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Alterations to the vision-associated transcriptome of zebrafish *(Danio rerio)* following developmental norethindrone exposure

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ABSTRACT

Synthetic sex steroids, like the synthetic progestin norethindrone (NET), can affect a wide variety of biological processes via highly conserved mechanisms. NET is prevalent in surface waters, yet the sub-lethal effects of NET exposure are not are net yet well characterized in aquatic biota. A few targeted gene expression and behavioral studies have concluded that NET affects the vision of adult fish; however, early life stage (ELS) fish are often more sensitive to contaminants. Furthermore, many species of fish rely heavily on visual perception for survival during development. The goal of the present study was to characterize the effects of developmental exposure to environmentally relevant concentrations of NET on the visual system of ELS zebrafish, using transcriptomics and histological methods. Results indicate that exposure to relatively low levels of NET in aquatic systems may be sufficient to affect the visual function of developing fish.

1. Introduction

It is well established that sex steroids are capable of affecting change in a wide variety of biological processes in vertebrates. These include processes involved in reproduction, brain function, apoptosis, inflammation, development, metabolism, and cell differentiation/proliferation (Ankley et al., 2012; Edwards, 2005; Hanna et al., 2010; Overturf et al., 2014). Modifications to these processes can be accomplished via steroid binding of nuclear steroid receptors (which then act as transcription factors), or through activation of signal transduction pathways (Edwards, 2005). A number of anthropogenic chemicals are capable of binding to/activating vertebrate sex steroid hormone receptors, albeit with varying degrees of affinity. These chemicals are capable of inducing changes in gene expression and signaling cascades that may cause adverse effects on whole organism health (Ankley et al., 2012; Lecomte et al., 2017; Overturf et al., 2014; White et al., 2011). Compounds that bind these receptors with a very high affinity are especially problematic for aquatic biota, as many of these contaminants have been shown to exert effects at concentrations in the ng/L range (Overturf et al., 2014; Ussery et al., 2018; Windsor et al., 2018). This is largely attributed to the chronic nature of exposure for these organisms (particularly in instances of persistence or pseudo-persistence), in combination with the complex and unique interactions that can be expected to occur in the presence of other aquatic contaminants (Windsor et al., 2018).

Synthetic progestins are among the most abundantly discharged pharmaceuticals in wastewater, due to their widespread medical use (e.g., birth control pills, treatment for the symptoms of menopause; (King et al., 2016; Kolodziej et al., 2009; Taitel and Kafrissen, 1995; Zeilinger et al., 2009). Norethindrone (NET), one of the earliest synthetic progestins, has been actively prescribed since the 1950's. Synthetic progestins, including NET are frequently detected in the low ng/L range in surface waters (Taitel and Kafrissen, 1995; King et al., 2016; Zeilinger et al., 2009). Although not classified as a persistent pollutant (due to a relatively short half-life), the continuous migration of NET into surface waters (from wastewater treatment plant effluent) has created a scenario of pseudo-persistence for these compounds in many aquatic ecosystems (King et al., 2016; Zeilinge et al., 2009).

NET was designed to target and tightly bind the progesterone receptor to inhibit luteinizing hormone and follicle stimulating hormone, preventing ovulation and pregnancy (Taitel and Kafrissen, 1995). The structures of sex-steroid hormone receptors, including the progesterone receptor, are highly conserved in vertebrates (Hanna et al., 2010). Consequently, the continuous discharge of low levels of NET into waterways can be expected to have a number of consequences for the health of aquatic vertebrates, both through altered gene expression and disruption of normal signal transduction cascades (Edwards, 2005; King et al., 2016; Overturf et al., 2014; Zeilinge et al., 2009; Zhao and Fent,

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2016).

Synthetic progestins, like NET, have been shown to disrupt the photo-transduction cascade in adult zebrafish following a 21-day waterborne exposure at environmentally relevant concentrations (Zhao et al., 2015; Zhao and Fent, 2016). Decreased visual function has important implications for survival of ELS fish in the wild, as vision is involved in the transition from endogenous to exogenous feeding (and the perception of food thereafter) (Blaxter, 1986; Carvalho and Tillitt, 2004; Guthrie, 1986; Magnuson et al., 2018). Furthermore, it is widely accepted that ELS fish are more sensitive to the effects of toxicant exposure (Bridges et al., 2017, 2018; Bridges et al., 2016; Magnuson et al., 2018; Ussery et al., 2018, 2019).

