Developmental and environmental plasticity in opsin gene expression in Lake Victoria cichlid fish

Running title: development of cichlid opsin expression

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Abstract

In many organisms, sensory abilities develop and evolve according to the changing demands of navigating, foraging and communication across different environments and life stages. Teleost fish inhabit heterogeneous light environments and exhibit a large diversity in visual system properties among species. Cichlids are a classic example of this diversity, generated by different tuning mechanisms that involve both genetic factors and phenotypic plasticity. Here, we document the developmental progression of visual pigment gene expression in Lake Victoria cichlids and test if these patterns are influenced by variation in light conditions. We reared two sister species of *Pundamilia* to adulthood in two distinct visual conditions that resemble the two light environments that they naturally inhabit in Lake Victoria. We also included interspecific first-generation hybrids. We then quantified (using RT-qPCR) the expression of the four

26 *Pundamilia* opsins (SWS2B, SWS2A, RH2A and LWS) at 14 time points. We find that
 27 opsin expression profiles progress from shorter-wavelength sensitive opsins to longer-
 28 wavelength sensitive opsins with increasing age, in both species and their hybrids. The
 29 developmental trajectories of opsin expression also responded plastically to the visual
 30 conditions. Finally, we found subtle differences between reciprocal hybrids, possibly
 31 indicating parental effects and warranting further investigation. Developmental and
 32 environmental plasticity in opsin expression may provide an important stepping stone in
 33 the evolution of cichlid visual system diversity.

34 **Keywords:** *Pundamilia*, vision, adaptation, light, heterochrony

35 **Research highlights:** In Lake Victoria cichlid fish, expression levels of opsin genes
 36 (encoding visual pigments) differ between developmental stages and between
 37 experimental light treatments. This plasticity may contribute to the evolution of cichlid
 38 visual system diversity.

39

40 **Introduction**

41 Animal sensory systems mediate interactions with the environment, contributing to
 42 foraging, navigation, predator avoidance and mate selection. Sensory systems are highly
 43 diverse within and between species, associated with differences in ecological niche and
 44 life history (Stevens, 2013). For example, the visual system rapidly adapts to variation
 45 in local light conditions, resulting in inter- and intraspecific variation in visual system
 46 properties (Bowmaker, 2008; Carleton, Dalton, Escobar-Camacho, & Nandamuri,
 47 2016; Chang et al., 2021; Veilleux & Kirk, 2014). In water, where light transmission is
 48 poorer than on land, there is substantial heterogeneity in light conditions, mediated by
 49 water depth and transparency. As a result, variation in visual properties is high across

50 aquatic vertebrates, compared to other groups (Bowmaker et al., 1994; Loew &
51 McFarland, 1990).

52 Cichlids are a family of teleost fish that has rapidly diverged into numerous species
53 across Africa, Asia, South America and Central America (Kocher, 2004; Salzburger,
54 2018; Seehausen, 2006). The species inhabit different photic environments, and their
55 visual system properties are highly diverse (Carleton & Yourick, 2020). Some of this
56 diversity is genetically encoded, such as variation in the opsin gene sequences that
57 affect the wavelength absorption properties of the visual pigments, and the subset of
58 opsin genes actually expressed (Carleton et al., 2016; Carleton, Parry, Bowmaker, Hunt,
59 & Seehausen, 2005; Carleton et al., 2008; Hofmann et al., 2009). Other aspects of the
60 visual system can be influenced by developmental or environmental plasticity, including
61 the expression levels of the opsin genes and the use of alternative chromophores
62 (Carleton et al., 2016; Carleton & Kocher, 2001; Halstenberg et al., 2005; Härer,
63 Torres-Dowdall, & Meyer, 2017; Hofmann et al., 2009). In this study, we examine the
64 developmental trajectory of relative opsin expression in two Lake Victoria cichlid
65 species (genus *Pundamilia*) and their hybrids and investigate the influence of light-
66 induced plastic variation in opsin expression.

67 Cichlids have seven distinct cone opsin genes: the short-wavelength-sensitive opsins
68 SWS1 (UV), SWS2B (violet), SWS2A (blue); the medium-wavelength-sensitive opsins
69 RH2A α , RH2A β , RH2B (green); and the long-wavelength-sensitive opsin LWS (red)
70 (Carleton et al., 2016; Carleton et al., 2005). During development, opsin expression
71 profiles may change, and species differ in their developmental patterns. This variation
72 (heterochrony) may provide a target for divergent selection and contribute to the
73 evolution of differences in opsin gene expression observed across species (Carleton et

74 al., 2008; Härer et al., 2017; O'Quin, Smith, Sharma, & Carleton, 2011; Sandkam et al.,
75 2020).

