

1 **Full title: Light induced changes in starry flounder (*Platichthys stellatus*) opsin expression**
2 **and its influence on vision estimated from a camouflage-based behavioural assay.**

3

4 **Short title: Rapid light induced changes in opsin expression.**

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15

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20

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22

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26 **Abstract**

27 Correlations between variation in opsin expression and variation in vision are often assumed but
28 rarely tested. We exposed starry flounder (*Platichthys stellatus*) to either broad spectrum sunlight
29 or green-filtered light in outdoor aquaria for seven weeks and then combined digital-PCR and
30 camouflage experiments to test two hypotheses: i) short-wavelength sensitive opsin expression
31 decreases in a green light environment, and ii) if observed, this change in opsin expression
32 influences colour vision as estimated using a camouflage-based behavioural assay. Of the eight
33 visual opsins measured, *Sws1* (UV sensitive) and *Sws2B* (blue sensitive) expression was
34 significantly lower in fish exposed to green light. However, opsin expression in fish transferred
35 to an arena illuminated with white LED light for three hours after the green light treatment did
36 not differ from broad spectrum controls. Changes in opsin expression in response to artificial
37 light environments have been reported before, but rapid changes over three hours rather than
38 days or weeks is unprecedented. We did not observe a significant difference in a flounder's
39 camouflage response based on light environment, although broad spectrum fish increased and
40 green-filter fish decreased the pattern contrast when on the blue-green substrate, and this
41 difference approached significance. This pattern is intriguing considering green-filter fish
42 expressed fewer UV and blue opsins and we recommend increased statistical power for future
43 experiments. Together, our results show that starry flounder opsin expression changes rapidly in
44 response to changes in light environment, however, there is no apparent effect on their visually
45 mediated camouflage.

46

47 **Introduction**

48 The ability to detect and discriminate among different wavelengths of light depends on the
49 diversity of opsins in the photoreceptors of the retina. Humans are considered to be trichromatic,
50 expressing short-wavelength sensitive (*OPNSW1*), middle-wavelength sensitive (*OPNIMW*), and
51 long-wavelength sensitive (*OPN1LW*) opsin genes in retinal cone cells, and rhodopsin (*RHO*) in
52 rod cells, which are used for scotopic (dim light) vision (1). Teleost fish typically have many
53 more visual opsins than other vertebrates (2,3), largely as a result of lineage-specific tandem
54 duplications (2,4). The advantage of large opsin repertoires, however, is not clear; humans can
55 discriminate between colours (wavelengths) that differ by less than one nm over much of the
56 visible spectrum (5).

57

58 It may be that a large visual opsin repertoire is a 'toolkit', with subsets of opsins used at different
59 stages of development, in different light environments, or indeed in particular regions of the
60 retina. Several authors have reported observations consistent with this hypothesis:
61 developmental variation (6), geographic distribution based on light environment (7), and regions
62 of the retina (8,9). Despite correlations between opsin repertoire or expression patterns and the
63 light environment, clear connections between variation in opsin expression and visual
64 performance have rarely been demonstrated outside of humans. Smith et al. (2012) manipulated
65 opsin expression in Lake Malawi Cichlids and showed that LWS expression variation account

66 for about 20% of the observed variation in optomotor response (10). In addition Sakai et al.
67 (2016) found that *Lws-3* expression increased in guppies grown in orange light and that fish with
68 higher levels of *Lws-3* expression had higher visual sensitivity to 600 nm light (11). Conversely,
69 Wright et al. (2020) found colour perception plays an important role in female cichlid mate
70 preference but opsin expression was only weakly correlated and a direct causal link between
71 expression and behaviour was lacking (12).

72
73 Adaptive camouflage in Pleuronectiformes was first described by Sumner (1911): turbot
74 (*Rhomboichthys podas* and *Lophopsetta maculata*), summer flounder (*Paralichthys dentatus*),
75 and winter flounder (*Pseudopleuronectes americanus*) changed patterns over a period of days in
76 response to various mottled sandy substrates and checkerboards (13). Juvenile plaice
77 (*Pleuronectes platessa*) change colour more quickly (14) and many bothids (e.g., left-eye
78 flounder) can camouflage to environment cues in seconds (15). Rapid changes in camouflage are
79 based on visual cues and can be used to infer visual performance. Flounder camouflage match
80 natural substrates well when modeled to mono-, di-, and tri-chromatic visual systems (16),
81 however, here we elected to use checkboards (as in Mäthger et al. 2006 (17)) to elicit an
82 exaggerated pattern response. We held starry flounder (*Platichthys stellatus*), a flatfish
83 possessing eight visual opsin genes (9), in outdoor aquaria exposed to either sunlight or green-
84 filtered light anticipating opsin expression would adjust to each environment as in Fuller &
85 Claricoates (2011) (18). We quantified the starry flounder's camouflage response to measure the
86 effect of opsin expression variation on vision.

87

88 Materials and Methods

89 *Fish Collection and Light Exposures*

90 Fish were collected by beach seine at low tide between 10:00 and 12:00 in May 2015 at Willows
91 Beach, Victoria, British Columbia, Canada. The seine net was deployed from a small aluminum
92 boat at a depth of approximately 3 m, or by hand at approximately 1 m depth. Sixteen starry
93 flounder were transported to the Outdoor Aquatics Unit at the University of Victoria, and held
94 under ambient light in 12°C recirculating seawater. These fish (TL = 172±28mm, m =
95 74.8±37.8g) were then distributed among eight experimental enclosures (**Fig 1**) on July 22, 2015.

96 Fish were exposed to either green-filtered or broad spectrum light for seven weeks. Light
97 transmission (%) for each filter (broad spectrum: Roscolux #3410; green: Roscolux #90) was
98 measured using Ocean Optics QE Pro at -10°C (sensor), integration time 100 µs, average of 3
99 for each spectrum, boxcar width (2), and electric dark current correction. Over the course of
100 seven weeks they were fed krill at 1% body weight per day, adjusted weekly at an estimated 2%
101 specific growth rate. Feeding occurred once daily through a 2-cm hole in the lid that was
102 otherwise sealed by a black rubber stopper to inhibit non-filtered light from entering the tanks.
103 All procedures were approved by the University of Victoria Animal Care Committee, which
104 abides by regulations set by the Canadian Council for Animal Care.