Mortality of ELS fish in the wild is very high, yet recruitment of young fish is necessary to maintain populations (Bailey and Houde, 1989). The likelihood that any given larval fish will survive to sexual maturity is determined by individual variations in fitness, including factors such as size, behavior, and/or visual acuity (Carvalho and Tillitt, 2004; Crowder et al., 1992; Fuiman and Delbos, 1998; Fuiman, 1994). Because the ability to catch prey items directly affects growth and development, costs to the visual function of ELS fish can have clear consequences for fitness (Carvalho and Tillitt, 2004).

Therefore, the goal of the present study was to determine the effects of exposure to a range of environmentally relevant concentrations of waterborne NET on the visual system of developing fish. To elucidate potential mechanisms by which NET may impact the vision of ELS zebrafish, we performed histological, and transcriptomic analyses of 28-dpf zebrafish that were continuously exposed to NET immediately following fertilization.

2. Materials and methods

2.1. Animal care

Fertilized wild type zebrafish embryos were obtained from breeding stock at the University of North Texas, Denton, TX (IACUC 1205-12). After hatch, larvae were transferred to 250-mL borosilicate glass beakers containing exposure solutions, at a density of 10 fish per beaker. Larvae were fed paramecium twice daily for the first 14 days of the exposure, followed by a mixture of paramecium and *Artemia nauplii* until test completion. Every 24-hours a 75% static renewal of exposure solutions was performed, with any remaining waste or debris carefully siphoned from tanks. Water quality parameters, including temperature (25 ± 2 °C), conductivity (300–700 µS), and pH (7.2–7.8), were monitored with probes (YSI Inc./Xylem Inc., Yellow Springs, Ohio). Ammonia and nitrates were monitored using API aquarium test strips. At the conclusion of the test, all larvae were rapidly sacrificed in buffered MS-222 according to the approved IACUC protocol.

2.2. Preparation of test solutions

Treatment solutions were prepared by diluting a master stock of NET in reconstituted moderately hard reconstituted water (RMHW; prepared with ultrapure 18 MΩ MilliQ, Instant Ocean® salts, and sodium bicarbonate) to the following nominal concentrations: 16, 32, 63, 125, 250, 500, or 1000-ng/L NET. Dimethylformamide (DMF) was used as an initial carrier solvent for NET, prior to subsequent dilutions with RMHW. The DMF concentration was held constant at 0.001% in all treatments, including the solvent control treatment (0-ng/L NET). A solvent-free control treatment was also included, to account for any effects on larval vision from DMF exposure alone.

2.3. Experimental design

Newly hatched larvae (0-DPH) were exposed to one of nine test solutions (one of seven NET treatments, a solvent control, or a control) in 250-mL borosilicate glass beakers for 28 days. Fish were maintained in test chambers at a density of 10 larvae per beaker, with five replicates per treatment (n = 5). At the conclusion of the test, replicates from each treatment were subsampled for various analyses.

RNAseq and qPCR analyses were performed on the 500 ng/L treatment and on solvent controls. It was necessary to use all larvae from the 500-ng/L dishes to ensure there was a sufficient quantity of RNA for both analyses (therefore, the 500 ng/L treatment was not sub-sampled for histology). Three 28-DPH larvae from each replicate in the 500 ng/L treatment were pooled into individual microcentrifuge tubes and snap frozen in liquid nitrogen. Next, frozen tissue samples were thoroughly homogenized with TRIzol reagent in a microcentrifuge tube using a motorized pellet pestle. RNA was then extracted from homogenates using a stepwise addition of reagents, punctuated by chilled centrifugation steps (13,000 x *g* at 40C). Briefly, a 24:1 chloroform: isoamyl alcohol mixture was added to homogenates, which were then vortexed and centrifuged. Isopropanol and 5 M NaCl were added to the top aqueous layer of samples (in clean microcentrifuge tubes) followed by a second chilled centrifugation step. After removal of the resulting supernatant, the RNA pellet was washed with 75% ethanol, vortexed, centrifuged, and evaporated. Pellets were reconstituted in RNA/DNA-free water and treated with DNase I.