76 Carleton et al. (2008) characterised the developmental patterns of opsin expression in
77 two Lake Malawi cichlid clades (sand-dwelling *Dimidiochromis compressiceps* and
78 *Tramitichromis intermedius* and rock-dwelling *Metriaclima zebra* 'gold', *Metriaclima*
79 *zebra*, *Metriaclima benetos*, and *Labeotropheus fuelleborni*) and an ancestral riverine
80 cichlid lineage (*Tilapia*, *Oreochromis niloticus*). *O. niloticus* expressed predominantly
81 short-wavelength-sensitive opsins as larvae (SWS1 and RH2B), increased the
82 expression of medium-wavelength-sensitive opsins as juveniles (SWS2B and RH2A)
83 and finally expressed high amounts of long-wavelength-sensitive opsins as adults
84 (SWS2A, RH2A and LWS) (Carleton et al., 2008). Rock-dwelling Lake Malawi larvae
85 and juveniles expressed short-and medium-wavelength-sensitive opsins (SWS1,
86 SWS2B and RH2A) and maintained this expression pattern until adulthood. Larvae and
87 juveniles of the sand-dwelling species expressed long-wavelength-sensitive opsins
88 (SWS2A, RH2A and LWS) and maintained this expression profile throughout
89 development. Carleton et al. (2008) hypothesised that the developmental pattern of
90 opsin expression in Lake Victoria cichlids resembles that observed in the Lake Malawi
91 sand-dwellers, but the available data comes from only a small number of Lake Victoria
92 juveniles sampled at a single time point (Carleton et al., 2005; Carleton et al., 2008).
93 Therefore, the developmental pattern of opsin expression in Lake Victoria cichlids
94 remains to be established.

95 *Pundamilia pundamilia* and *Pundamilia nyererei* are two closely related cichlid species
96 that co-occur at several rocky islands in southeast Lake Victoria, in the Mwanza and
97 Speke Gulfs (Seehausen et al., 2008). Water transparency differs between sites, with
98 turbid waters in the south and clearer waters in the north (Castillo Cajas, Selz,

99 Ripmeester, Seehausen, & Maan, 2012). Both species are consistently depth-segregated
100 at all locations but with more overlap in more turbid locations. *P. pundamilia* is
101 restricted to shallow waters (0 - 2 m), while *P. nyererei* is most abundant at 3 - 10 m
102 depth, where the light spectrum is shifted towards longer wavelengths and is largely
103 lacking short-wavelength light (Seehausen et al., 2008). Anatomically, both species are
104 similar, but they differ in male coloration. *P. pundamilia* males are metallic blue with a
105 blue dorsal fin, and *P. nyererei* males are red with yellowish flanks. Females of both
106 species are cryptic yellow/grey.

107 In this study, we use fish from Python Island, where until recently, the blue and red
108 species were thought to be *P. pundamilia* and *P. nyererei*, respectively. However, Meier
109 et al. (2017; 2018) showed that the population at Python Island represents a separate
110 speciation event and is, therefore, referred to as *P. sp. 'pundamilia-like'* and *P. sp.*
111 *'nyererei-like'*. Across *Pundamilia* populations, and consistently linked to male colour,
112 depth, and water clarity, the LWS opsin gene has sequence variations that alter pigment
113 sensitivity (Hofmann et al., 2009; Seehausen et al., 2008; Wright et al., 2019). Wild
114 populations also differ in opsin expression within and between locations (Hofmann et
115 al., 2009; Wright et al., 2019). In the laboratory, we mimicked the natural shallow
116 (broad-spectrum) and deep (red-shifted) light environments of Python Island and
117 observed evidence of opsin expression plasticity: adult fish expressed more LWS and
118 less SWS2A when reared in red-shifted light conditions, compared to their counterparts
119 from broad spectrum light conditions (Wright et al., 2020).

120 Here, we characterise the developmental patterns of opsin expression in *P. sp.*
121 *'pundamilia-like'*, *P. sp. 'nyererei-like'* and their hybrids. We hypothesise that the
122 developmental patterns of both species will resemble those of the Lake Malawi sand-
123 dwellers and that the hybrids will be intermediate. We also explore the extent of

environmental plasticity during development by rearing the fish in broad-spectrum and red-shifted light conditions (Maan, Seehausen, & Groothuis, 2017; Wright et al., 2017). We expect longer wavelength sensitivities in the red-shifted light condition compared to the broad-spectrum light condition. Moreover, a study in neotropical Midas cichlids suggests that the transition to the adult expression profile may be delayed in short-wavelength-rich light environments and accelerated in long-wavelength-rich environments (Härer et al., 2017). Therefore, we predict that LWS expression increases faster in the red-shifted light condition. For both overall opsin expression and its developmental pattern, we expect hybrids to respond more strongly to the light treatments than the parental species. This is because they are expected to be heterozygous for species-specific alleles influencing opsin expression, which may allow for greater environmental effects on the phenotype.