105

106 **Fig 1** Schematic of the study design. Upper panels, Light exposure conditioning (7 weeks): Four
107 tanks ($lwh = 49'' \times 19'' \times 25''$) were maintained outdoors on a 12°C closed, recirculating sea water
108 system. Each tank was partitioned with an opaque plastic sheet with perforations to allow sea
109 water to cycle through, but limit light transmission between treatments. Half of each tank was
110 wrapped in Roscosun 1/8 CTO cinematic gel filter (Roscolux #3410) and the other half in Dark
111 Yellow Green cinematic gel filter (Roscolux #90). Two fish were held in each enclosure, one
112 fish was immediately euthanized after 7 weeks exposure, and the other fish proceeded to the
113 camouflage assay for three hours prior to being euthanized. Light transmission (%) for each filter
114 (top right: Roscolux #3410, yellow line; Roscolux #90, green line) was measured using Ocean
115 Optics QE Pro. Bottom panels, Camouflage assay under white light (3 hours): one fish per
116 enclosure was randomly selected for the behavioural assay. The individual was placed
117 sequentially on five different substrates, illuminated with white LEDs, for 30 minutes per
118 substrate. RAW images were captured on a Nikon DSLR and camouflage analysis was run on
119 randomly selected images with methodologically selected regions of interest (ROI). Each ROI
120 was “filtered” through seven bandpass filters (bottom right panel) and the pixel energy at each
121 spatial scale is measured to quantify the camouflage pattern.

122

123 **Behavioural Assay**

124 The behavioural assay was conducted in a dark room with a sea-tray table containing 12°C
125 seawater on a recirculating system. The arena was comprised of a 30 cm diameter, 50 cm tall
126 plastic tube with four XLamp Neutral White 4000K LEDs (Cree, Inc.) mounted on top of the
127 tube in pairs at opposite ends. Neutral white foam core was used to reflect light into the arena to
128 reduce hotspots and shadows. Laminated checkerboard substrates were inserted vertically and
129 horizontally on the inside of the tube. The substrates were designed in Adobe Photoshop CS6.
130 The colour space used was CMYK US Web Coated SWOP v2. The printer was an Epson Stylus
131 Pro 9890 with Epson UltraChrome K3® Ink package, and substrates were printed on Epson
132 Premium Luster Photo Paper (206). A total of five substrates were printed, one uniform grey and
133 four checkerboards (i.e., blue-green, blue-red, red-green, and black-white). The pigments used
134 for the colourful checkerboards were selected with the saturation resulting in equal percent-
135 reflectance of total photons. Equal reflectance of photons resulted in reflected light intensity
136 being equal across the checkerboard, limiting the spectral signal from the checkerboards to hue
137 (or wavelength), and reducing the role of contrast (intensity) as an explanation for a camouflage
138 response. Percent-reflectance was calculated using an USB2000 Spectrophotometer
139 (OceanOptics Inc.) and the reflectance software in OOOIBase. Images were captured using a
140 Nikon D3100 Digital SLR camera and an AF Nikkor 50mm 1:1.4D lens. The camera settings
141 remained constant for the duration of the experiment (aperture = F5.6, shutter speed = 1/5", ISO
142 = 200, exposure compensation = +0.7). The camera was mounted on a tripod approximately 1.5
143 meters above the behavioural arena.

144

145 Beginning on September 9, 2015, two fish from a randomly selected enclosure were selected on
146 each of eight days. Diel rhythms in opsin expression have been previously reported in fish
147 (19,20), therefore, individuals were chosen by balanced design, alternating between broad
148 spectrum and green-filtered enclosure at the same time daily. One fish was selected (by coin
149 toss) for the behavioural trial and transferred to the experimental arena. Fish were acclimated to
150 the arena for 30 minutes before the five-substrate assay began. The other fish was euthanized and
151 whole retinas dissected to provide baseline opsin expression, unaffected by the bright white
152 lighting in the behavioural experiment. All behavioural trials began at 9:00AM to control for the
153 effects of circadian rhythms. Fish were exposed to five substrates in the following order at
154 intervals of 30 minutes (starting time in brackets): acclimatization period (9:00AM), grey
155 (9:30AM), blue-green (10:00AM), blue-red (10:30AM), red-green (11:00AM), and black-white
156 (11:30AM). Photos were captured at intervals of 30 seconds (60 photos per substrate, 300 photos
157 total per individual).

158

159 ***Image Analysis***

160 Images were captured in RAW file format. A total of six randomly selected images were
161 analysed per substrate per individual (6 images per substrate, 30 images per individual, 240
162 images total). Multispectral images were generated from RAW files and analyzed using the
163 Image Calibration and Analysis Toolbox (21). All images were calibrated to a standard (PTFE
164 sheet). Fish camouflage response was characterized from a cropped image. Cropping was
165 performed by a person who was not aware of the study design. Image cropping followed a
166 standard protocol, in short: a polygon representing the region of interest (ROI) was created
167 starting at the base of the anal fin near the caudal peduncle. Points of the polygon were selected
168 at the base of every third ray of the anal fin extending anterior the caudal fin. The pelvic fin,
169 operculum, head, and pectoral fin were excluded from the polygon. The polygon extended
170 posteriorly along the base of the dorsal fin (points at every third ray) back to the caudal peduncle
171 and the polygon was closed off completing the ROI.

172

173 Granularity analysis similar to that used to quantify cuttlefish camouflage (22) and avian egg
174 pattern (23) was used to get a single measurement for camouflage pattern; cropped images were
175 filtered using each of seven spatial frequency bands, or bandpass filters (i.e., 2, 4, 8, 16, 32, 64,
176 128 pixels). The pattern of individual fish was estimated using the standard deviation of
177 luminance, which measures the overall contrast within an image modelled to human vision.
178 Higher standard deviation of luminance equates to more light-and-dark contrasting patterns (i.e.,
179 disruptive or mottle camouflage), whereas low values equate to low pattern contrast (i.e.,
180 uniform camouflage).