2.4. RNA sequencing and bioinformatics

Samples (n = 3 biological replicates) of purified RNA from the 500 ng/L and solvent control treatments were hand-delivered on dry ice to the Next Generation Sequencing Core at the UT Southwestern Medical Center (Dallas, TX). RNA samples were assessed for quality using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), and quantitated using a Qubit fluorometer (Invitrogen, Carlsbad, CA). DNAse-treated RNA (4-µg) was prepared using a TruSeq® Stranded Total RNA LT Sample Prep Kit from Illumina (Illumina, Inc., San Diego, CA) according to the manufacturer's instructions, and poly-A RNA was purified and fragmented before strand specific cDNA synthesis. Resulting cDNA were A-tailed, and indexed adapters were ligated prior to amplification via PCR. Amplified cDNA was then purified with AMPure XP beads (Beckman Coulter Life Sciences, Indianapolis, IN) prior to another round of validation on the Agilent 2100 Bioanalyzer. Single read 1 × 50 bp sequencing was performed using an Illumina HiSeq 2000 (all samples were run in duplicate).

All data analyses were performed at the University of North Texas (Denton, TX). After verification that data met Illumina® quality control (QC) requirements (Phred score of 30 for at least 80% of the data), the Tuxedo software suite was used to filter and trim the reads, align and map reads to the zebrafish genome, and perform the quantitative/differential expression analyses. The resulting transcript list was sorted according to adjusted p-values (adjusted using the Benjamini-Hochberg procedure), and DAVID v6.8 (Huang da et al., 2009) was used to perform a pathway analysis of the sorted transcript list.

2.5. qPCR gene expression analysis

To validate the transcriptomics data, the expression of a subset of vision-associated genes in 28-DPH larvae were also evaluated by quantitative polymerase chain reaction (qPCR). Briefly, cDNA was synthesized from purified RNA samples (n = 5 biological replicates) using an iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA), with a Biometra Thermocycler (LABREPCO, Göttingen, Germany). Primers were supplied by Thermo Fisher Scientific (Waltham, MA). Genes were selected for their importance in the phototransduction pathway, and relevance to the RNAseq results.

A QuantiTect™ SYBR™ Green PCR Kit (Qiagen, Louisville, KY) was used to prepare reactions (according to the manufacturer's specifications) and a Rotor-Gene (Corbett Research, Mortlake, Australia) was used for qPCR analysis. Non-template controls were run for all primers, and all samples were run in triplicate. Expression of target genes was

normalized to 18 s rRNA, and ΔΔCt values were calculated. Treatment effects on gene expression were evaluated with a statistical analysis of ΔΔCt values.

2.6. Histology

Replicates from all remaining treatments were each subsampled for 5 larvae, which were then prepared for histological analysis using the methods described in Magnuson et al. (2018). Briefly, 28-DPH larvae were dehydrated using a series of ethanol solutions, and then embedded in paraffin blocks. A microtome was used to cut 5-μm sections along the frontal plane of the larvae, sections were placed on poly-L-lysine coated slides and stained with hematoxylin and eosin.

Slides were imaged with an Axio imager A1 Zeiss compound microscope, and ImageJ (version 1.47) (Rasband, 1997) was used to measure the diameter of the whole retina (WR), lens (L), ganglion (G), inner plexiform (IP), inner nuclear (IN), outer plexiform (OP), outer nuclear (ON), photoreceptor layer (PL), and pigmented epithelium (PE) in the left eye of each fish (n = 5 fish per treatment). Mean diameter was determined by averaging individual layer measurements from three consecutive slides for every individual and normalizing the average against total retinal thickness to generate each data point. Treatment differences were evaluated using a single factor ANOVA, with concentration as a factor.

3. Results and discussion

3.1. Transcriptional responses to NET exposure

Exposure to 500-ng/L NET significantly (FDR p_adj < 0.05) affected the expression of 1277 genes in 28-DPH zebrafish. A pathway analysis indicated consequences for several physiological functions in NET-exposed larvae including effects on pathways involved in steroid biosynthesis and glycerolipid metabolism (Fig. 2). However, the two pathways predicted to be most significantly (p_adj < 0.05) impacted by ELS NET exposure were protein processing in the endoplasmic reticulum (17 genes), and phototransduction (8 genes). A similar direction and degree of fold change was confirmed via qPCR analysis (one-way ANOVA, $p < 0.05$, $DF = 4$, $F = 12.7$) for three of the phototransduction genes, including *pde6a, gnat 1,* and *arr3a* (Fig. 3).