136

137 **Material and methods**

138 Fish species and breeding

For this experiment, we used first (F1) and second (F2) generation aquarium-reared offspring of wild caught *P. sp. 'pundamilia-like'* and *P. sp. 'nyererei-like'* collected from Python Island (-2.6238, 32.8567) in 2014.

For breeding, we housed several females with a single male. All fish were tagged (PIT tags, Passive Integrated Transponder, Biomark, ID, Idaho; USA, and Dorset Identification, Aalten, Netherlands). Haplochromine cichlids are maternal mouthbrooders; about five days after fertilization, the eggs were removed from the mother's buccal cavity and divided equally between light treatments (described below).

147 We reared both species, as well as their reciprocal hybrids, in both light environments.
148 The analysed samples came from 33 aquarium-reared F1 and F2 families, with 24 dams
149 and 16 sires (Table S1). The animal experiments that we performed for this study were
150 approved by the animal ethics committee of the University of Groningen (DEC 6205B;
151 AVD105002016464).

152

153 Housing and light conditions

154 All fish were maintained at 25 ± 1 °C, on a 12:12h day:night light cycle. In both light
155 treatments (mimicking the shallow and deep waters at Python Island), the tanks were lit
156 with halogen bulbs (Philips Halogen Masterline ES, 35W) that were differently filtered
157 depending on the treatment. In the deep treatment, the light was filtered with yellow
158 (no. 15 by LEE, Andover, UK) and green filters (LEE no. 243), generating a red-shifted
159 light spectrum (Fig. S1). In the shallow treatment, the light was filtered with the green
160 filter only. Halogen bulbs have a limited short-wavelength radiance, so the short-
161 wavelengths were supplemented with blue lights (Paulmann 88090 ESL Blue spiral
162 15W) in the broad-spectrum light environment (Fig. S1). Further details on the light
163 treatments and a comparison to the spectral conditions at Python Island are provided in
164 the supplementary information.

165

166 Samples

167 Fish were sampled at 14 different time points, ranging from 10 to 180 days post
168 fertilization (dpf) (Table S2; *Pundamilia* reach sexual maturity at ~8 months of age; i.e.,
169 ~240 dpf). To control for variation between families, we aimed to include at least two
170 different families at each point. Based on previous studies (Carleton et al., 2008; O'Quin

et al., 2011) and on pilot trials of total RNA isolation from fish of various ages, the number of eyes sampled differed between time points: we pooled both eyes from 2 individuals at 10 - 20 dpf, both eyes from 1 individual at 30 - 60 dpf, and used 1 eye from a single fish from 70 dpf onwards. Whole eyes were used up to 90 dpf; from 120 dpf onwards we used retinal tissue.

Fish were euthanized using an overdose of MS-222 buffered with sodium bicarbonate, and the eyes were removed, preserved in RNALater (Ambion®, Foster City, CA, USA) and frozen until qPCR analysis (detailed below). Previous studies have shown a circadian rhythm in opsin expression (Halstenberg et al., 2005). To minimize variation and maximize yield, all samples were collected between 16:00 and 18:00.

Quantification of opsin expression by RT-qPCR

Following previous studies (Wright et al., 2019; Wright et al., 2020), we isolated total RNA with Trizol (Ambion) and reverse-transcribed one microgram of RNA from each sample with Oligo (dT)₁₈ (100µM) (Thermo Scientific, Life Technologies) and RevertAid H minus RT reverse transcriptase (Thermo Scientific, Life Technologies) at 45 °C.

We performed real time quantitative PCR (RT- qPCR) to quantify the relative expression of the four opsins present in adult Lake Victoria cichlids (SWS2B, SWS2A, RH2A and LWS (Hofmann et al., 2009)), using gene-specific TaqMan® primers and probes (Table S2). RH2A α and RH2A β were analysed together as RH2A because they are highly similar in sequence and function (Parry et al., 2005). Fluorescence was monitored during 50 cycles with a BIO-RAD C1000 Thermal Cycler (CFX96 Real Time System) (95 °C for 2 minutes, 95 °C for 50 seconds and 60 °C for 1 minute).

