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Wilwert, Elodie; Etienne, Rampal S.; van de Zande, Louis; Maan, Martine E.

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Visual system plasticity is differently mediated by cone *opsin* expression and chromophore composition in closely related cichlid species

Elodie Wilwert · Rampal S. Etienne ·
Louis van de Zande · Martine E. Maan

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Abstract Phenotypic plasticity allows organisms to rapidly adjust to environmental changes. Cichlid fish inhabit a wide range of light environments and show a large diversity in visual system properties, which makes them a good model system to address the role of phenotypic plasticity in visual adaptation. Cichlid retinal cone pigments consist of opsin proteins bound to Vitamin A₁ or A₂-derived chromophores. Plasticity in expression has been shown for cichlid *opsin* genes, but less is known about the contribution of *cyp27c1*, the enzyme that converts Vitamin A₁ into A₂. Here, we studied both *opsin* and *cyp27c1* expression patterns for three closely related cichlid species from different visual habitats in Lake Victoria, across different light treatments. We found differences in *cyp27c1* as well as in *opsin* expression patterns between the three species. Experimental light treatments affected the developmental trajectory of *cyp27c1* expression

in one species and *opsin* expression in all three species. Within each species, we found large individual variation in *cyp27c1* expression levels and no consistent association with *opsin* expression levels. These results indicate that visual system plasticity of even closely related species can be differentially mediated by *opsin* and *cyp27c1* expression, possibly associated with species differences in visual niche.

Keywords Local adaptation · *Paralabidochromis sauvagei* · *Pundamilia* sp. “*pundamilia-like*” · *Pundamilia*. sp. “*nyererei-like*” · Sensory drive

Introduction

Phenotypic plasticity, the ability of a single genotype to express different phenotypes depending on environmental conditions, is a key determinant of organismal performance and greatly influences ecological interactions. Its role in evolution is also increasingly acknowledged (Bradshaw, 1965; Schlichting & Pigliucci, 1998; Agrawal, 2001; Pigliucci, 2001; West-Eberhard, 2003; DeWitt & Scheiner, 2004; Van Snick Gray & Stauffer, 2004; Fusco & Minelli, 2010; Pfennig et al., 2010). Plasticity might weaken selection pressure for genetic change and thus slow down or prevent adaptive evolution (Huey et al., 2003; Price et al., 2003; DeJong, 2005), but it might also promote adaptive evolution by allowing a broad tolerance to environmental conditions (Schlichting, 1986,

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E. Wilwert (✉) · R. S. Etienne · L. van de Zande ·
M. E. Maan
Groningen Institute for Evolutionary Life Sciences
(GELIFES), University of Groningen, Nijenborgh 7,
9747 AG Groningen, The Netherlands
e-mail: elodie.wilwert@gmail.com

West-Eberhard, 1989, Scheiner, 1993). Therefore, promotion or inhibition of evolution by plasticity may depend on the trait involved. In the present study, we explore the contribution of phenotypic plasticity to visual system variation in cichlid fish.

Organisms often rely on their visual system to assess and exploit their environments (e.g. foraging and social interaction) (Stevens, 2013) and may differ in visual sensitivity as a result of genetic variation and/or phenotypic plasticity (Collin & Shand, 2003; Smith et al., 2012; Hornsby et al., 2013; Dalton et al., 2015). The visual system is often key in local adaptation, as it is fine-tuned to environmental features. Evidence for visual adaptation to local environments has been documented in numerous taxa, particularly in aquatic species (Cummings & Endler, 2018), probably because aquatic environments constitute highly heterogeneous visual habitats with substantial variation in spectral composition and light intensity (Boughman, 2002; Seehausen et al., 2008; Warrant & Johnsen, 2013). For several fish species, there are indications that divergent visual adaptation to different light environments may contribute to, or even initiate, population divergence (Endler, 1992; Seehausen et al., 1997; Boughman, 2002; Spady et al., 2005; Fuller & Noa, 2010; Maan & Seehausen, 2010). Cichlid fish inhabit a large diversity of visual environments (Schelly et al., 2006) and have highly diverse visual system properties (Carleton & Kocher, 2001; Seehausen et al., 2008; Terai et al., 2017). Moreover, several traits have been shown to be plastic in cichlids (reviewed in Schneider & Meyer, 2017) including the visual system. Here, we address the role of plasticity in cichlid visual system variation by exploring the contributions of both developmental and environmental plasticity to different components of the visual system in three closely related species of Lake Victoria cichlids.

As in other vertebrates, cichlid visual pigments consist of an opsin protein covalently bound to a Vitamin A-based chromophore. Cichlid fish have one rod *opsin* gene (*Rh1*), involved in dim light vision, and seven cone *opsin* genes that are expressed in either one of two morphological cone types (Fernald & Liebman, 1980): single cones: three short-wavelength-sensitive opsins (*SWS1*, *SWS2a*, *SWS2b*) and double cones: three medium-wavelength-sensitive opsins (*Rh2b*, *Rh2a α* , *Rh2a β*) and one long-wavelength-sensitive opsin (*LWS*) (reviewed in Carleton

et al., 2016). Variation in the coding sequence (that may alter the wavelength spectrum absorbed by the pigment) and changes in expression patterns of these *opsins*, determine differences in visual sensitivity across species. In addition, *opsin* gene expression has been shown to be plastic (i.e. to be affected by environmental conditions) in cichlids, with varying degrees of plasticity among species (Carleton & Kocher, 2001; Carleton et al., 2005, 2008, 2016; Terai et al., 2006; Hofmann et al., 2010; Nandamuri et al., 2017; Wright et al., 2020). Pigment absorption patterns not only depend on *opsins*, but also on the type of chromophore. Fish (as well as amphibians and some reptiles) use two types of chromophores, based on either Vitamin A₁ (11-cis-retinal) or Vitamin A₂ (11-cis 3,4-didehydroretinal). Chromophore-based tuning involves changes in the proportion of these two forms in the retina. Switching from A₁- to A₂-results in a shift in absorption maxima towards longer wavelengths, with a stronger shift when coupled with longer-wavelength-sensitive opsins (Bridges, 1972; Hárosi, 1994; Parry & Bowmaker, 2000). Chromophore composition varies among species and is associated with variation in light conditions (Bridges & Yoshikami, 1970; Reuter et al., 1971; Provencio et al., 1992; van der Meer & Bowmaker, 1995; Toyama et al., 2008; Morshedien et al., 2017). Underwater light spectra in freshwater habitats are on average more red-shifted than in marine habitats. Consistent with this, freshwater and coastal species tend to use A₁/A₂ mixtures or only Vitamin-A₂-based chromophores, while marine fish use A₁-based chromophores (Toyama et al., 2008). In some species, chromophore ratios change during the lifetime of an individual (Munz & McFarland, 1977), often related to seasonal migration (Temple et al., 2006) or developmental alteration in diet (Giovannucci & Stephenson, 1999). In cichlids, microspectrophotometry (MSP) of retinal tissues suggests that chromophore ratios may differ between species: species from clear waters (e.g. Lake Malawi) mainly use A₁-derivatives (Carleton et al., 2000; Parry et al., 2005; Sugawara et al., 2005), while cichlids from turbid waters (entailing red-shifted visual conditions; e.g. Lake Victoria, Lake Nicaragua and Lake Managua) tend to have a higher proportion of A₂-based pigments (Terai et al., 2006). In zebrafish, conversion of Vitamin A₁ to Vitamin A₂ is mediated by the enzyme Cyp27c1 (Enright et al., 2015). In line with this, studies in