181

182 Two-way repeated measures ANOVA was run in R version 3.2.4 using the “nlme” package and
183 Tukey multiple comparisons was run using the “multcomp” package. Analyses were based on
184 standard deviation of luminance from a total of eight fish, held for seven weeks in either broad

185 spectrum sunlight or green-filtered light, on five chromatically different substrates. The mixed
186 effects model tested was: camouflage ~ light environment + substrate + light environment ×
187 substrate + (1|individual) + ϵ .

188

189 ***RNA isolation and digital PCR***

190 Eyes were removed and a razor blade was used to cut the cornea exposing the lens and retina.
191 The lens was removed and the retina extracted. Retinas were frozen in liquid Nitrogen and stored
192 at -80°C. Retinas were then homogenized in TriZol (Invitrogen) with zirconia beads using a mini
193 beadbeater (BioSpec products) for 30 seconds. RNA was isolated following the TriZol
194 manufacturer's protocol, with slight modification. The RNA pellet was washed twice (rather than
195 once) with >75% ethanol. DNA, if present, was digested using RNase-free DNase I
196 (ThemoFisher Scientific, EN0521). Total RNA was quantified using Qubit® RNA Broad Range
197 Assay Kit (ThemoFisher Scientific, Q10210). 1 μ g of RNA from each sample was reverse-
198 transcribed in 40 μ l using iScript™ cDNA Synthesis Kit (BioRad).

199

200 Digital-PCR (dPCR) was run on QuantStudio® 3D Digital PCR System (Life Technologies)
201 using locus-specific primers and TaqMan probes for all eight visual opsins found in the starry
202 flounder transcriptome (Table 1). Opsins were multiplexed using FAM and VIC reporter dyes.
203 cDNA, primers, probes, and master mix were loaded onto a QuantStudio® 3D Digital PCR 20K
204 v2 Chip and sealed with immersion oil to prevent evaporation. After equilibrating at room
205 temperature for 15 minutes, PCR was performed on a ProFlex™ 2x Flat PCR System (step 1:
206 94°C × 30 sec; step 2: 55°C × 2 min, 94°C × 30 sec (39 cycles); step 3: 55°C × 2 min, 10°C
207 hold). Chips were read using the QuantStudio® 3D Digital PCR instrument. Sample
208 concentrations were adjusted to ensure that transcripts per microliter fall within the digital range
209 of the 3D system (i.e., 200 – 2000 copies \cdot μ l-1). cDNA template varied from 0.1 to 100 ng per
210 chip. Opin expression was normalized using the alpha subunit of transducin (*Gnat2*), the G-
211 protein activated by cone opsins (19). Patterns in expression were tested using a paired student's
212 t-test. All statistical tests were evaluated at $\alpha = 0.05$ level of significance.

213

214 **Table 1:** Primers and TaqMan probes used for starry flounder digital-PCR.

Gene	Oligo	Sequence (5' to 3')
<i>Lws</i>	Forward	AACTCCGTCACCCACTGAAC
	Reverse	TCTCCCAGGAGATGATGGAC
	Probe	FAM -TTCTGGACACCCGATGTGCA-QSY
<i>Sws1</i>	Forward	TGTTCTCAGTGAGCCAGGTG
	Reverse	GGCTCCGAATGGTTACAGA
	Probe	FAM -TGGAATCTGCCATGGGCTCGA-QSY
<i>Sws2B</i>	Forward	GCTCTTCACCTGCTTACTG
	Reverse	CTATGGCATGGCTGGATTG
	Probe	FAM -TACAGCGACTGTTGGTCCAATGGTCAG-QSY

Rh1	<i>Forward</i>	CTTGGCTGCAACCTAGAAGG
	<i>Reverse</i>	CCCTCAGGGATGTAACGAGA
	<i>Probe</i>	FAM-TTTCAGCCTCTGCTTGC-GC-QSY
Rh2A-1	<i>Forward</i>	CGTCCACTTCTTCCTTCCAG
	<i>Reverse</i>	AAGACCATCAGGACGCACAT
	<i>Probe</i>	VIC-GGTGCTGACAGTCAAAGCTGCTGC-QSY
Rh2A-2	<i>Forward</i>	ACGGCTCCTGTCTTCACAAT
	<i>Reverse</i>	AGCTACCAGGAAGCCAATGA
	<i>Probe</i>	VIC-CATTCTGACAGTCAAAGCCGCTGC-QSY
Sws2A-1	<i>Forward</i>	GTGACACTTGGTGGATGGT
	<i>Reverse</i>	CATCCGAACAGAGGTGGAGT
	<i>Probe</i>	VIC-GGCTTGTCACTGCAAGCCATTAGGT-QSY
Sws2A-2	<i>Forward</i>	GCATCAACACCCCTGACCATT
	<i>Reverse</i>	ACCATACTCCGAGTGTGC
	<i>Probe</i>	VIC-TGGTGAATTGGCTGTGGCGA-QSY
Gnat2	<i>Forward</i>	AGCCAGATTACCTCCCCACT
	<i>Reverse</i>	GGTCACACCCTCGAAACAGT
	<i>Probe</i>	VIC-TGTGCTGCGTTCCCGAGTCAA-QSY

215

216 **Results**

217 **Experimental animals**

218 Fish varied in size but this variation was distributed among treatment and control aquaria. Light
219 treatment did not influence growth over the seven-week exposure (broad spectrum: Δ TL =
220 7.5 ± 5.9 mm; green-filtered: Δ TL = 4 ± 7.2 mm) or mass (broad spectrum: Δ mass = 1.2 ± 9.3 g;
221 green-filtered: Δ mass = 0.9 ± 9.2 g). There was no statistical difference in length and mass of
222 baseline fish (i.e., those immediately euthanized after seven weeks of conditioning) and fish used
223 in the behavioural assay (i.e., fish exposed to bright white LED for 3 hours after conditioning)
224 (baseline: TL = 165.20 ± 24.20 mm and mass = 61.60 ± 29 g; time 3 hours: TL = 190.80 ± 29.42
225 mm and mass = 89.97 ± 36.20 g; TL: $t = -1.8925$, $p = 0.08006$ and mass: $t = -1.7296$, $p = 0.1067$).
226