The downregulation of eight genes involved in the phototransduction cascade in the present study may have profound effects on fish vision. Only ten genes, which are divided into seven different functional groups, have been established as key components of this process in zebrafish (Zhao and Fent, 2016). These include the opsin (*opn*) and rhodopsin (*rho*) gene families (which initiate the first steps in the phototransduction cascade), and the transducin (*gnat, gng*) and phosphodiesterase (*pde6*) families, which subsequently transfer the signal from *opn* and *rho* (Brockerhoff et al., 2003; Rinner et al., 2005a; Zhao and Fent, 2016).

Five of the phototransduction genes that were significantly downregulated by exposure to NET are included among these families, including *opn1mw4, pde6a, pde6b, gngt1,* and *gnat1* (Fig.4). The remaining genes in the phototransduction cascade regulate the expression of those in key families (Allison et al., 2010; Bilotta et al., 2001; Rinner et al., 2005b; Zhao and Fent, 2016), including G protein-coupled receptor kinase (*grk*), guanylate cyclase activator (*guca*), and recoverin (*rcvrn*) (Allison et al., 2010; Bilotta et al., 2001; Rinner et al., 2005b; Zhao and Fent, 2016). All of the aforementioned genes were also significantly downregulated by NET-exposure in the present study.

Zhao and Fent (2016) also used qPCR to examine changes in the expression of several vision-associated gene families in adult zebrafish. While that study did find treatment effects on the expression of many of the same vision genes affected in our study, the change was not always in the same direction. During weeks 2–4 of development the zebrafish eye begins to transition from the larval to the adult mosaic, a process that involves rearranging the photoreceptor pattern and altering the fixed ratios at which the different cone types occur (Allison et al., 2010; Bilotta et al., 2001; Fadool and Dowling, 2008).

The age of ELS fish sampled for RNA sequencing in the present study falls within the window of time that this transition is expected to occur in zebrafish, and provides a possible explanation for the dissimilar direction of change observed in vision genes in the Zhao and Fent (2016) study (Takechi and Kawamura, 2005; Allison et al., 2004). Furthermore, it is well established that waterborne exposure to sex steroids can affect the timing and trajectory of a number of developmental processes (Frankel et al., 2016; Nagarajan et al., 2013; Overturf et al., 2014), and it is possible that developmental NET-exposure also alters the timing of the mosaic transition. Decreased expression of several vision genes (e.g., *opn1mw4, gnat1, pde6a, and pde6b)* in the transcriptome of NET-exposed zebrafish provide additional evidence to support the hypothesis that developmental NET exposure impacts the transition from the larval to the adult photoreceptor mosaic pattern.

Green sensitive opsins typically become twice as abundant after the transition to the adult zebrafish mosaic (Allison et al., 2010), an ontogenic modification thought to be driven by thyroid hormone (TH) (Brown, 1997). The green sensitive cone pigment *opn1mw4* was significantly downregulated in NET-exposed larvae, yet no significant alterations were observed in the transcripts of genes involved in TH production. Similarly, no effects on the transcription of TH-associated genes were observed when pre-metamorphic *Xenopus* tadpoles were exposed to environmentally relevant concentrations of NET; however, complete thyroid gland inactivity was observed early in development (Lorenz et al., 2018).

Downregulation of *gnat1* and *pde6a&b* were also observed, suggesting a possible delay in the functional divergence of rods and cones, which is also expected to occur between weeks 2–4 of development (Bilotta et al., 2001; Branchek, 1984; Rinner et al., 2005b). Both *gnat1* and *pde6a&b* play a key role in coupling cGMP to rhodopsin in rods (Fig. 4), a process which leads to membrane hyperpolarization and neurotransmitter release from closure of cGMP-gated ion channels (Brockerhoff et al., 2003). Mutations in *gnat1* and *pde6a&b* result in night blindness and retinitis pigmentosa (Brockerhoff et al., 2003; Xu et al., 2016). Similarly, reduced synthesis of the proteins encoded by *gnat1* and *pde6a&b* is expected to decrease initiation of the phototransduction cascade in low light conditions, with costs to scotopic vision (Fig. 4). NET-exposed fish from the present study also had significantly decreased expression of *grk7*, *rcvrn,* and *guca, all* of which code for proteins that are essential to cone photorecovery (Rinner et al., 2005b). Larval fish deficient in the aforementioned proteins are expected to adapt poorly to low light conditions and be unable to adequately respond to subsequent visual stimuli (Rinner et al., 2005a, 2005b). These results have clear consequences for the survival of ELS fish that rely on light stimuli to obtain food and avoid predators (Fleisch and Neuhauss, 2006).