195 We used LinRegPCR® to determine the critical threshold for each sample. This
196 program examines the log-linear part of the PCR curve for each sample to determine the
197 upper and lower limit of the “window of linearity”. Linear regression analysis is then
198 used to calculate the intercept (i.e., the estimated initial concentration) (Ramakers,
199 Ruijter, Deprez, & Moorman, 2003). We used the same approach to calculate the slope
200 and the intercept of a serially diluted construct of the four opsin genes ligated together.
201 We used the following equation to calculate the relative opsin gene expression:

$$\frac{N_0}{N_{all}} = \frac{e^{\left(\frac{Cti-b}{m}\right)}}{\sum e^{\left(\frac{Cti-b}{m}\right)}}$$

202 where N_0/N_{all} represents the expression of each opsin gene, relative to total opsin
203 expression. Cti is the threshold cycle number for the focal opsin, b is the intercept of the
204 mean Cti of each diluted point of the ligated standard, and m the slope of this ligated
205 standard. All samples were run twice.

206

207 Data analysis

208 We discarded samples with PCR efficiencies below 1.75 and above 2.25 and when the
209 Cq standard deviation between replicates was greater than 0.5. To avoid unwarranted
210 exclusion of low expression levels, we did not apply these rules for opsins with < 1% of
211 the total expression (efficiencies decrease and error rates increase at such low
212 concentrations). The relative expression of each opsin is defined in relation to the other
213 three opsins, so discarding a sample for one opsin means discarding the entire sample.
214 The RT-qPCR from the discarded samples was repeated once for any opsin that fell
215 outside of the parameters mentioned above. After applying these quality thresholds, 26
216 of 239 samples were discarded.

217 Prior to statistical analysis, we performed an outlier check with Tukey’s method
 218 identifying the outliers that fell above and below the 1.5 interquartile range. For this
 219 procedure, we divided the dataset by species and treatment. Moreover, due to the
 220 change in opsin expression with developmental time (see results), the data was divided
 221 by age of sampling (10 to 30 dpf and 40 dpf onwards). This analysis was performed to
 222 ensure that the dataset did not contain artefacts from the sampling, RT-qPCR or the
 223 methodology (as discussed in Carleton et al., 2005; Hofmann et al., 2009; Wright et al.,
 224 2019; Wright et al., 2020). Thirty-four outliers were removed, leaving 179 samples for
 225 the analyses. We used linear mixed modelling (in R, R Development Core Team 2017,
 226 lmer, package lme4), separately for each opsin, with the time (dpf), species (*P. sp.*
 227 ‘*pundamilia-like*’, *P. sp.* ‘*nyererei-like*’ or hybrid) and light treatment (broad-spectrum
 228 or red-shifted) as fixed effects, and mother and father ID as random effects
 229 ($expression \sim treatment * species * time + (1/momID) + (1/dadID)$). We used stepwise
 230 backward selection based on statistical significance (in R, ‘MASS’ package version 7.3-
 231 45; (Venables & Ripley, 2002)) to determine the minimum adequate model (retaining
 232 parental identities in all models to account for pseudo replication). Finally, we
 233 performed “KRmodcomp” to estimate the parameter effects, P-values, and degrees of
 234 freedom based on the Kenward-Roger approximation (in R, package ‘pbkrtest’ version
 235 0.4-6; Halekoh and Højsgaard (2014)).

236

237 **Results**

238 *Pundamilia* opsin expression throughout development

239 The expression of all four opsins changed significantly over time, as evidenced by the
 240 significant effect of time in all models - particularly during early development (10 - 90

241 dpf; Fig. 1; Table 1). Throughout development, *Pundamilia* expressed high proportions
242 of LWS, followed by RH2A, SWS2A and SWS2B. With time, RH2A expression
243 decreased from ~25% to below 20%, and LWS increased from ~60% to ~75%. The
244 expression of both SWS2 opsins was low in early development (~5%). SWS2A
245 increased to about 20%, while SWS2B decreased to nearly 0 at 50 dpf. The expression
246 of all four opsins stabilized after 200 dpf, reaching the levels previously established for
247 adult fish (Fig. 1).

248

249 **Table 1. Parameter estimates from the models explaining variation in opsin expression.** Time
250 represents the days post fertilization, treatment represents the light conditions (broad-spectrum or red-
251 shifted) and species represents the different crosses (*P.sp. 'pundamilia-like'* or *P. sp. 'nyererei-like'* or
252 their hybrids). We used the *Anova* function ('car' package) to estimate the parameter effects, degrees of
253 freedom and P-values of the significant factors in the minimum adequate model. For the removed factors,
254 we used "KRmodcomp" to compare the minimum adequate model with a model containing the removed
255 factor(s).