227 **Image analysis**

228 Fish patterns changed in response to the substrate. The mixed effects model for the camouflage
229 indicated substrate (checkerboard) was significantly associated with the camouflage pattern ($F =$
230 4.552, $p = 0.0071$). When placed on a blue-green substrate, fish exposed to broad spectrum light
231 displayed greater pattern contrast than fish from the green light treatment, but the difference was
232 not significant ($F = 5.767$, $p = 0.0532$) and the interaction between substrate and light
233 environment did not significantly influence camouflage ($F = 1.209$, $p = 0.3327$). Tukey multiple
234 comparisons indicated that the pattern of fish from broad spectrum light on the black-white
235 substrate was significantly different than on: i) broad spectrum, blue-red substrate ($z = 3.135$, $p =$
236 0.0493), ii) green-filtered, grey ($z = 3.506$, $p = 0.015$), iii) green-filtered, blue-green ($z = 3.876$, p

237 < 0.01), and iv) green-filtered, blue-red ($z = 3.338$, $p = 0.0264$). Green-filtered, black-white was
238 significantly different than green-filtered, blue-green ($z = 3.3132$, $p = 0.0498$). Overall, contrast
239 (i.e., black-white substrate) results in the greatest pattern change in both treatments and the effect
240 of light environment approached significance, based on Tukey multiple comparisons the
241 difference was driven by differential camouflage response on the blue-green substrate ($z = -$
242 3.110 , $p = 0.0536$) (Fig 2).

243

244 **Fig 2** Camouflage pattern measured as the standard deviation of luminance across seven spatial
245 frequency bands (granularity analysis) of starry flounder (shapes represent individuals) on five
246 different substrates as depicted on the x-axis (left to right: grey, blue-green, blue-red, green-red,
247 and black-white). Fish were conditioned for seven weeks to either broad spectrum sunlight
248 (yellow bars) or green-filtered light (green bars).

249

250 **Digital-PCR**

251 Fish that were immediately euthanized after being removed from the 7 week light treatment (e.g.,
252 the ‘baseline fish’) had significantly different opsin expression; individuals held in green-filtered
253 light had lower expression of UV sensitive (*Sws1*) and short-wavelength sensitive (*Sws2B*)
254 opsins compared to those exposed to broad spectrum light (student’s t-test, $t = 3.9414$, $p =$
255 0.01121 and $t = 1.1458$, $p = 0.004792$, respectively) (Fig 3). Opsin gene expression levels were
256 the same in fish from the broad spectrum and green-filtered light exposure that were transferred
257 to the behavioural arena and exposed to white LED light for three hours (i.e., the duration of the
258 behavioural assay) (Fig 3).

259

260 **Fig 3** *Gnat2* normalized opsin expression of starry flounder held in either broad spectrum
261 sunlight (x-axis, C) or green-filtered sunlight (x-axis, G) for seven weeks. Fish ($n=4$) were
262 euthanized immediately after being removed from the light environments (the “7 Week
263 Exposure” panel, top) or 3 hours after being transferred to the behavioural arena (the “7 Weeks +
264 3 hours White LED” panel, bottom) illuminated with four white LED lights ($n=4$). Asterisk
265 denote significant differences between light exposure (*: $p = 0.01121$; **: $p = 0.004792$).

266

267 **Discussion**

268 ***Opsin expression plasticity in response to light environment***

269 Transcripts of eight distinct visual opsins are expressed in the eyes of juvenile starry flounder.
270 Microspectrophotometry data indicate that all are translated and that just one type of
271 chromophore is used (9). We predicted opsin expression would be modified by a seven-week
272 exposure to distinct light environments and that changes in opsin expression over that length of
273 time would influence vision. We used a camouflage-based assay to assess visual performance.
274 Opsin expression in the starry flounder retina did change in response to the light treatment, and
275 then changed again within three hours under white LED light.

276

277 Experiments designed to influence opsin expression have succeeded in the past, but the time
278 scale observed here is unprecedented. Killifish reared in clear or tea stained water were
279 monitored over four weeks and opsin expression differences were observed within 1-3 days (18).
280 Opsin expression from clear and tea stained water were concordant with natural killifish
281 populations and suggest opsin plasticity is used to tune vision to the light environment (18,24).
282 Here we show that *Sws1* (UV) and *Sws2B* (blue) expression was lower in the individuals that
283 spent 7 weeks in an environment lacking wavelengths below 450 nm compared to those in broad
284 spectrum light. The six other visual pigments have wavelengths of maximum absorbance within
285 the light available in the green-filtered tank, and were expressed at the same level in all fish
286 despite the overall light intensity being markedly different between the green and broad spectrum
287 tanks. The difference in *Sws1* and *Sws2B* expression, induced by the absence of short-
288 wavelength light, was lost after only three hours of exposure to white light in the camouflage
289 trials. Although the white LEDs do not emit UV light, they do emit near-UV and blue light,
290 which was enough to induce higher expression of *Sws1* and *Sws2B* opsins. Development can
291 play a role in UV opsin expression. In Salmonids UV opsin is one of the first opsins expressed in
292 the larval fish and is subsequently lost as they develop and transition into an active lifestyle
293 following smoltification (25). However, the differences observed here were not due to ontogeny,
294 as *Sws1* and *Sws2B* expression was not correlated with fish length or mass. Further, opsin
295 expression changes occur more rapidly in single cones than double cones (26), and both opsins
296 rapidly affected here are found in single cones.