Interestingly, other studies have also observed significant downregulation of *gnat1, pde6a&b, and grk7* genes in ELS fish developmentally exposed to polycyclic aromatic hydrocarbons (PAHs; Xu et al. 2016; Huang et al; 2013). Predicted effects of PAH exposure on fish vision were confirmed using behavioral assays, which revealed altered phototactic behaviors and abnormal swimming. The authors attributed the observed behavioral alterations to abnormal visual perception/suppressed phototransduction (Huang et al., 2013). Results of those studies also predicted significant effects on steroid biosynthesis and metabolism and lipid metabolism (Xu et al., 2016), all of which are predicted to be significantly altered in NET-exposed fish in our pathway analysis (Fig. 1).

There is evidence that some progestins affect the expression of CYP1A1in fish, the transcription of which is determined by aryl hydrocarbon receptor (AhR) activity (Hou et al., 2017). Some PAHs also bind the AhR with a high affinity (Oziolor et al., 2014), providing a plausible explanation for the similar effects of NET and PAH exposure

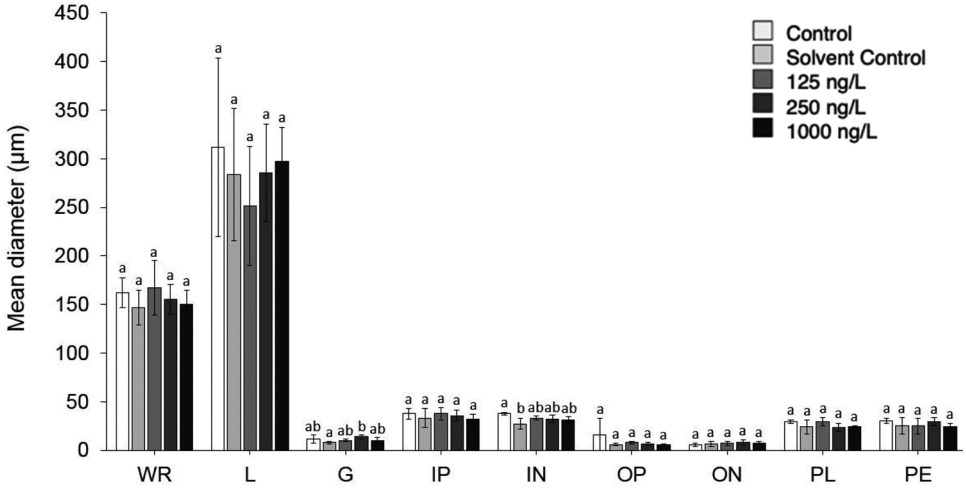

Fig. 1. Developmental NET exposure did not significantly ($p > 0.05$) affect the mean diameter of the following layers in 28-DPH larvae: lens (L), ganglion (G), inner plexiform (IP), inner nuclear (IN), outer plexiform (OP), outer nuclear (ON), photoreceptor layer (PL), and pigmented epithelium (PE). The diameter of each layer was normalized against the diameter of the whole retina (WR) for each individual (N = 5). Bars with matching letters are statistically similar, with error bars representing ± 1 SD.

on the transcriptome of ELS fish. Furthermore, progestins and some PAHs are antagonists for estrogenic activity (Lerner, 1964; Matthews and Gustafsson, 2006), which can lead to effects on a wide variety of biological processes (primarily through nuclear receptor-mediated gene expression changes (Dahlman-Wright et al., 2006).

3.2. Eye histology

Altered visual function may occur through a variety of pathways, including changes in expression of genes involved in the phototransduction pathway, direct interference with molecules involved in the signaling cascade, or through a variety of physiological abnormalities (Edwards, 2005; Magnuson et al., 2018; Xu et al., 2016; Zhao and Fent, 2016). To account for the possibility of morphological abnormalities in the eye structure, we performed a histological analysis to look for treatment effects on the thickness of various eye layers (L, G, IP, IN, OP, ON, PL, and PE relative to the WR). Results of this analysis revealed no significant changes in the endpoints evaluated (Fig.1). Although the 500 ng/L treatment was not histologically analyzed, no significant changes were observed in either of the adjacent treatment concentrations (250 or 1000-ng/L; Fig. 1).