SWS2B	F	Df	Df resid	P
time	119.0229	1	168.23	< 0.001***
treatment	91.2334	1	155.25	< 0.001***
time:treatment	5.8824	1	156.45	0.016*
Removed factors	F	ndf[†]	ddf[*]	P
time:species	0.0396	2	116.518	0.96
treatment:species	1.1234	4	30.1961	0.36
species	0.9696	2	11.2216	0.41
SWS2A	F	Df	Df resid	P
time	14.9456	1	167.706	< 0.001***
treatment	4.7524	1	153.903	0.03*
species	1.3549	2	10.255	0.30
time:treatment	17.5882	1	154.815	< 0.001***
time:species	2.8392	2	157.995	0.06•
Removed factors	F	ndf	ddf	P
treatment:species	1.8521	2	152.1398	0.16

RH2A	F	Df	Df resid	P
time	21.7602	1	170.707	< 0.001***
treatment	9.5157	1	157.131	< 0.01**
species	41.3711	2	10.154	< 0.001***
treatment:species	4.8666	2	157.615	< 0.01**
Removed factors	F	ndf	ddf	P
time:species	0.6172	2	156.1669	0.54
time:treatment	2.0496	1	156.2806	0.15
LWS	F	Df	Df resid	P
time	30.2459	1	167.906	< 0.001***
treatment	0.2872	1	156.235	0.59
species	25.8555	2	9.853	< 0.001***
time:treatment	4.0317	1	156.362	< 0.05*
treatment:species	3.2048	2	156.597	< 0.05*
Removed factors	F	ndf	ddf	P
time:species	0.3604	2	153.7226	0.7

† Numerator degrees of freedom

‡ Denominator degrees of freedom

256

257 Species differences

258 Expression levels of LWS and RH2A differed significantly between the species groups
259 (Table 1, Fig. 2), and post hoc tests showed that all pairwise comparisons were
260 significant (all $P < 0.001$). LWS expression was highest in *P. sp. 'pundamilia-like'*,
261 lowest in *P. sp. 'nyererei-like'* and the hybrids were intermediate. RH2A showed the
262 opposite pattern: it was highest in *P. sp. 'nyererei-like'*, lowest in *P. sp. 'pundamilia-*
263 *like'* and the hybrids were in-between. Expression levels of the short wavelength
264 sensitive opsins were similar for both species and their hybrids (Table 1).

265 Despite the differences in relative opsin expression levels, there were no differences in
266 the developmental patterns between species: the interaction between species and time
267 did not significantly affect the expression level of any of the opsins (Table 1; there was
268 a trend for SWS2A).

269

270 Effects of the light environment

271 We found significant effects of the light treatments on SWS2B, SWS2A and RH2A (see
272 Fig. 3 and Table 1). In broad-spectrum light, fish expressed more SWS2B and less
273 SWS2A and RH2A compared to their counterparts from the red-shifted light
274 environment.

275 The developmental patterns of opsin expression were similar between light conditions
276 (Fig. 3), but the rate of change in opsin expression differed significantly. Overall,
277 SWS2B decreased with age in both light conditions, but in broad-spectrum light,
278 SWS2B expression was initially higher and decreased faster than in the red-shifted light
279 condition (Fig. 3). SWS2A, on the other hand, increased in both light conditions, but the
280 rate of change also differed (Table 1). In broad-spectrum light, SWS2A expression was
281 initially lower, but it rapidly increased (during 10 - 50 dpf) until the expression levels
282 were similar in both light conditions. The increase in SWS2A expression was less steep
283 in the red-shifted light condition, where expression remained relatively stable
284 throughout development. LWS expression was lower in early development in the red-
285 shifted light condition and increased more steeply than in the broad-spectrum light
286 environment. The developmental pattern of RH2A expression was not affected by the
287 light treatments.

We also examined whether the two *Pundamilia* species and their hybrids responded differently to the light treatments. We found significant interaction effects (species by treatment) for RH2A and LWS expression (Table 1, Fig. 3). *P. sp. 'nyererei-like'* and the hybrids had higher RH2A expression in the red-shifted light environment, while *P. sp. 'pundamilia-like'* RH2A expression did not differ between conditions. LWS showed the opposite pattern: in *P. sp. 'nyererei-like'* and the hybrids it did not differ between light conditions, but *P. sp. 'pundamilia-like'* had higher LWS expression in the red-shifted light environment.

Hybrids

We also explored whether the reciprocal hybrids differed in opsin expression (Table 2, Fig. 4). For this analysis, we used samples ≤ 90 dpf, as we lacked older samples for the NP hybrids (NP: *P. sp. 'nyererei-like'* mother, *P. sp. 'pundamilia-like'* father; PN: *P. sp. 'pundamilia-like'* mother, *P. sp. 'nyererei-like'* father). Figure 4 suggests that hybrids tend to resemble their maternal species more than their paternal species, particularly in SWS2A and RH2A expression.

This effect was statistically significant only for RH2A, showing an interaction of time and cross type, indicating a difference in developmental pattern between PN and NP hybrids. Both hybrid types had similar RH2A expression at 90 dpf, but the NP hybrids started with high expression and rapidly decreased, similar to *P. sp. 'nyererei-like'*. The PN hybrids exhibited a more stable and lower expression level, similar to *P. sp. 'pundamilia-like'*.