297
298 The rapid plasticity of opsins on the order of hours, rather than days, has implications for the
299 visual ecology of starry flounder and the study of opsin expression in natural populations more
300 broadly. The changes could function as a way of tuning the retina to varying light conditions,
301 rapidly setting the machinery in place to restructure the retina if novel light conditions persist.
302 Increasing populations of photoreceptors sensitive to the light in the environment could improve
303 visual sensitivity and confer benefits for predator avoidance and prey capture. Future studies
304 should investigate whether plasticity is a persistent phenomenon throughout starry flounder
305 ontogeny, or if the retina is plastic at certain stages of development. Juvenile starry flounder are
306 found in shallow, nearshore waters and as adults descend to depths of more than 200 meters, but
307 occasionally migrate kilometers up river (27). These three environments (e.g., coastal shallows,
308 benthic depths, and river) are spectrally dramatically different, and a tunable retina even at later
309 ontogenetic stages could be adaptive. Given the logistical constraints of sampling wild
310 populations of fish, the rapid change in opsin expression has implications for studies moving
311 forward. One must consider both the time until preservation and the light conditions one is
312 sampling in. We recommend as standard practise to limit ambient light while collecting samples
313 in the field and to perform dissections under red light. Where ever possible, gear that limits
314 introducing novel ambient light should be used (e.g., a closing cod-end on a trawl net).

315

316 Varying light environments, driven by water depth or season, affect opsin expression in several
317 species of damselfish, whereas other species appear to have more stable expression patterns (28).
318 In stickleback, opsin expression is shifted toward longer wavelengths in freshwater populations
319 relative to marine populations, and these shifts correlate with differences in the light available
320 (29). Furthermore, there is evidence for local adaption to light among benthic and limnetic
321 ecotypes within a lake. These aforementioned differences in opsin expression were maintained in
322 laboratory rearing experiments under fluorescent light illumination, ergo stickleback opsin
323 expression is primarily under genetic control, a result of standing genetic variation (29). Why
324 some species appear to possess plastic opsin expression while others do not warrants further
325 investigation.

326
327 A change in opsin expression may not immediately reflect the opsin proteins present in the outer
328 segment of a cell. Photoreceptors are terminally differentiated; they are long lived, and the
329 cellular components must be regularly turned over to prevent a loss of function (30). Outer
330 segment membranes, the light sensitive region of a photoreceptor, are shed distally and the
331 addition of new membranes at the base renews the outer segment components. In mouse, rat, and
332 frog, radioactively labelled amino acids accumulated at the base of the outer segment within 24
333 hours. Furthermore, in rods the labelled amino acids proceeded as a “reaction band” to the distal
334 point of the outer segment in approximately ten days (31). Similar observations were observed in
335 rhesus monkey and cat cone cells (32). For the aforementioned reasons we made the duration of
336 the initial light treatment (i.e., seven weeks) sufficiently long to allow for protein-level changes
337 throughout the outer segments of the starry flounder retina. Additionally, we do not expect the
338 three hour period in which opsin expression returned to baseline to be enough time to make
339 functional changes at the protein-level. Protein-level changes are observed over longer periods of
340 time, such as ontogenetic shifts in opsin expression in coho salmon resulted in protein-level
341 shifts as measured by microspectrophotometry (34). Recently, we observed parallel opsin
342 switches within the outer segments of double and single cones of starry flounder. The proportion
343 of outer segments containing co-expressed opsins were greater in juvenile fish, with shorter
344 wavelength-sensitive opsins expressed at the distal tip of the outer segment (33). Given the delay
345 from expression of opsin mRNA to translated and localized visual pigments, the starry flounder
346 used in the behavioural assay are better represented by the baseline opsin expression data than
347 the data collected from their retinas after three hours under a white LED light. If the *Sws1* and
348 *Sws2B* expression patterns were consistent with the baseline over seven weeks, then we predict
349 that the opsin protein populations in the retina would reflect those changes. However, it would be
350 valuable to complement future behavioural assays with immunohistochemistry, or a survey of
351 the retina using microspectrophotometry to confirm our prediction.

352
353 ***Empirical evidence for active camouflage in starry flounder***
354 The behavioural assay presented here contributes to a large body of empirical evidence that
355 flatfish can change their skin pattern in response to substrate changes. The fish tested showed

356 noticeable changes within 10 seconds, and the body pattern was stable within minutes, indicating
357 direct neural input. Camouflage in fish is the aggregate response of millions of chromatophores
358 in the skin. Unlike cephalopod molluscs (35,36), fish camouflage involves the physical
359 movement of pigment, rather than muscular contraction and expansion of the cell itself (37).
360 Therefore, it is not surprising that starry flounder camouflage is relatively slow compared to the
361 remarkably fast change observed in cephalopods and other more specialized flatfish, which in
362 some cases can occur in as little as two seconds (15,38).

363

364 Light environment may have affected visual performance of starry flounder camouflaging on the
365 blue-green checkerboard. Broad spectrum fish increased pattern contrast and green-filter fish
366 decreased pattern contrast and the difference approached significance (**Fig 2**). Higher standard
367 deviation of luminance equates to more light-and-dark contrasting patterns (i.e., disruptive or
368 mottle camouflage), whereas low values equate to low pattern contrast (i.e., uniform
369 camouflage). A fish camouflaging with greater contrast would indicate that the fish can see a
370 difference between the blue and green checkers. The broad spectrum fish deployed more mottle
371 camouflage compared to green-filtered fish, and may therefore detect a greater difference
372 between the blue and green checkers. Differential visual performance on the blue and green
373 substrate is supported by gene expression, with broad spectrum fish expressing more UV- and
374 blue-sensitive opsins, possibly conferring greater visual sensitivity to short-wavelength light.
375 These data suggest a positive correlation with the *Sws1* and *Sws2B* opsin expression and an
376 ability to detect a difference between blue and green hues, however the behavioural experiment
377 did not have power to detect a significant difference in camouflage. Power analysis indicates if
378 light environment truly impacts performance, a sample size of 20 fish would be required to
379 detect it reliably.

380

381 An alternative explanation is that the *Sws1* and *Sws2B* opsins are not main drivers of the
382 camouflage response. As with double cones that contribute chiefly to luminance vision, motion
383 detection (39), and polarization vision (40), specialization in either double or single cones may
384 exist that contribute to camouflage not affected by variation in UV and blue opsin expression.
385 Future studies using different filters attempting to change opsin expression in different
386 photoreceptors (e.g, green and red cones) would be informative. Previous research on starry
387 flounder found a preponderance of unequal double cones in the dorsal retina, which receives
388 reflected light from the substrate (9), and it may be functionally important for the camouflage
389 response. In cichlids, the pattern of expression in double cones was reversed dorso-ventrally in
390 response to red light illumination from below (41). If similar bottom-up illumination of starry
391 flounder “flips” the double cone opsin expression dorso-ventrally, we could test whether the
392 unequal double cones play an important role in camouflage.