In accordance with our findings, Zhao and Fent (Zhao and Fent, 2016) also saw no morphological differences in the eye organization of adult zebrafish exposed to similar concentrations of progesterone (P4) for 21 days. In combination with the results presented here, these studies suggest molecular level changes as a more likely explanation for the potential effects of progestins on fish vision. Nonetheless, changes at the molecular level often initiate a cascade of events that result in effects at higher levels of organization (Feswick et al., 2016; Villeneuve et al., 2010). It also remains possible that morphological differences between treatments do exist, but are not being captured with the histological methods used in the present study.

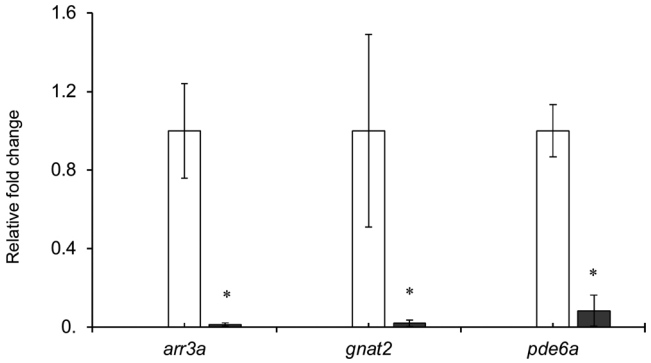

Fig. 3. The qPCR analysis of a subset of vision-associated genes confirmed significant ($p < 0.05$) downregulation in 28-DPH zebrafish larvae developmentally exposed to 500 ng/L NET (n = 3). An asterisk (*) indicates significant differences in the expression of the denoted gene in larvae exposed to 500 ng/L NET, relative to controls (error bars are ± 1 SD).

4. Conclusions

There is an increasing weight of evidence that suggests synthetic progestins pose a risk to the health of aquatic biota. Given the pseudo-persistence of NET in many aquatic systems, there is a pressing need to characterize its effects on non-target organisms, like ELS fish. Here, we show that an environmentally relevant concentration of waterborne NET leads to changes in the expression of genes involved in several important pathways in developing fish, including steroid biosynthesis, glycerolipid metabolism, and visual function. Based on the transcriptional responses of NET-exposed ELS zebrafish (in conjunction with findings from other studies), we hypothesize that ELS NET exposure may affect and/or delay the processes necessary to develop the adult photoreceptor mosaic in zebrafish. Our histological analyses did not

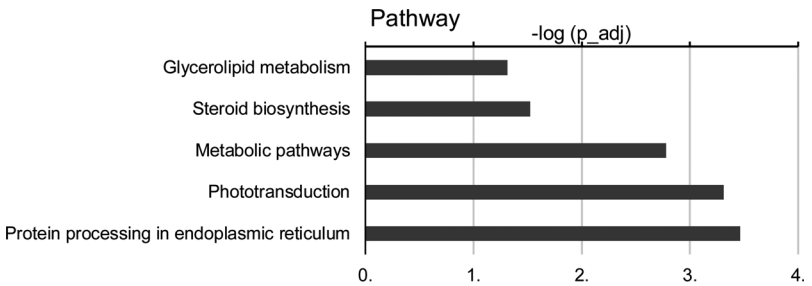

Fig. 2. Predicted biological pathways altered by developmental (0-DPH through 28-DPH) exposure to 500 ng/L NET in larval zebrafish. The x-axis represents the negative log of the adjusted p-value for each pathway.