Table 2. Parameter estimates from the models explaining variation in opsin expression among the reciprocal hybrids until 90 dpf. Time represents days post fertilization, treatment represents the light conditions (broad-spectrum or red-shifted) and cross denotes NP (*P. sp. 'nyererei-like'* mother, *P. sp. 'pundamilia-like'* father) or PN (*P. sp. 'pundamilia-like'* mother, *P. sp. 'nyererei-like'* father). We used

the *Anova* function to estimate the parameter effects, degrees of freedom and the P-values of the significant factors in the minimum adequate model. For the removed factors, we used a “KRmodcomp”.

SWS2B	F	Df	Df resid	P
time	38.687	1	64.318	< 0.001 ***
treatment	52.458	1	60.929	< 0.001 ***
Removed factors	F	ndf[†]	ddf[‡]	P
time:treatment	0.0905	1	60.1676	0.76
treatment:cross	0.5921	2	9.81	0.57
time:cross	2.0146	1	58.2417	0.16
cross	0.0812	1	4.1869	0.79
SWS2A	F	Df	Df resid	P
time	13.5463	1	63.36	< 0.001 ***
treatment	3.2938	1	61.152	0.07•
cross	4.8588	1	3.549	0.10
time:treatment	9.81	1	61.328	< 0.01 **
Removed factors	F	ndf	ddf	P
time:cross	0.1285	1	63.4182	0.72
treatment:cross	1.7816	1	62.5275	0.19
RH2A	F	Df	Df resid	P
time	15.1876	1	65.191	< 0.001 ***
treatment	8.5974	1	61.882	< 0.01 **
cross	1.7027	1	3.696	0.27
time:cross	4.7041	1	65.345	0.03 *
Removed factors	F	ndf	ddf	P
time:treatment	0.0002	1	60.5536	0.99
treatment:cross	0.6981	1	63.9932	0.41
LWS	F	Df	Df resid	P
time	11.915	1	68.32	< 0.001 ***
Removed factors	F	ndf	ddf	P
treatment:cross	1.4291	3	15.0737	0.27
time:treatment	1.3407	1.3407	63.332	0.25
treatment	3.4179	1	63.2963	0.07•
time:cross	2.6334	1	25.3959	0.12
cross	0.0664	1	3.7826	0.81

[†] Numerator degrees of freedom

[‡] Denominator degrees of freedom

319 Discussion

320 In this study, we characterised the developmental pattern of opsin expression in two
321 Lake Victoria haplochromines, *P. sp. 'pundamilia-like'* and *P. sp. 'nyererei-like'*, and
322 their hybrids in two distinct light conditions. Our results show that opsin expression
323 changes over developmental time and is also influenced by environmental light.

324

325 *Pundamilia* opsin expression throughout development

326 We hypothesised that the relative opsin expression in *Pundamilia* cichlids (SWS2B,
327 SWS2A, RH2A and LWS) would remain fairly constant across development, with
328 relatively high long-wavelength sensitivity throughout, as previously documented in
329 sand-dwelling cichlids from Lake Malawi (Carleton et al., 2008). Our results are partly
330 consistent with this hypothesis: throughout development, expression of the long-
331 wavelength sensitive opsin (LWS) was high, compared to the medium- and short-
332 wavelength sensitive opsins (RH2A, SWS2B and SWS2A). However, contrary to the
333 Lake Malawi sand-dwellers, opsin expression in *Pundamilia* changed over
334 development. This resembles the developmental pattern of *O. niloticus* (Carleton et al.,
335 2008; O'Quin et al., 2011). Therefore, the developmental pattern of *Pundamilia* cichlids
336 falls in-between *O. niloticus* and Lake Malawi sand-dwellers. When comparing our
337 present findings to the opsin expression levels we documented in adults (Wright et al.,
338 2020), we observe that the opsin expression profile stabilizes around 200 dpf.

339 At 10 dpf, SWS2B expression was relatively high but quickly decreased to zero at ~100
340 dpf. A possible adaptive explanation for high SWS2B expression in young fish might be
341 an ontogenetic shift in the vertical distribution in the water column. In several
342 haplochromine species from Lake Victoria, larvae and juveniles have shallower depth

distributions than adults (Goldschmidt, Witte, & De Visser, 1990) and shallow waters are relatively rich in short-wavelength light (captured by SWS1, SWS2B and SWS2A). The depth distribution of *Pundamilia* juveniles is unknown, but high SWS2B expression may suggest that they inhabit relatively shallow waters. SWS2B expression may also contribute to foraging efficiency; cichlids and other teleost fish change their foraging strategies as they develop, and zooplankton is an important component of larval and juvenile diets (Fryer & Iles, 1972). Short-wavelength vision particularly aids the detection of small and translucent objects (Britt, Loew, & McFarland, 2001; Carleton et al., 2008; Loew & Wahl, 1991; Novales-Flamarique & Hawryshyn, 1994; Novales Flamarique, 2013).