393

394 Camouflage may be visually mediated through achromatic channels (e.g., cuttlefish have only
395 one visual pigment). The most pronounced pattern changes were observed on the black and

396 white checkerboard. That is not to say colour vision is unimportant to camouflage behaviour.
397 Gulf flounder (*Paralichthys albiguttata*) and ocellated flounder (*Acanthopsetta ommata*) of family
398 Paralichthyidae preferred to settle on blue and green substrates after being adapted to the same
399 colour (42). Mäthger et al. (2006) created a series of checkerboards that were white and green,
400 with the white checker position getting progressively darker until the final substrate was black
401 and green (17). At some point the green checker matched the luminance of the grey checker, and
402 cuttlefish deployed a uniform camouflage pattern when placed on top. Thus, cuttlefish
403 camouflage is achromatic. A similar experimental design, or perhaps substrates designed based
404 off of Ishihara plates, would be useful to confirm whether starry flounder camouflage is driven
405 by luminance, colour, or both.

406

407 The experiment did not control for the difference in overall light intensity between the two
408 environments. The green-filtered environment allowed approximately 12% sunlight through
409 compared to 88% in the broad spectrum environment. Although it is possible the differences in
410 opsin expression could be due to light intensity, the evidence contrary to that point is two-fold.
411 One, the opsins that were expressed significantly lower correspond to the wavelengths of light
412 omitted by the filters. Two, the other opsins were not noticeably affected by the significantly
413 lower light intensities in the green-filtered tank. Our rational behind selecting the green filter was
414 that it approximated the light environments starry flounder encounter at depths in the turbid,
415 coastal waters around Vancouver Island, Canada. With that said, follow-up studies that match
416 light intensity, but vary colour, would enhance the present study.

417

418 **Concluding remarks**

419 We found significantly greater UV and blue opsin expression after seven weeks in starry
420 flounder held under broad spectrum light compared to green-filtered light. Surprisingly, that
421 difference was lost after three hours under white LED light, indicating more rapid plasticity in
422 opsin expression than previously reported. The timescale of change has relevance to both the
423 visual ecology of fishes and the logistics and possible bias of studying opsin expression in
424 natural systems. By using starry flounder's visually-mediated adaptive camouflage, we were able
425 to quantify visual performance on a variety of substrates, though we did not find statistically
426 significant differences among fish from different light environments and recommend greater
427 statistical power for future behavioural experiments.

428

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435

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437

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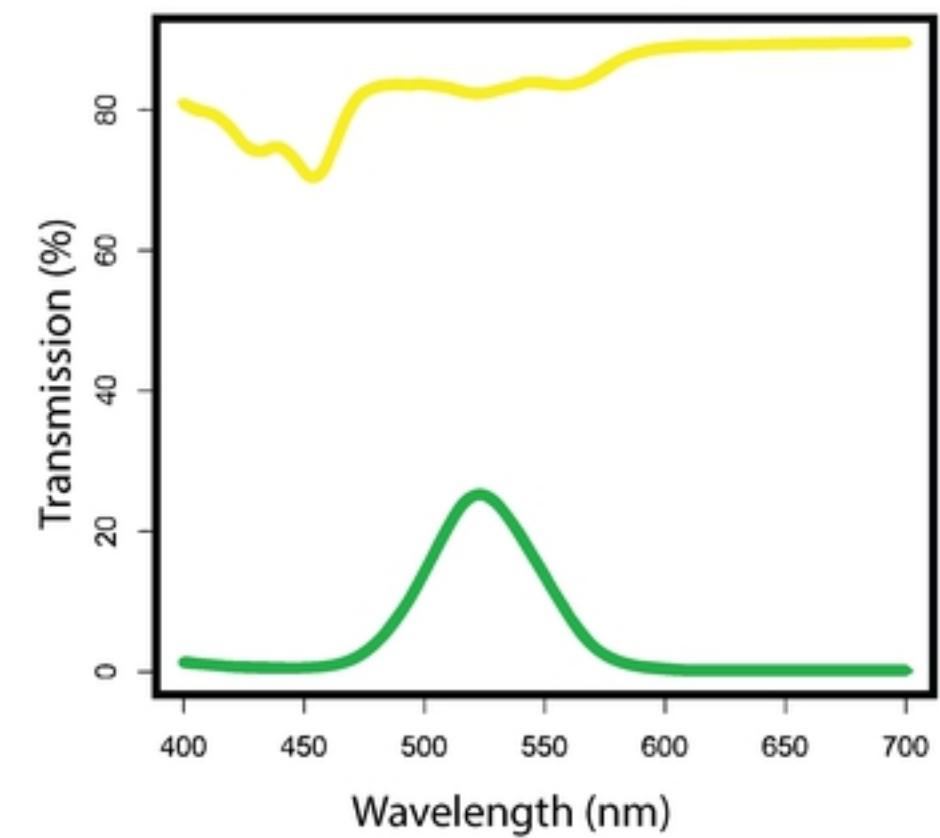
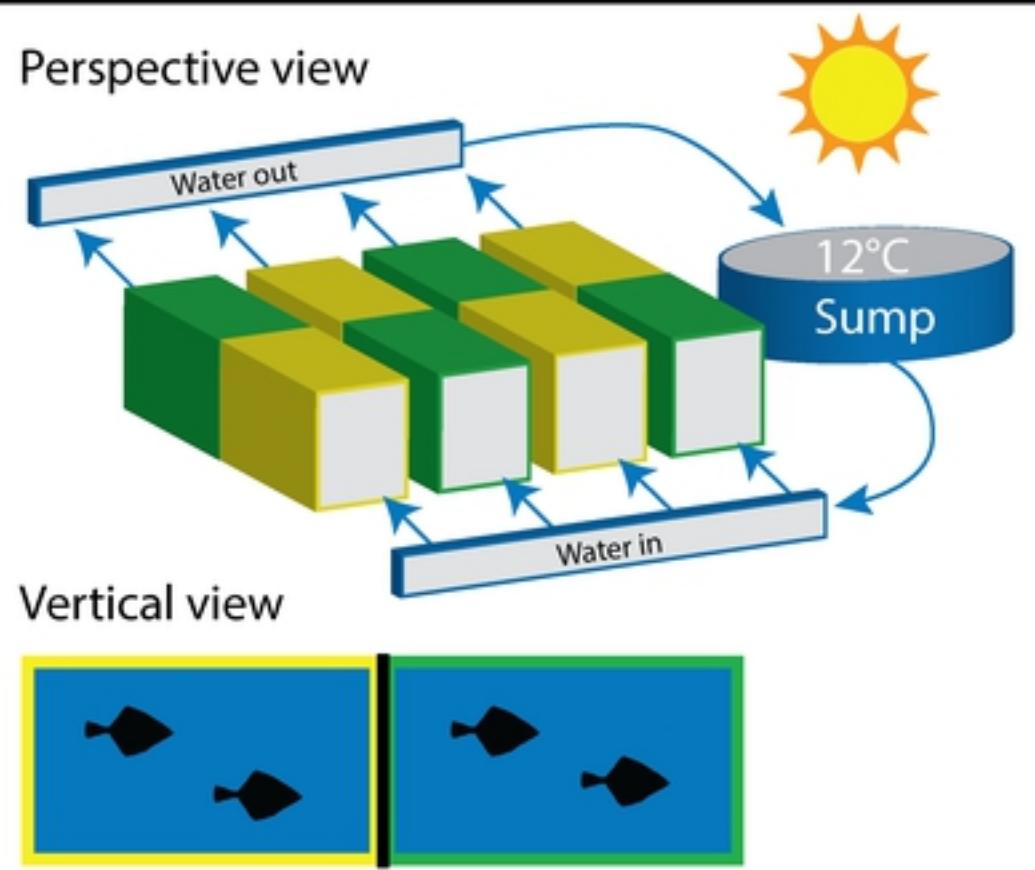
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552