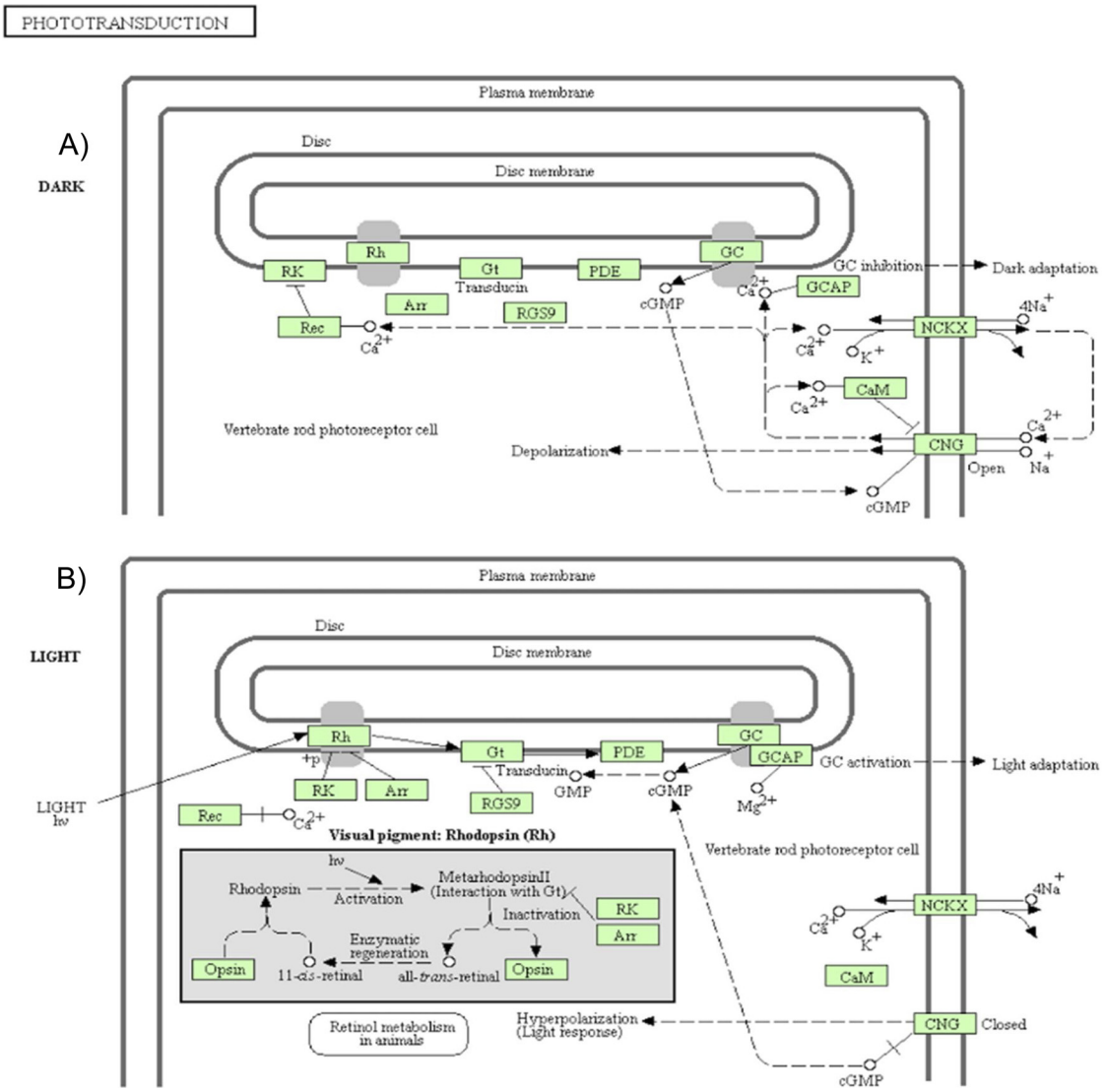

Fig. 4. Proteins (and their associated *genes)* involved in the phototransduction cascade under A) dark, and B) light conditions. Genes coding for opsins (*opn1mw4)*, transducins (*gngt1,* and *gnat1*), PDE (*pde6a & pde6b),* RK (*grk*), GCAP (*guca*), and Rec (*rcvrn*) proteins are significantly downregulated in 28-DPH zebrafish larvae exposed to 500 ng/L NET during development.

reveal changes in the endpoints chosen; however, future studies (using more complex imaging methods) are needed to fully characterize the effects of developmental NET exposure on the timing and trajectory of retinal development in ELS zebrafish. Regardless of the mechanisms by which NET affects visual function, costs to the visual function of ELS fish can have clear consequences for individual fitness in the wild. Many species of fish rely heavily on visual perception for growth and survival during early life stages (e.g., through the perception of food, and predator detection/evasion). Given the prevalence of NET in surface waters, it is possible that such species may be at an increased risk of experiencing reduced recruitment of new fish to breeding populations, an outcome that can have implications at the community level.

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Conflict of Interest

The authors declare no conflict of interest.

Animal use

All procedures described in the present manuscript were approved by the University of North Texas Institutional Animal Care and Use Committee under protocol #1205-12.

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