bullfrog and lamprey have documented positive correlations between *cyp27c1* expression levels and A_2 proportions in retinal pigments (Enright et al., 2015; Morshedian et al., 2017). This suggests that *cyp27c1* expression levels can be used as a proxy for A_2 proportions. In a previous study, we observed that this gene is expressed in two closely related haplochromine *Pundamilia* cichlid species from Lake Victoria, with higher *cyp27c1* expression in populations from turbid waters than from clear waters in one of the two species (Wilwert et al., 2021). However, cichlid species and populations from such red-shifted environments not only tend to have higher A_2 to A_1 ratios, but also higher expression of *opsin* genes that absorb longer wavelengths (i.e. *Rh2* and *LWS*; Hofmann et al., 2009; Carleton et al., 2020). This suggests that *opsin* expression patterns and chromophore usage can jointly contribute to visual performance.

In addition to environmental effects, *opsin* gene expression is developmentally plastic: individuals from several species shift from high levels of short-wavelength-sensitive opsin expression as larvae and juveniles to high long-wavelength-sensitive *opsin* expression as adults (Takechi & Kawamura, 2005; Carleton et al., 2008; O'Quin et al., 2011; Dalton et al., 2015; Irazábal-González et al., 2022). These patterns likely reflect differences between life stages in water depth occupation and foraging behaviour (Fryer, 1972; Novales-Flamarique & Hawryshyn, 1994; Jordan et al., 2004). Environmental and developmental plasticity in gene expression is widely documented for *opsin* genes, but less is known about plasticity at the level of chromophore usage.

In this study we explored (1) patterns of *cyp27c1* and cone *opsin* gene expression across three Lake Victoria haplochromine cichlid species, (2) the developmental trajectory and extent of environmental plasticity of *cyp27c1* expression level and (3) the association between *cyp27c1* and cone *opsin* expression patterns.

Methods

Cichlid species

We studied three closely related haplochromine cichlid species, inhabiting different light environments in Lake Victoria (Fig. S1): *Pundamilia*

sp. "pundamilia-like" (P), *Pundamilia* sp. "nyererei-like" (N) and *Paralabidochromis sauvagei* (S). *Pundamilia pundamilia* (Seehausen 1998) and *Pundamilia nyererei* (Witte-Maas & Witte, 1985) are two closely related cichlids species. Males of the *Pundamilia* species pair differ in nuptial coloration (blue vs. red) (Seehausen, 1996). Until recently all *Pundamilia* populations with blue males were classified as *P. pundamilia* and all populations with red males as *P. nyererei*. However, population genomic analyses revealed that populations from the southern and western Mwanza Gulf (Kissenda and Python islands) represent a separate speciation event; they are therefore referred to as *P. sp. "pundamilia-like"* and *P. sp. "nyererei-like"* (Meier et al., 2017, 2018). For both *Pundamilia* species, we used first- and second-generation offspring of wild-caught individuals from Python Island in the Mwanza Gulf of Lake Victoria (− 2.6237, 32.8567). At this location, *P. sp. "pundamilia-like"* occurs in shallow waters (0–2 m) and encounters broad-spectrum light. *P. sp. "nyererei-like"* extends to greater depths (0–5 m), but is most abundant between 2.5 and 3.5 m (Seehausen et al., 2008). High water turbidity at Python Island results in heterogeneous visual conditions across depth ranges, with a shift of the light spectrum towards longer wavelengths with increasing depth, such that *P. sp. "nyererei-like"* experiences little short-wavelength light (Seehausen et al., 2008). *Pa. sauvagei* was collected from Makobe Island (Speke Gulf; − 2.3654, 32.9228), where water transparency is higher than at Python (Seehausen et al., 2006; Castillo Cajas et al., 2012). The species has a relatively broad depth distribution (1.5–14 m; Seehausen, 1996) and therefore encounters a range of light conditions, from broad-spectrum light in shallow waters to red-shifted conditions at the deeper end of its depth distribution (Fig. S1). Families were established opportunistically with 13 dams and 9 sires for *P. sp. "pundamilia-like"*, 15 dams and 10 sires for *P. sp. "nyererei-like"* and 15 dams and 8 sires for *Pa. sauvagei*. In total, eighty-nine crosses resulted in 166 test fish (Table S2). Test fish were randomly selected from these crosses. For individual identification we inserted a microchip in the abdominal cavity (PIT tags, Passive Integrated Transponder, Biomark, ID, Idaho; USA, and Dorset Identification, Aalten, Netherlands) of each test fish. All haplochromines are maternal mouthbrooders; eggs were removed

approximately 6 days after fertilization from brooding females and split evenly between two light conditions (see below). Fish were housed at 25 ± 1 °C on a 12L:12D light cycle and fed twice a day with commercial cichlid pellets and frozen raw food (Artemia, spirulina and krill). For gene expression analysis, fish were sacrificed by applying a lethal dose of buffered MS-222 (1 g/L) and subsequent cutting of the vertebral column in the late afternoon (16:00–18:00; synchronized to maximize RNA yield and reduce effects of circadian variation in *opsin* and *cyp27c1* expression; Halstenberg et al., 2005; Yourick et al., 2019). Eyes were extracted, preserved in *RNAlater*TM (Ambion) and frozen (– 20 °C).

Light conditions

Fish were reared in two different light conditions, mimicking shallow (i.e. broad-spectrum light) and deep (i.e. red-shifted light) water conditions in Lake Victoria (described in Maan et al., 2017; Fig. S1). For both light conditions, we used halogen light bulbs with a green light filter (LEE #243). For the broad-spectrum light condition, we added blue light bulbs (Paulmann, 8809). For the red-shifted light condition we added a yellow filter (LEE #015) to reduce short-wavelength light. Rearing light conditions were designed to mimic the spectral differences between depths and partly recreated intensity differences encountered in the lake (i.e. the red-shifted light environment had a light intensity of ~70% of the shallow light condition; see Fig. S1).