Light exposure conditioning (7 weeks)



Camouflage assay under white light (3 hours)

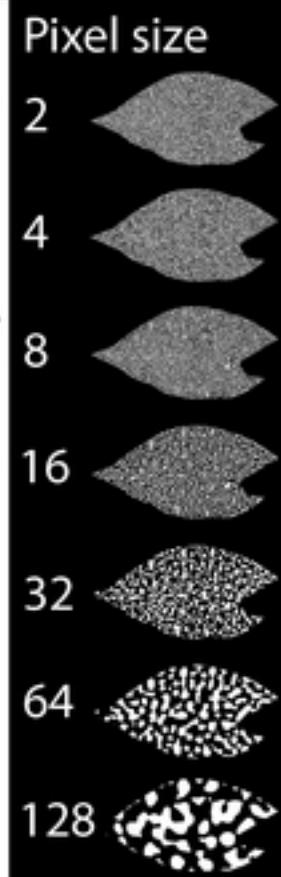
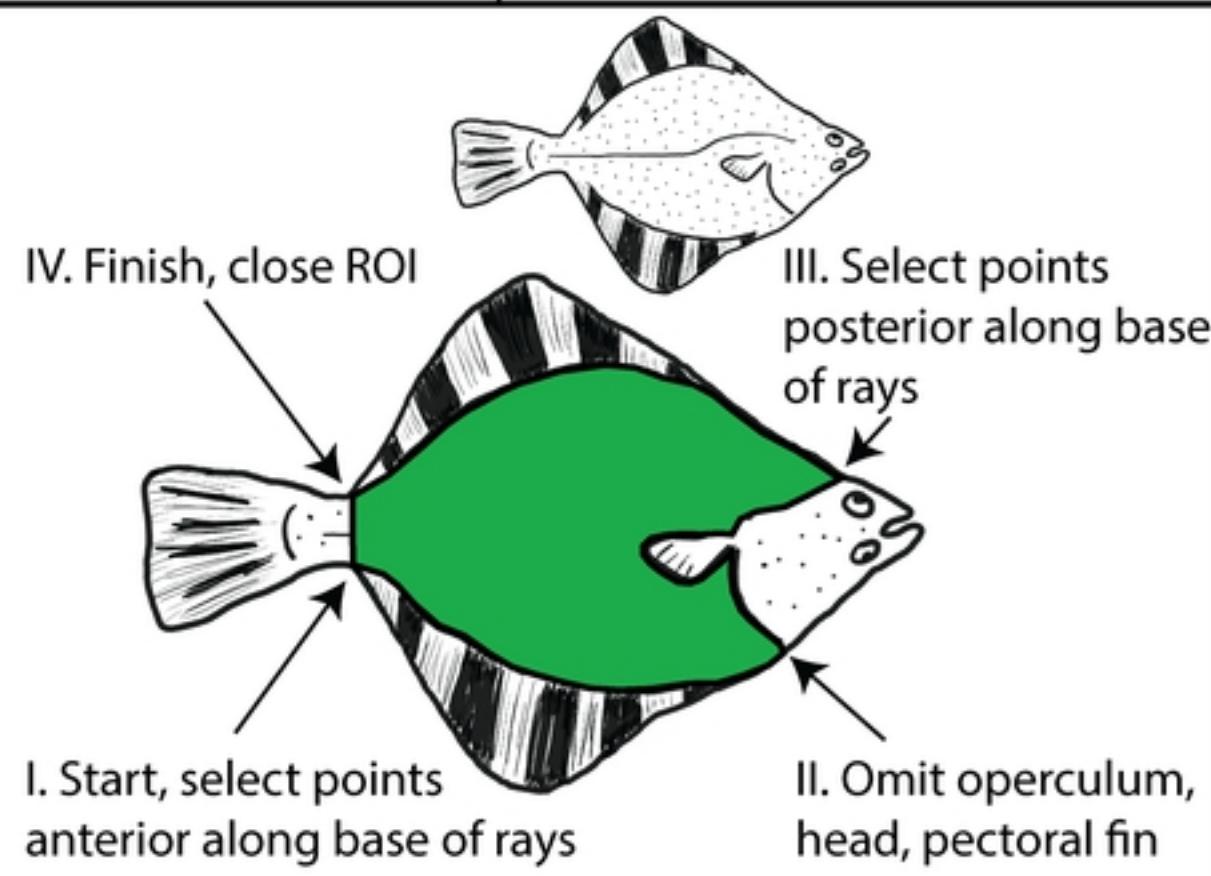
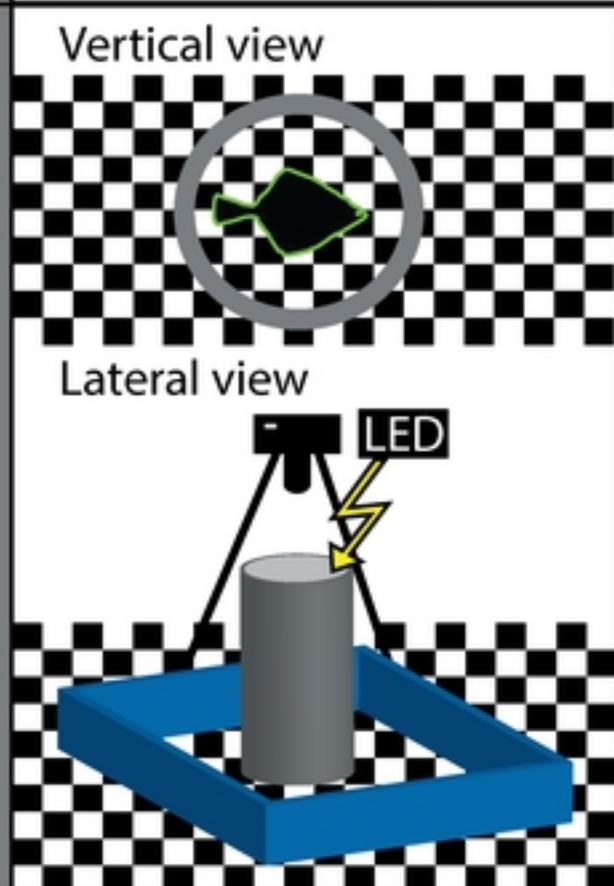