353

354 Species differences

The parental species differed in LWS and RH2A expression: *P. sp. 'pundamilia-like'* had higher LWS expression than *P. sp. 'nyererei-like'*, while RH2A exhibited the opposite pattern. This is in line with earlier studies of wild caught (Wright et al., 2019) and laboratory-reared fish (Hofmann et al., 2009, Wright et al., 2020). As hypothesised, the developmental patterns of opsin expression were similar in both species.

360

361 Effects of the light environment

The fish were reared in two light environments mimicking the natural shallow (broad-spectrum) and deep (red-shifted) light environments of Lake Victoria. These light treatments influenced opsin expression. SWS2B expression was higher in broad-spectrum light than in red-shifted light, while SWS2A and RH2A followed the opposite pattern. Higher SWS2A expression in the red-shifted condition contrasts with what was

367 previously observed in *Pundamilia* adults (Wright et al., 2020); we discuss this further
368 below.

369 In addition to the overall expression levels, the developmental patterns of LWS,
370 SWS2A, and SWS2B were affected by our light treatments. LWS expression increased
371 faster in the red-shifted light condition, similar to the pattern observed in Midas cichlids
372 (Härer et al., 2017). However, the SWS opsins showed a different effect, changing
373 faster in the broad-spectrum light conditions: SWS2A increased faster and SWS2B
374 decreased faster. Thus, our findings do not correspond to the general pattern suggested
375 for neotropical cichlids, where progress towards the adult phenotype was accelerated in
376 light conditions rich in long wavelengths (Härer et al., 2017). Rather, it seems that LWS
377 and SWS2A increase faster in the light conditions where these opsins confer greater
378 photon capture. To test this interpretation, experiments are needed to quantify fish'
379 visual performance in different visual conditions and at different developmental stages.

380 When analysing the interaction between environmental light and species (*P. sp.*
381 '*pundamilia-like*', *P. sp.* '*nyererei-like*' or hybrids), we found that LWS and RH2A
382 expression were both species- and environment-specific (Fig. 3). *P. sp.* '*pundamilia-*
383 *like*' reared in the red-shifted light environment expressed more LWS than their
384 counterparts raised in broad-spectrum light. *P. sp.* '*nyererei-like*' and hybrid LWS
385 expression did not differ between the treatments. RH2A showed the opposite expression
386 pattern between species: *P. sp.* '*nyererei-like*' and the hybrids had higher RH2A
387 expression in red-shifted light, while *P. sp.* '*pundamilia-like*' expression did not differ
388 between the treatments. These results suggest that both *Pundamilia* species respond
389 plastically to the light treatments, but in different ways. In the adults, LWS and RH2A
390 were also differently expressed between species in the two light environments (Wright
391 et al., 2020). However, contrary to what we observed here, adult *P. sp.* '*nyererei-like*'

expressed more LWS in the red-shifted condition than in the broad-spectrum condition. Hybrids only differed in SWS2A expression and *P. sp. 'pundamilia-like'* did not show any significant differences. The mismatch between our results and the data from the adults might be explained by the lack of data points between 180 dpf and the adult stage (200 dpf and older). Possibly, *P. sp. 'nyererei-like'* increase their LWS expression during this time. Additional sampling during this developmental period, as well as transfer experiments (e.g.(Härer, Karagic, Meyer, & Torres-Dowdall, 2019)), are required to establish the sensitive window for the effects of light conditions on opsin expression and potential species differences in this regard.

Heterochronic changes are modifications in developmental pattern compared to the ancestral state (McKinney & McNamara, 1991). For haplochromine cichlids, *O. niloticus* is considered to represent the ancestral pattern of opsin expression development (Carleton et al., 2008; O'Quin et al., 2011). Rock- and sand-dwelling species from Lake Malawi differ greatly from *O. niloticus*, and differences in their developmental patterns can be interpreted as adaptations to different light environments. *P. sp. 'pundamilia-like'* and *P. sp. 'nyererei-like'* also differ from *O. niloticus*. Yet, even though the two *Pundamilia* species inhabit very different natural light environments, there is little difference in their developmental patterns. We do find that they respond somewhat differently to the light treatments. This may represent a starting point for heterochronic shifts in *Pundamilia*, possibly providing a target for selection and allowing the evolution of species-specific developmental trajectories.