Sampling

Fish were sacrificed at several developmental time points, ranging from 10 to 1391 days post fertilization (dpf). Lake Victoria haplochromines reach adulthood at ~180 dpf (6 months). Accordingly, fish samples were classified into two categories: juveniles (ranging from 10 to 180 dpf, 5 timepoints: 10; 40; 90; 170; 180 dpf) and adults (older than 190 dpf). We included individuals from at least two families for each timepoint. To obtain sufficient retinal RNA (Carleton et al., 2008), we pooled both eyes from one individual for time points 10–40 dpf and one whole eye from one individual at 90 dpf onwards. From 120 dpf onwards, one retina (instead of the whole eye) per individual was extracted (Table S3).

Measuring expression levels

Real-time quantitative polymerase chain reaction (qRT-PCR) was used to quantify *cyp27c1* and *opsin* gene expression. Total RNA was isolated using Trizol (Ambion) followed by a DNase treatment to remove genomic DNA. 1 µg of RNA was reverse-transcribed into cDNA using Oligo(dT)₁₈ primer (Thermo Fisher Scientific) and RevertAid H Minus (Thermo Fisher Scientific) at 45 °C. cDNA was diluted to a final concentration of 10 ng/µl.

Cyp27c1 expression

As a reference for *cyp27c1* expression, we used three housekeeping genes (HKGs): *L-lactate dehydrogenase 1 (ldh1)*, *Glyceraldehyde-3-phosphate dehydrogenase 2 (gapdh2)* and *β-actin* (Jin et al., 2013; Torres-Dowdall et al., 2017). Stability of HKG expression was confirmed using RefFinder (Xie et al., 2012). After a denaturation step (95 °C for 3 min), qRT-PCRs were run for 45 cycles (95 °C for 15 s, 60 °C for 25 s, 72 °C for 30 s). Each reaction mixture (20 µl) contained the gene-specific primer pair (Table S1), diluted cDNA sample and SYBR Green PCR Master Mix (BioRad). Fluorescence was monitored on a StepOnePlus Real-Time PCR System (Applied Biosystems). To test for specificity of amplification, qPCR was followed by a melting curve analysis. We used LinRegPCR (Ruijter & Ramakers, 2003) to determine the initial concentration (N_0) of *cyp27c1* and the HKGs. Expression levels were based on two technical replicates. We applied the following quality control thresholds: PCR efficiency 1.75–2.25 and standard deviation between duplicates ≤ 0.5 . We used the mean of the duplicate N_0 estimates to calculate relative expression levels. The following equation was used to calculate the relative *cyp27c1* expression E_{cyp} for each sample separately:

$$E_{cyp} = \frac{N_{0cyp}}{N_{0Reference}}$$

where N_{0cyp} is the initial concentration of *cyp27c1* cDNA (or mRNA) and $N_{0Reference}$ is the geometric mean of the starting cDNA (or mRNA) concentration of the three HKGs.

Opsin gene expression

Opsin gene expression was quantified only for adults. Expression levels of the *opsin* genes (*SWS2b*, *SWS2a*, *Rh2*, *LWS*) were determined in relation to total *opsin* gene expression, using previously described methods (Wright et al., 2019). Expression was quantified using gene-specific Taqman primers and probes (Table S1). *Rh2aα* and *Rh2Aβ* were combined as *Rh2A*, as they are more than 99% similar in sequence and function (Parry et al., 2005). *Opsin* gene expression (*SWS2a*, *SWS2b*, *Rh2* and *LWS*) was monitored on StepOne-Plus Real-Time PCR System (Applied Biosystems™ StepOnePlus™ Real-Time PCR System) for (95 °C for 2 min, 95 °C for 50 s, 60 °C for 1 min). Using LinRegPCR (Ruijter & Ramakers, 2003), we determined the initial concentrations (N_0) and the critical threshold cycle numbers (Ct) of all four *opsin* genes. This approach determines the upper and lower limits of a “window-of linearity” by examining the log-linear of the PCR curve of each sample (Ruijter & Ramakers, 2003). On each plate, we included a serial dilution of a construct composed of one fragment of each of the four *opsin* genes ligated together. We used the mean of the technical replicates N_0 estimates to calculate relative expression levels. Individual opsin gene expression was quantified relative to total opsin expression, using the following equation:

$$E_{opsin} = \frac{N_0}{N_{0,all}} = \frac{e^{\frac{ct_i - b}{m}}}{\sum_i e^{\frac{ct_i - b}{m}}},$$

where $N_0/N_{0,all}$ is the expression of a given *opsin* gene relative to the total expression of all measured opsin genes, Ct is the critical threshold cycle number of the sample, b the intercept and m the slope value derived from the construct linear regression (Gallup, 2011).

Data analysis

All analyses were performed in R (v4.1.2; R Development Core Team, 2021). Prior to statistical analysis, relative gene expression data were tested for outliers (1.5 * the interquartile range), separately for each species, light treatment and age class (juveniles and adults). Outlier removal resulted in 149 samples for *cyp27c1* expression (15 removed) and 83 samples for *opsin* gene expression (7 removed). To analyse the effects (and interaction) of species differences

and light treatments on *cyp27c1* expression across life stages, we modelled expression as: relative gene expression ~ species * treatment * age + (1|mother ID) + (1|father ID). To analyse the relationship between *opsin* and *cyp27c1* expression in adult fish, we included *opsin* expression data of an additional 38 *Pundamilia* adults, reported in Wright et al., 2019. These data were obtained using the same protocol as applied in our study. In addition, to describe changes at the cone type level, we calculated ratios of *SWS2a/SWS2b* (single cones) and *LWS/Rh2* (double cones). We used linear mixed effects modelling (lmer, R package lme4) for each gene and ratio separately. To determine minimum adequate models, we used stepwise backward selection based on statistical significance. We used Anova (car package; Fox et al., 2017) of the minimum adequate model to estimate the parameter effects, degrees of freedom, and P values of the significant factors with Kenward–Roger degrees of freedom (Kenward & Roger, 1997; Halekoh & Højsgaard, 2014). To estimate parameter effects, P values and degrees of freedom, we performed ‘KRmodcomp’ (pbkrtest package). In case of more than two categories, we used Tukey tests (glht – multcomp package, Hothorn et al., 2008) to estimate parameter effects and P values. Finally, principal component analysis (PCA) was used to obtain a visual representation of species- and treatment-specific expression profiles in terms of both *opsin* gene expression levels and *cyp27c1* expression levels.

