machinery is continuous OS renewal through growth and shedding.⁶⁰ In support of this hypothesis, a recent study has challenged the general assumption that the ROS is a functionally homogenous compartment by proposing that PDE6 is progressively depleted, perhaps by oxidative stress, along the length of the ROS toward the distal tip.⁶¹ Highly localized spots of light targeted at points along the length of the ROS demonstrated that the efficacy of the phototransduction machinery is 5 to 10 times higher at the base than at the tip of the ROS. With mathematical modeling and assuming a relatively high intracellular cGMP diffusion constant, it was proposed that a limited number of PDE6 molecules are available at the ROS tip and that illumination causes a diffusion gradient of cGMP from the ROS tip toward the light-activated PDE6 localized to the middle and base of the ROS.⁶¹ In following with this hypothesis and the results presented in this study, a possible mechanism for initiation of the shedding event may begin with the loss of functional PDE6 at the tip of the ROS. Following illumination, a diffusion gradient of cGMP is established, which initiates the diurnal shedding event that occurs a couple of hours after illumination. Thus, when PDE6 is inhibited, as in this study with sildenafil and vardenafil or at night while the two zebrafish paralogues of the rod PDE6 inhibitory subunits, *pde6ga* and *pde6gb*, are most highly expressed,⁶² cGMP does not diffuse away from the tip and shedding is not initiated.

To our knowledge, our data are the first to reveal a molecular mechanism that directly regulates ROS shedding in vivo. Other studies have shown an increase in ROS length, but these studies did not directly assess which component of ROS renewal was affected (growth or shedding). The mouse *Mak*-knockout retina has increased cilia length,⁶³ and deletions of *Gas6* and *Protein S* are associated with increased ROS length.⁶⁴ It is likely that *Gas6* and *Protein S*, along with phosphatidylserine exposure, directly regulate ROS shedding since they are expressed by photoreceptors and are ligands for the Mer tyrosine kinase receptor (*Mertk*), which is expressed by RPE cells and is established as having a key role in phagocytosis.⁶⁴⁻⁶⁹ Future studies are needed to determine if PDE6 activity or cGMP levels regulate the exposure of *Gas6*, *Protein S*, and phosphatidylserine at ROS tips.

Whereas ROS shedding is triggered by light onset, COS shedding is triggered after lights are turned off.^{16,17,41,42} We hypothesize that COS shedding may be increased with PDE6 inhibitor treatment. Sildenafil and vardenafil are similarly effective at inhibiting purified bovine cone PDE6 as purified PDE5 and approximately 2.3-fold more effective at inhibiting purified bovine cone PDE6 than rod PDE6.²¹ By measuring total green COS length, we found that sildenafil and vardenafil do not significantly influence total green COS length at the lowest concentrations tested. However, we observed significant green COS degeneration and green and ultraviolet cone loss at concentrations that did not cause significant rod cell loss, indicating that ultraviolet and green cones are more

sensitive than rods to the toxic effects of sildenafil and vardenafil.

The degree of inherent variability in ROS growth and shedding rates between ROS within the same retina was unexpected (Supplementary Fig. S1), given our prior understanding that ROS have a predictable and steady maintenance.^{4,16,17} However, the processes seem to be stochastic between rods within a retina; for example, it is reported that shedding is likely initiated in only approximately 25% of rods with each onset of daily illumination.¹⁸ The linear mixed effects modeling accounts for the multiple levels of variability between ROS within a retina and between individual fish, for which calculation of mean and standard deviation does not sufficiently account. We show the spread of data with box plots so that we can detect shifts in the distribution of data in treated fish as compared to control. Variability between fish is likely due to individual differences in physiology or fitness. While we control for variability between fish by rearing individuals from the same treatment condition within the same treatment tank or dish, we cannot control for decreased feeding, for example, which we observe causes a decrease in ROS growth rate (Campbell LJ, Jensen AM, unpublished observation, 2012). The variability between fish is slightly greater in larvae than in adults, which may be due to variability in overall fitness resulting from yolk quality during development. A major contributing factor to variability within retinas may be that new rods are continuously added to the zebrafish retina. This is most pronounced in larval and juvenile stages where rod differentiation initially spreads across the retina in a wave-like fashion. Additional rods are produced to fill in the rod population, which reaches the three or four layers of rod cell bodies seen in the adult retina (see Figs. 2B, 2F).^{26,48,70} Finally, it takes approximately a month for the zebrafish ROS to reach mature length (Campbell LJ, Jensen AM, unpublished observation, 2015). Even in adulthood, new rods are added from precursor cells interspersed among the photoreceptor nuclear layer.⁷¹ Thus, the variability in age of individual rod photoreceptors may contribute to the variability in D^S and D^G.

Since progressive ROS degeneration is a common characteristic of retinitis pigmentosa and other blinding diseases,⁹ a significant inquiry is whether ROS degeneration can be prevented, arrested, or reversed by stimulating growth or inhibiting shedding. Constant darkness is an impractical option; however, a drug that reduces or inhibits shedding may be useful. Certainly, drugs that inhibit components of the phototransduction cascade are probably not useful. For example, sildenafil and vardenafil raise issues for those with cone or rod PDE6 mutations. The *rd1*^{+/-} mouse contains a heterozygous nonsense mutation in the β subunit of rod PDE6; these mice demonstrate normal retinal function but have a lower threshold for stress such that 1 hour of sildenafil treatment significantly reduces photoreceptor function as determined by electroretinogram.⁷² Furthermore, visual perturbations have been reported in people who take Viagra (sildenafil) and Levitra (vardenafil).⁷³⁻⁷⁷ Nevertheless, the identification of effectors downstream of PDE6 that influence shedding, but not phototransduction, could prove therapeutically beneficial. Ultimately, understanding the molecular regulation of ROS renewal will help us understand the misregulation of photoreceptor maintenance that leads to degeneration, photoreceptor death, and blindness. The transgenic HA-mCherryTM marker of ROS in zebrafish provides a tool to investigate growth and shedding rates, which are inseparable when one is simply measuring full OS length. Combined with inducible, cell-specific investigations made possible with other tools,^{28,31,78} we can investigate these mechanisms with greater resolution in time and space.

Acknowledgments

The authors thank Maura Benson, Jessica Capri, Frank Carellini, Victoria Guarino, Patrick Hartnett, Amir Mina, and Kathryn Thomas for assistance with cell measurements and Michael Lavine, PhD (Department of Mathematics and Statistics, University of Massachusetts Amherst) and Fangzhou Liu for guidance on statistical analyses. The authors also thank John Willoughby, PhD, for producing the *Tg(opn1sw1:mCherry)* zebrafish and Megan West for fish care.

Supported by National Eye Institute Award R21 EY023694.

Disclosure: **L.J. Campbell**, None; **A.M. Jensen**, None

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