413

414 Hybrids

415 The developmental profiles of hybrids fell in-between the parental species. The level of
 416 environmental plasticity in opsin expression was similar between hybrids and parental
 417 species, in contrast to our prediction that hybrids might show greater plasticity, but in
 418 line with the earlier data on adults (Wright et al., 2020). We used the reciprocal hybrids
 419 (≤ 90 dpf only) to test for potential parental effects on opsin expression. Our results
 420 suggest that PN (*P. sp. 'pundamilia-like'* mothers) and NP hybrids (*P. sp. 'nyererei-*
 421 *like'* mothers) may have different developmental profiles: RH2A expression decreased
 422 faster in NP hybrids than in PN hybrids. This might be interpreted as a parental effect,
 423 as PN hybrids showed more similar expression patterns to *P. sp. 'pundamilia-like'*
 424 (higher SWS2A and lower RH2A), while NP hybrids showed more similar patterns to
 425 *P. sp. 'nyererei-like'*. Thus, while our study was not designed to fully explore the
 426 influences of maternal or paternal effects on opsin expression, it provides some exciting
 427 first indications that warrant further investigation.

428

429 **Conclusion**

430 The evolutionary importance of heterochrony lies in the fact that certain phenotypes
 431 appear during development and are beneficial in a given environment, which can then
 432 become targets of selection, potentially leading to arrested or accelerated development.
 433 Likewise, environmentally-induced phenotypes, throughout development, are 'seen' by
 434 natural selection and can provide stepping stones for evolutionary adaptation (West-
 435 Eberhard, 2003).

436 In this study, we documented the developmental pattern of opsin expression in two
 437 species of Lake Victoria cichlids and their reciprocal hybrids.

438 We found that *Pundamilia* cichlids progress from high levels of short-wavelength-
 439 sensitive opsin expression as larvae and juveniles to high levels of long-wavelength-
 440 sensitive opsin expression as (sub)adults. This pattern may reflect differences between
 441 life stages in water depth distribution, where larvae and juveniles reside in more shallow
 442 waters, with broad-spectrum light, compared to adults. Developmental patterns were
 443 similar between the species, but the overall opsin expression levels differed between
 444 them, consistent with prior work. The developmental trajectory of opsin expression also
 445 responded plastically to the visual conditions. Finally, we found preliminary evidence
 446 for parental effects in the developmental patterns of opsin expression that warrants
 447 additional research.

448

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454

455

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576

577 **Figures**

578 **Figure 1. *Pundamilia* opsin expression throughout development.** The relative
579 expression of all four opsins (**SWS2B**, **SWS2A**, **RH2A**, and **LWS**) changed during
580 development, as highlighted by the significant effect of time (dpf, days post-
581 fertilization) in all models. Each point represents an individual (2 individuals pooled for
582 time points 10 and 20 dpf), with shaded areas indicating 95% CI. The opsin expression
583 profiles of lab-reared adults (> 200 dpf) are provided for reference (from Wright et al.,
584 2020). Error bars represent the 95% CI.

585 **Figure 2. Opsin expression throughout development for *P. sp. 'pundamilia-like'*, *P.***
586 ***sp. 'nyererei-like'* and their hybrids.** LWS and RH2A expression differed
587 significantly between the species groups, but the developmental patterns were similar.

588 **Figure 3. Effects of the light treatments on the developmental patterns of**
589 ***Pundamilia* opsin expression.** (A) The opsin expression patterns of fish housed in
590 broad-spectrum (left panel) vs. red-shifted light (right panel). The expression of
591 SWS2B, SWS2A and RH2A were significantly affected by the light treatments. The
592 developmental patterns of SWS2B, SWS2A and LWS differed significantly between
593 light environments. Shaded areas indicate 95% CI. The opsin expression profiles of lab-
594 reared adults (> 200 dpf) is provided for reference (from Wright et al., 2020). (B) There
595 were significantly different effects of the light treatments on RH2A and LWS
596 expression in *P. sp. 'pundamilia-like'*, *P. sp. 'nyererei-like'* and their hybrids. Error bars
597 represent the 95% CI.

598 **Figure 4. Differences in the developmental patterns of opsin expression between**
599 **reciprocal hybrids.** RH2A was the only opsin that differed across time for the
600 reciprocal hybrids; the NP hybrids had a faster decrease in their expression. The *P. sp.*
601 *'pundamilia-like'* and *P. sp. 'nyererei-like'* panels show that the PN hybrids resemble *P.*
602 *sp. 'pundamilia-like'* and the NP hybrids resemble *P. sp. 'nyererei-like'*. Shaded areas
603 indicate 95% CI.

604

Pundamilia sp. 'pundamilia-like'

Hybrids

Pundamilia sp. 'nyererei-like'

A **B**