Results

Species differences in *cyp27c1* and *opsin* gene expression

Cyp27c1 expression levels differed significantly between species (juveniles and adults combined; $F_{2,12.1} = 29.18$, $P < 0.001$; $n = 146$): expression was highest in *Pa. sauvagei* (compared to *P. sp. “pundamilia-like”*: $z = 4.133$, $P < 0.001$; compared to *P. sp. “nyererei-like”*: $z = 3.72$, $P = 0.001$), while the two *Pundamilia* species showed similar levels of *cyp27c1* ($z = -0.37$, $P = 0.983$). For *opsin* expression patterns (adults only, $n = 90$), we found that proportions of *LWS* ($70.9\% \pm 1.3$) and *Rh2* ($16.6\% \pm 1.2$) were high across species, together making up $87.5\% \pm 2.5$ of total *opsin* expression, while

the expression levels of *SWS2a* ($11.1\% \pm 0.5$) and *SWS2b* ($1.4\% \pm 0.03$) were low. Independent of light treatments, we found species differences in *opsin* gene expression (*Rh2*: $F_{2,11.5} = 45.40$, $P < 0.001$; *LWS*: $F_{2,7.04} = 47.38$, $P < 0.001$). Consistent with earlier work (Wright et al., 2019), *P. sp.* "pundamilia-like" expressed a higher proportion of *LWS* ($z = 3.08$, $P = 0.011$) and lower *Rh2* ($z = 5.17$, $P < 0.001$) compared to *P. sp.* "nyererei-like" (Fig. 2a). *Pa. sauvagei* had lower *LWS* expression than both *Pundamilia* species (compared to *P. sp.* "pundamilia-like": $z = -10.09$, $P < 0.001$; compared to *P. sp.* "nyererei-like": $z = 5.39$, $P < 0.001$), and higher *Rh2* expression (compared to *P. sp.* "pundamilia-like": $z = 12.16$, $P < 0.001$; compared to *P. sp.* "nyererei-like": $z = -5.31$, $P < 0.001$). Proportions of *SWS2a* and *SWS2b* did not differ between species (*SWS2a*: $F_{2,7.516} = 2.81$, $P = 0.122$; *SWS2b*: $F_{2,18.9} = 0.83$, $P = 0.451$; Fig. 2a).

Developmental pattern and effect of light conditions on *cyp27c1* expression

Cyp27c1 expression levels changed with age ($F_{1,114.4} = 106.75$, $P < 0.001$; $n = 146$), but the developmental pattern of *cyp27c1* expression differed between species (i.e. significant species by age interaction; $F_{3,120} = 4.88$, $P = 0.003$). In the *Pundamilia* species *cyp27c1* expression slightly decreased with age (*P. sp.* "pundamilia-like": $F_{1,18.3} = 15.88$, $P = 0.001$; *P. sp.* "nyererei-like": $F_{1,24.8} = 25.53$, $P = 0.001$) and did not differ between rearing light conditions (*P. sp.* "pundamilia-like": $F_{1,41.56} = 1$, $P = 0.408$; *P. sp.* "nyererei-like": $F_{1,44.23} = 1$, $P = 0.455$; Fig. 1a). In *Pa. sauvagei*, however, the developmental pattern of *cyp27c1* expression was significantly influenced by the light conditions ($F_{1,41.1} = 12.14$, $P = 0.001$): in both light conditions, *cyp27c1* was expressed at low levels in early life stages (10–90 dpf), but in the broad-spectrum light condition it increased with age while it remained low in the red-shifted light condition (Fig. 1). This was also reflected by a significant species by light treatment interaction ($F_{2,127.9} = 2.88$, $P = 0.038$), indicating that species responded differently to the different light treatments. *Pa. sauvagei* individuals reared in broad-spectrum light tended to express higher *cyp27c1* compared to their siblings reared in red-shifted light ($z = 2.36$, $P = 0.072$), while in the *Pundamilia* species,

expression levels did not differ between light conditions (*P. sp.* "pundamilia-like": $z = 0.63$, $P = 0.950$; *P. sp.* "nyererei-like": $z = 0.54$, $P = 0.972$) (Note that we did not document the developmental patterns in *opsin* expression in *Pa. sauvagei*; for the *Pundamilia* species the developmental is reported in Irazabal et al., 2021).

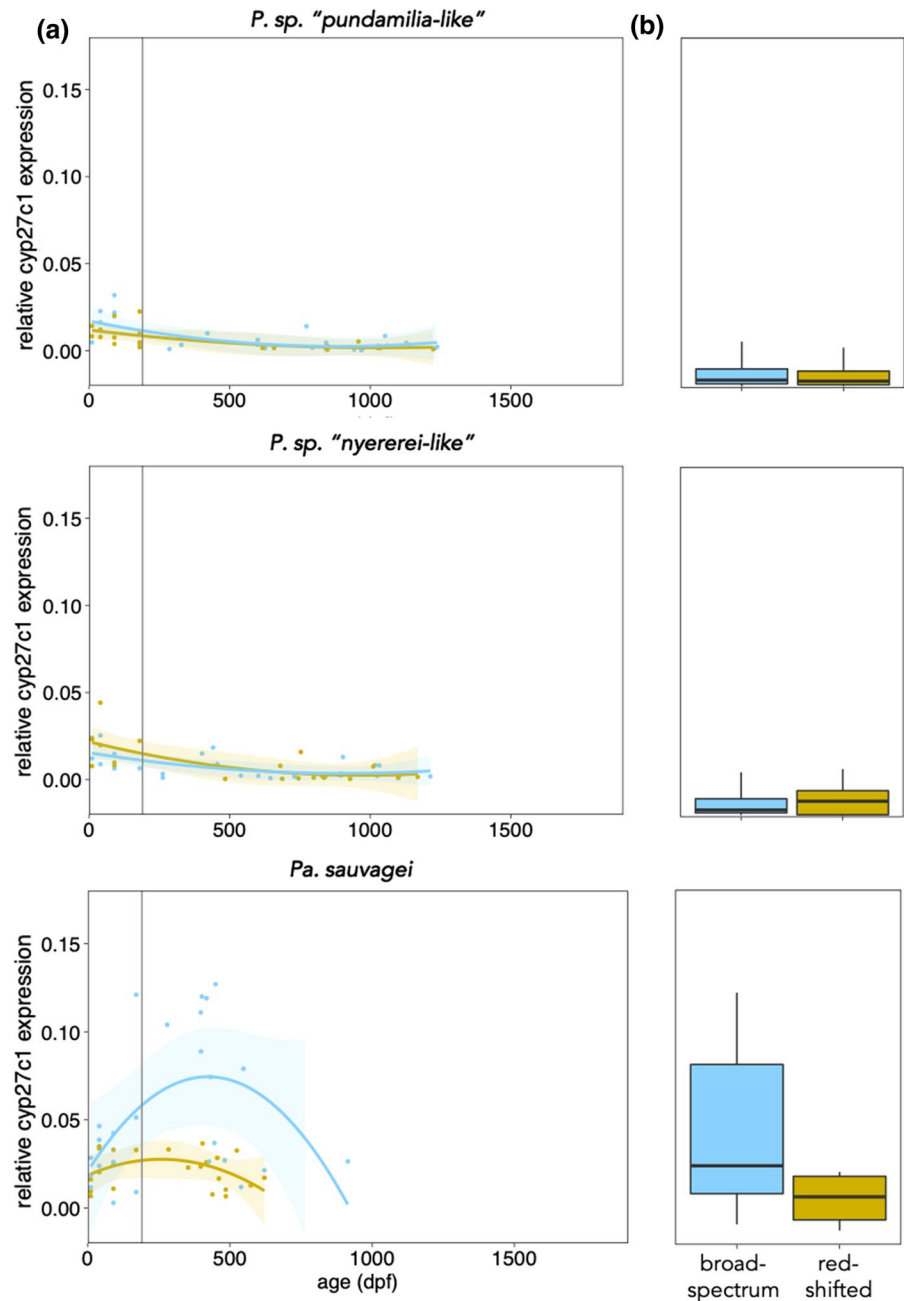
Effects of light conditions on *cyp27c1* and *opsin* gene expression in adult stage

To explore the effects of light conditions on *cyp27c1* expression at the adult stage, we used the same dataset as above, but now only included adults ($n = 93$). We found a similar pattern as described in the previous section: the different rearing light conditions did not induce a plastic response in *cyp27c1* expression in the *Pundamilia* species (*P. sp.* "pundamilia-like": $z = 1.41$, $P = 0.407$; *P. sp.* "nyererei-like": $z = 1.18$, $P = 0.559$), while *Pa. sauvagei* reared in broad-spectrum light expressed higher *cyp27c1* compared to siblings reared in red-shifted light ($z = 3.03$, $P = 0.007$; Fig. 1b). For *opsins* ($n = 89$), we found a significant effect of rearing light on the relative expression levels of *SWS2b* ($F_{1,22.1} = 22.11$, $P < 0.001$) and a tendency for an effect on *SWS2a* ($F_{1,70.8} = 2.83$, $P = 0.097$) and *LWS* ($F_{1,73.9} = 3.51$, $P = 0.065$) in adult fish. No effect on *Rh2* expression was observed ($F_{1,73.2} = 1$, $P = 0.349$) (Fig. 2c). We found that *P. sp.* "nyererei-like" individuals reared in the red-shifted environment expressed lower proportions of *SWS2b* ($z = -3.08$, $P = 0.008$) and *SWS2a* ($z = -3.32$, $P = 0.003$) and higher proportions of *LWS* ($z = -2.87$, $P = 0.016$), compared to their siblings in the broad-spectrum light condition. Similar to *P. sp.* "nyererei-like", *Pa. sauvagei* reared in the red-shifted light condition expressed lower *SWS2b* proportions compared to their siblings reared in the broad-spectrum light condition ($z = -5.89$, $P < 0.001$). *P. sp.* "pundamilia-like" individuals showed no difference in *opsin* expression profiles between rearing light conditions (*SWS2b*: $z = 0.84$, $P = 0.864$; *SWS2a*: $z = -0.05$, $P = 0.999$; *Rh2*: $z = 0.25$, $P = 0.998$; *LWS*: $z = -0.53$, $P = 0.971$).

No clear association between *cyp27c1* and *opsin* gene expression in adult fish

To visualize the relationship between species differences in *opsin* expression and species differences in

Fig. 1 *Cyp27c1* expression. (a) Over development and (b) in adulthood (> 190 dpf). *Cyp27c1* expression decreased with age in the *Pundamilia* species, but increased in *Pa. sauvagei* individuals reared in broad-spectrum light. Irrespective of the light conditions, *cyp27c1* expression was higher in *Pa. sauvagei* than in the *Pundamilia* species. Each symbol represents an individual (for 10 dpf two individuals were pooled). The grey line indicates the transition from juvenile state to adulthood. Shaded areas indicate $\pm 95\%$ confidence intervals. Boxes represent 25–75th percentiles intercepted by the median. Error bars indicate 95% confidence intervals. Colours indicate light treatments (blue = broad-spectrum light; yellow = red-shifted light)



cyp27c1 expression, we calculated the ratios of *opsin* expression within single cones (*SWS2a/SWS2b*) and double cones (*LWS/Rh2*) and plotted these against mean *cyp27c1* expression levels, for each species (Fig. 2b, 2c). This shows that *Pa. sauvagei* combines low *SWS2a/SWS2b* and *LWS/Rh2* ratios with a high *cyp27c1* expression level, while *P. sp. "pundamilia-like"* combines high *SWS2a/SWS2b* and *LWS/Rh2*

ratios with a low *cyp27c1* expression level. In *P. sp. "nyererei-like"*, *SWS2a/SWS2b* ratio, *LWS/Rh2* ratio and *cyp27c1* expression were low. Thus, *Pa. sauvagei* and *P. sp. "pundamilia-like"* differ in both *cyp27c1* expression and *opsin* ratios, while *P. sp. "nyererei-like"* differs from *Pa. sauvagei* mostly in *cyp27c1* expression level. In line with this, PCA indicated that the cluster dominated by *Pa. sauvagei* had positive

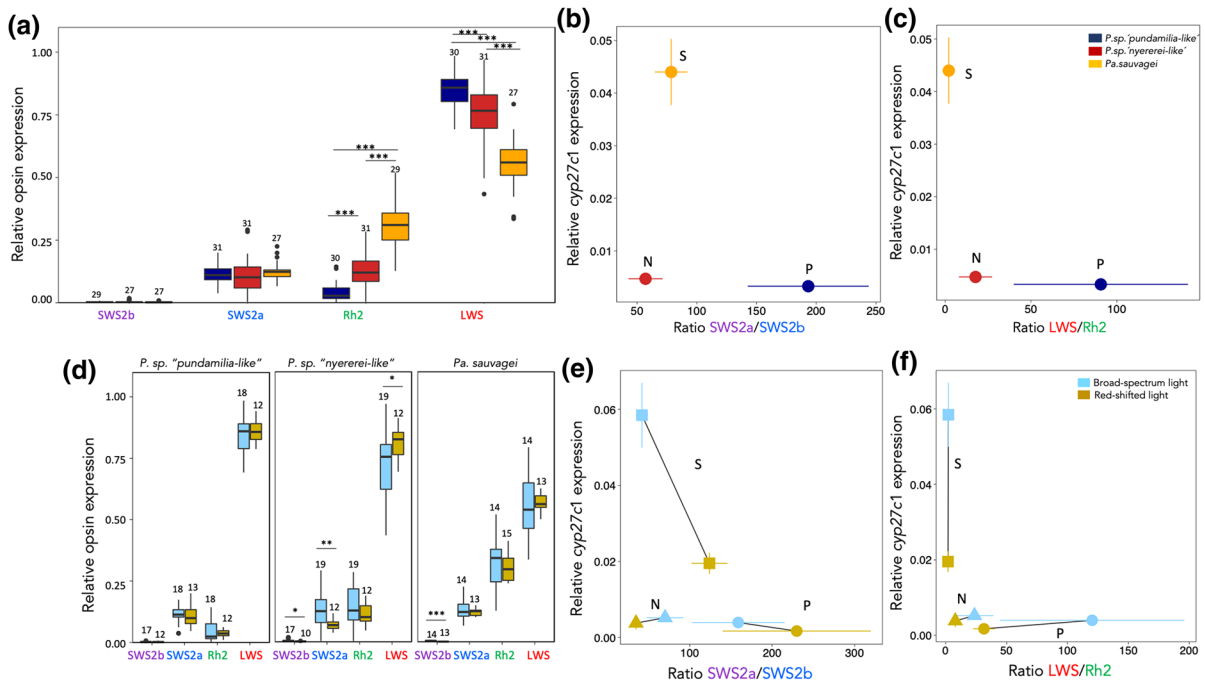


Fig. 2 Relationship between *opsin* gene and *cyp27c1* expression. (a) *Opsin* expression in adult *P. sp. "pundamilia-like"*, *P. sp. "nyererei-like"* and *Pa. sauvagei*, (b–c) Relationship between *opsin* gene expression (SWS2a/SWS2b and LWS/Rh2 ratio) and *cyp27c1* expression across species. (d) Light-induced changes in *opsin* expression, (e–f) Relationship between light-induced changes in *opsin* and *cyp27c1* expres-

sion across species. Boxes represent 25–75th percentiles, intercepted by the median and black symbols are outliers. Shapes in (e–f) indicate species (*Pa. sauvagei* (■), *P. sp. "nyererei-like"* (▲) and *P. sp. "pundamilia-like"* (●)). Sample sizes are indicated above each boxplot and error bars represent $\pm 95\%$ confidence interval. *** indicates $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$

PC1 values (indicating high *cyp27c1* and *Rh2*, but low *LWS*), while the cluster dominated by *P. sp. "pundamilia-like"* had negative PC1 values (indicating low *cyp27c1* and *Rh2*, but high *LWS*; Fig. 3a). The cluster dominated by *P. sp. "nyererei-like"* shows that *P. sp. "nyererei-like"* overlaps primarily with *P. sp. "pundamilia-like"*, but also with *Pa. sauvagei*.

Using the same cone-class-specific *opsin* expression ratios, we visualized the light-induced changes in *opsin* and *cyp27c1* expression for each species (Fig. 2d–f). We observed that in response to the different light conditions, *P. sp. "pundamilia-like"* did not change *opsin* or *cyp27c1* expression, while *P. sp. "nyererei-like"* changed SWS2a/SWS2b ratios, but not LWS/Rh2 ratios or *cyp27c1* expression levels. *Pa. sauvagei* in red-shifted light conditions did not change LWS/Rh2, but increased SWS2a/SWS2b ratio and decreased *cyp27c1* expression. In line with this, PCA indicated more pronounced plastic responses in the cluster dominated by *Pa. sauvagei* and *P.*

sp. "nyererei-like" than *P. sp. "pundamilia-like"* (Fig. 3b).

At the individual level, we found no consistent relationship between *cyp27c1* and *opsin* gene expression (Fig. 4; Fig. S2). There was substantial individual variation, but the nature and extent of variation differed between species. For instance, we observed large individual variation in *cyp27c1* expression in *Pa. sauvagei*, particularly in individuals reared in broad-spectrum light conditions, but not in the *Pundamilia* species.

Discussion

Phenotypic plasticity allows organisms to rapidly adjust to environmental changes, but its potential role in evolutionary change is unclear. Here, we explored the extent of phenotypic plasticity in multiple components of the visual system in three closely related

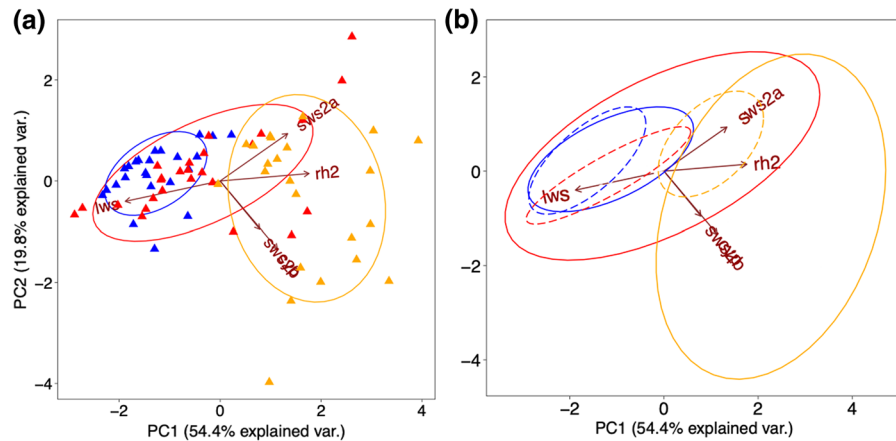


Fig. 3 Expression profiles of *cyp27c1* and opsins (a) form two clusters: the cluster dominated by *Pa. sauvagei* (▲) was characterized by positive PC1 scores, indicating high expression levels of *cyp27c1* and *Rh2*, but low expression levels of *LWS*. The cluster dominated by *P. sp. "pundamilia-like"* was characterized by negative PC1 scores, indicating low expression levels of *cyp27c1* and *Rh2*, but high expression levels of *LWS*, while the cluster dominated by *P. sp. "nyererei-like"* overlaps

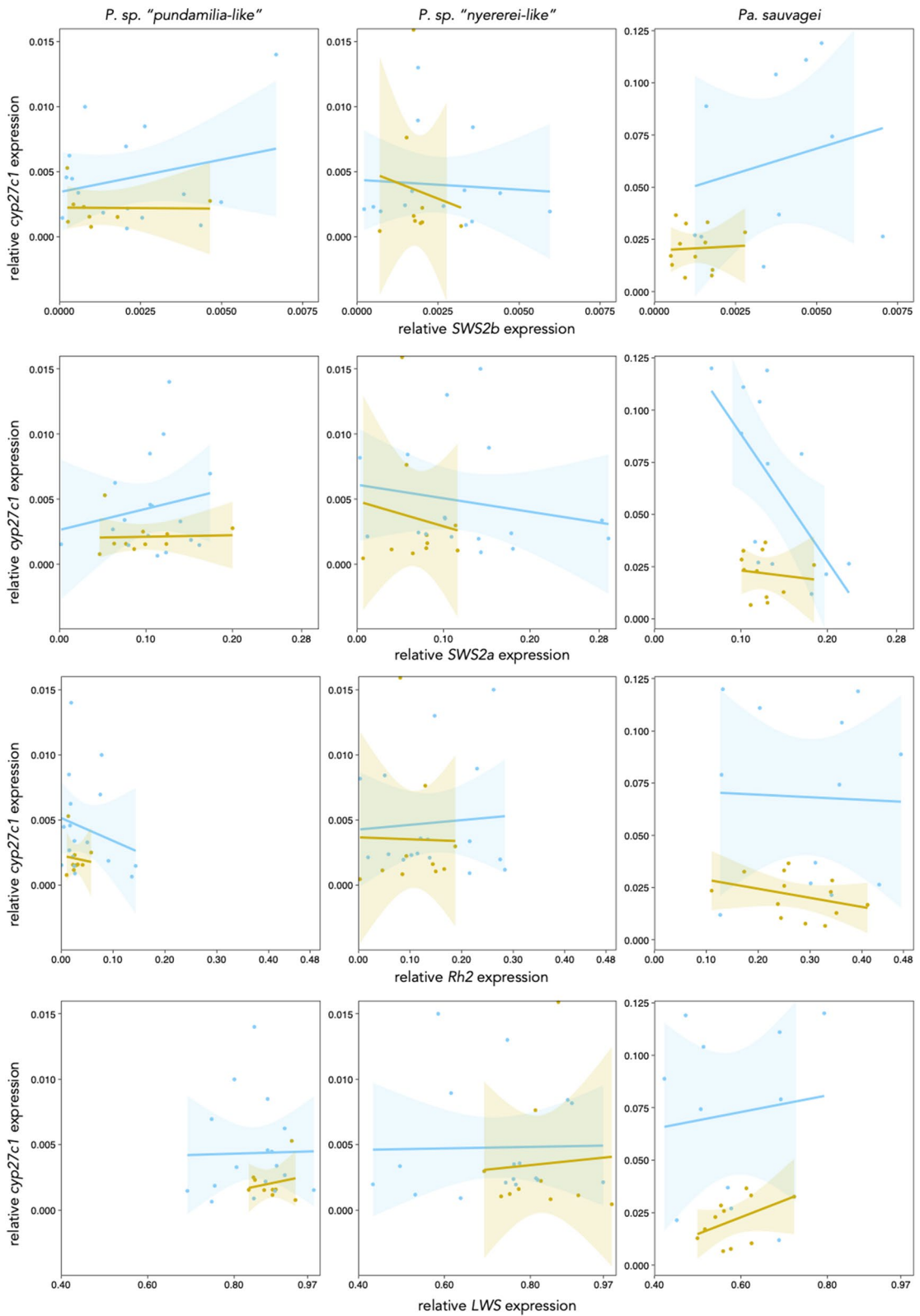
with *Pa. sauvagei* and *P. sp. "pundamilia-like"*. Each symbol represents an individual. (b) Plastic responses in *cyp27c1* and opsins expression were more pronounced in the two species experiencing a broader visual niche (i.e. *P. sp. "nyererei-like"* and *Pa. sauvagei*). Colours indicate species: *P. sp. "pundamilia-like"* (blue) *P. sp. "nyererei-like"* (red) and *Pa. sauvagei* (orange). Linetypes indicate light treatment: broad-spectrum light (solid) and red-shifted light (dashed)

haplochromine cichlid species from distinct photic environments in Lake Victoria, to start to understand the role of plasticity in cichlid visual system variation. We explored variation across species, environments and life stages by measuring the expression levels of opsins genes and, as a proxy for chromophore usage, the *cyp27c1* gene (Enright et al., 2015). We have previously shown that *cyp27c1* is expressed in two of these species (*P. sp. "pundamilia-like"* and *P. sp. "nyererei-like"*), although at very low levels, with variation in expression levels across species and populations (Wilwert et al., 2021). In the present study, we added a third, more distantly related haplochromine species (*Pa. sauvagei*) and explored environmental and developmental plasticity in *cyp27c1* expression of all three species. We also explored the relationship between *cyp27c1* expression and opsins gene expression profiles.

Species differences in *cyp27c1* and opsins gene expression

We observed species differences in *cyp27c1* expression levels, with *Pa. sauvagei* expressing higher levels than the two *Pundamilia* species. Possibly, this relatively low expression in *Pundamilia* species

indicates that it is not relevant for visual adaptation in these species. For opsins gene expression profiles, we found that *P. sp. "pundamilia-like"* expressed higher *LWS* and lower *Rh2* than *P. sp. "nyererei-like"*, in line with previous work in wild-caught and laboratory-reared *Pundamilia* (Wright et al., 2019, 2020). In *Pa. sauvagei*, we found substantially lower *LWS* and higher *Rh2* proportions compared to the two *Pundamilia* species. Together, these findings show that, even when reared under the same light conditions, Lake Victoria cichlids show species-specific expression levels of opsins and *cyp27c1*, supporting a genetic basis of species differences in visual system gene expression profiles. Thus, *Pa. sauvagei* has a markedly different expression profile for both opsins and *cyp27c1*. There are at least two not mutually exclusive explanations for these observations. First, the three species inhabit different visual niches. This implies that species occupying similar visual niches (*Pundamilia*) show similar visual system properties, compared to a species with a more diverse visual niche (*Pa. sauvagei*). Second, species differences might be related to their phylogenetic history: The two *Pundamilia* species are very closely related and even hybridize incidentally, while *Pa. sauvagei* is less closely related to both *Pundamilia* species. Thus,



◀**Fig. 4** Relationship between *opsin* gene and *cyp27c1* expression at individual level. Symbols represent individual fish. Shaded areas indicate $\pm 95\%$ confidence intervals. ** $P < 0.01$ and * $P < 0$

visual system properties in *Pundamilia* may resemble each other more not because of current adaptative significance, but due to shared evolutionary history. To evaluate the contributions of each of these, assessing expression patterns in additional species is needed.

Effects of environmental light on *cyp27c1* and *opsin* gene expression

Adaptive evolution and diversification of the visual system may be facilitated by phenotypic plasticity. Given the red-shifted sensitivity associated with increased A_2 , one would expect higher *cyp27c1* expression levels in fish reared under red-shifted light conditions. We found no such effect. In *Pundamilia*, expression levels were low and did not change in response to light manipulation. This is in line with our previous study (Wilwert et al., 2021), where we also observed very low *cyp27c1* expression in both *Pundamilia* species, and hypothesised that *cyp27c1* may not contribute significantly to visual functioning in these species. The absence of phenotypically plastic responses to light manipulation may indicate that *cyp27c1* also contributes little to individual adjustment to prevailing light conditions in these species. In contrast, in *Pa. sauvagei*, the different light treatments induced a strong plastic response in *cyp27c1* expression. However, against the expectation, fish reared in broad-spectrum light expressed more *cyp27c1* than fish reared in red-shifted light. Because *Cyp27c1* is not a structural component of the pigments, it could be that in broad-spectrum light, the expression of *cyp27c1* ensures a buffer of the enzyme to be employed when the light suddenly changes towards longer wavelengths. This could yield a swifter response than altering *opsin* expression levels (Munz & McFarland, 1977; Collin & Shand, 2003). In red-shifted light, the *opsin* expression profile may ensure sufficient visual flexibility, precluding the need for high expression levels of *cyp27c1*. It is important to note that the plastic response observed in *Pa. sauvagei* does not fully account for the observed species difference (i.e. *Pundamilia* vs. *Pa. sauvagei*) in *cyp27c1* expression. Even when comparing within

the same light condition, *Pa. sauvagei* expressed higher levels of *cyp27c1* than *Pundamilia*, suggesting a genetic difference in the regulation of *cyp27c1* expression between the species. Together with its stronger response to the light treatments, this suggests that chromophore-level variation may be more important in visual system functioning in *Pa. sauvagei* than in *Pundamilia*, or that *Pundamilia* species have a different pathway to modulate chromophore ratios. Further studies are needed to investigate the effects of *cyp27c1* and *opsin* expression levels on actual visual functioning in cichlids.

In line with previous work (Wright et al., 2019), light conditions significantly influenced *opsin* expression patterns (i.e. *SWS2b*, *SWS2a* and *LWS*) in *P. sp. "nyererei-like"*, but not in *P. sp. "pundamilia-like"*, however it should be noted that part of the *opsin* expression data reported in this study (22 out of 64 of *Pundamilia* individuals) came from Wright et al., 2019. In *Pa. sauvagei*, we observed that these different light conditions induced a change in *SWS2b* proportions. Apparently, *P. "nyererei-like"* respond to different light conditions by changes in *opsin* expression levels, while *Pa. sauvagei* respond by changes in *cyp27c1* and *SWS2b* expression levels. We discuss this further below.

Cyp27c1 expression across life stages

Over the course of an individual's lifetime, *opsin* gene expression profiles may change to match developmental changes in water depth occupancy and feeding behaviour (Carleton et al., 2008; Dalton et al., 2015; Härer et al., 2018). Haplochromine cichlid larvae and juveniles typically feed on zooplankton (Fryer, 1972), naturally occurring in shallow waters where the light spectrum is still relatively rich in short-wavelength light (Goldschmidt et al., 1990). Over development fish change their foraging strategies (Fryer, 1972) and explore greater depths encountering different photic conditions. Prior work has shown that the *Pundamilia* species pair shifts from shorter-wavelength sensitive to longer-wavelength sensitive *opsins* with increasing age (Irazábal-González et al., 2022). Here, we find that *cyp27c1* expression slightly decreases with age in *Pundamilia*. If this decrease is functionally relevant, it would be consistent with the expected decrease in long-wavelength sensitivity with age, adding to the pattern observed for *opsins*. In *Pa. sauvagei*, *cyp27c1*

expression levels were also low at the start of development, but increased significantly with age in the broad-spectrum light condition, while remaining low in the red-shifted light condition. Establishing whether or not these species differences in developmental and environmental plasticity reflect different ecologies requires detailed observation of juvenile behaviour in (semi-) natural conditions. In addition, the developmental pattern of *opsin* expression in *Pa. sauvagei* must be determined, to assess whether this also differs from that of *Pundamilia* species and whether it covaries with light conditions.

Possibly, the observed differences are related to the different visual niches experienced by the three species. *P. sp. "pundamilia like"* inhabits a narrow visual niche (0–2 m) occurring exclusively in rock crevices at shallow depths (Seehausen et al., 1998). The two other species, *P. sp. "nyererei like"* and *Pa. sauvagei*, experience broader visual niches and are also more responsive to the different light manipulations (Fig. S1): *P. sp. "nyererei like"* inhabits shallow and deep waters between 0 and 5 m, while *Pa. sauvagei* extends to even greater depths (1.4–15 m) (Seehausen et al., 1998).

Based on our findings, we can formulate three possible ways in which *opsin* and *cyp27c1* expression together may contribute to visual performance. First, *opsin* expression and chromophore usage may act in an additive fashion, where red-shifted light conditions select for increased expression of both *LWS* opsin and *cyp27c1*. This has been observed in Neotropical Midas cichlids, where populations from turbid waters showed increased *LWS* and *cyp27c1* expression compared to populations from clear waters (Torres-Dowdall et al., 2017; Härer et al., 2018). A second scenario could involve a compensatory mechanism, where reduced long-wavelength sensitivity (i.e. lower *LWS* proportions) is counteracted by higher Vitamin A₂ usage (i.e. higher *cyp27c1* expression) and vice versa, as was observed in populations of *A. cf. citrinellus* from turbid waters (Härer et al., 2018). Third, there might be no functional relationship at all between *opsin* genes and *cyp27c1* expression. Our observations could be explained by two of these scenarios: first, the high expression of *cyp27c1*, but low *LWS* in *Pa. sauvagei* and the low *cyp27c1*, yet high *LWS* proportions in *P. sp. "pundamilia-like"* suggests a compensatory effect. Second, the absence of a clear relationship between expression levels of *opsin* genes

and *cyp27c1* at individual level, despite substantial inter-individual variation, is consistent with the third scenario in which there is no relationship between the two, suggesting that other factors are more important in regulating visual system properties.

An implicit assumption of this study is that *cyp27c1* expression levels represent A₁/A₂ ratios. Previous studies in zebrafish and bullfrog have shown that the expression of *cyp27c1* is correlated with the presence of Vitamin A₂. In cichlids, however, only a few data points are available to substantiate this pattern (Torres-Dowdall et al., 2017). The fact that our findings are different from previous findings (Härer et al., 2018), shows that we do not have a full understanding of the mechanism by which *cyp27c1* may or may not mediate chromophore composition and, how its expression interacts with other genes involved in visual system functioning. In zebrafish and bullfrogs *cyp27c1* is expressed in the retinal pigment epithelium (Enright et al., 2015). However, in cichlids, Vitamin A₁/A₂ conversion might be regulated differently and does not necessarily have to occur in the retina. Hence, to interpret *cyp27c1* expression levels, direct measurements of Vitamin A₁/A₂ ratios directly in the visual pigments of Lake Victoria cichlids are required.

Our current picture of cichlid visual adaptation and its role in species divergence is primarily based on opsin level variation. This study illustrates that visual adaptation is more complex, as we not only observe developmental and environmental plasticity of *cyp27c1* expression, but also find that even closely related haplochromine cichlids may use different visual strategies. Ultimately, a better understanding of the molecular basis of the fish visual system will enhance its value as a model system for evolutionary research. This will allow us to exploit it for understanding how organisms adapt, how fast they can respond to environmental changes and how the components of the visual system and phenotypic plasticity contribute to local adaptation.

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Author contributions MEM designed the study, together with RSE, LVDZ and EW. EW designed the qPCR protocol for

cyp27c1 and completed the laboratory work. EW performed the analysis, with assistance from MEM, RSE and LVDZ. EW wrote the manuscript with contributions of MEM, RSE and LVDZ. All authors approved the contents of this manuscript.

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Data availability Once the manuscript is accepted, we will submit our data and R-scripts to Dryad.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was conducted under the approval of the Institutional Animal Care and Use Committee of the University of Groningen.

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