Figure 1

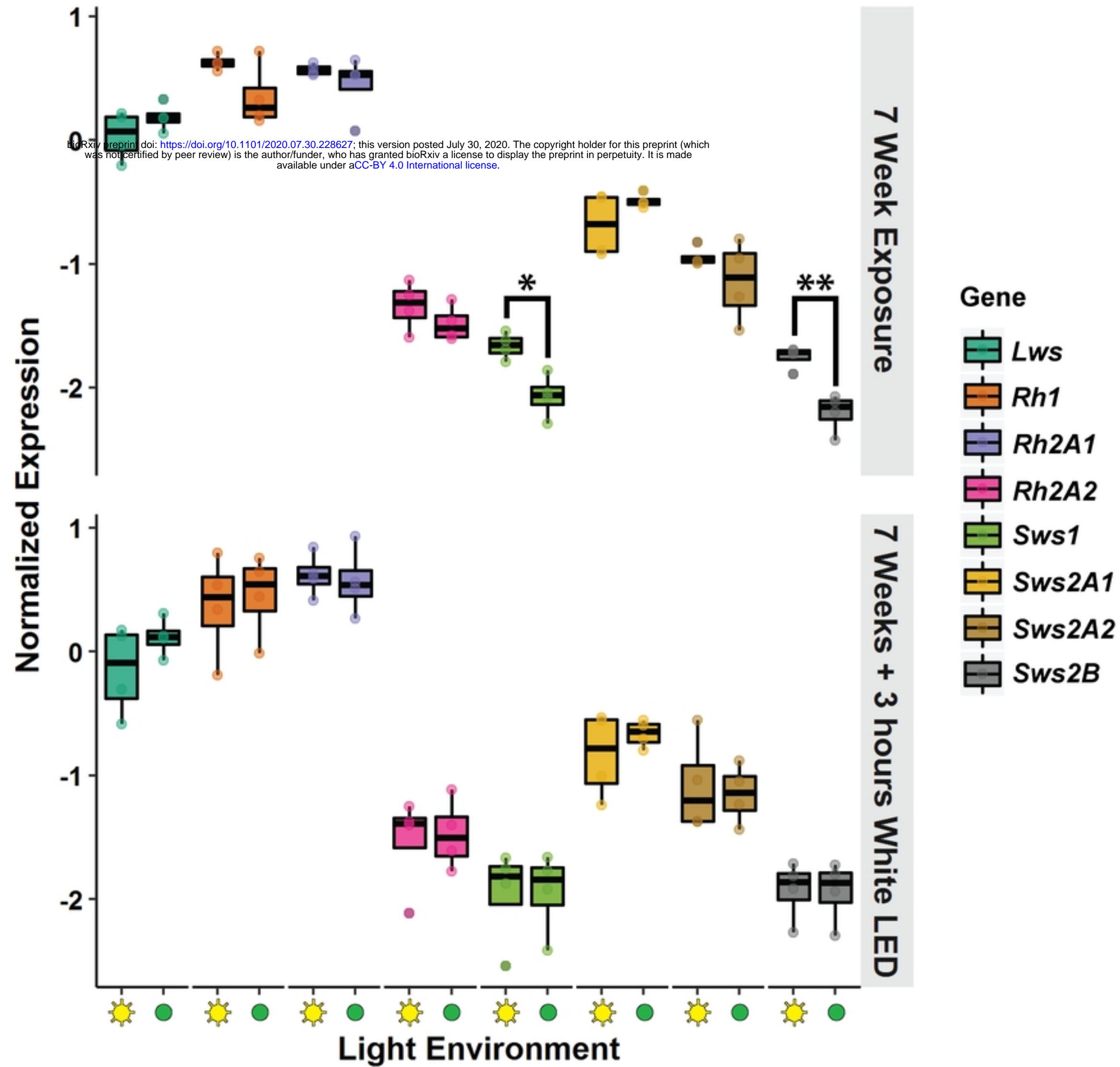


Figure 3

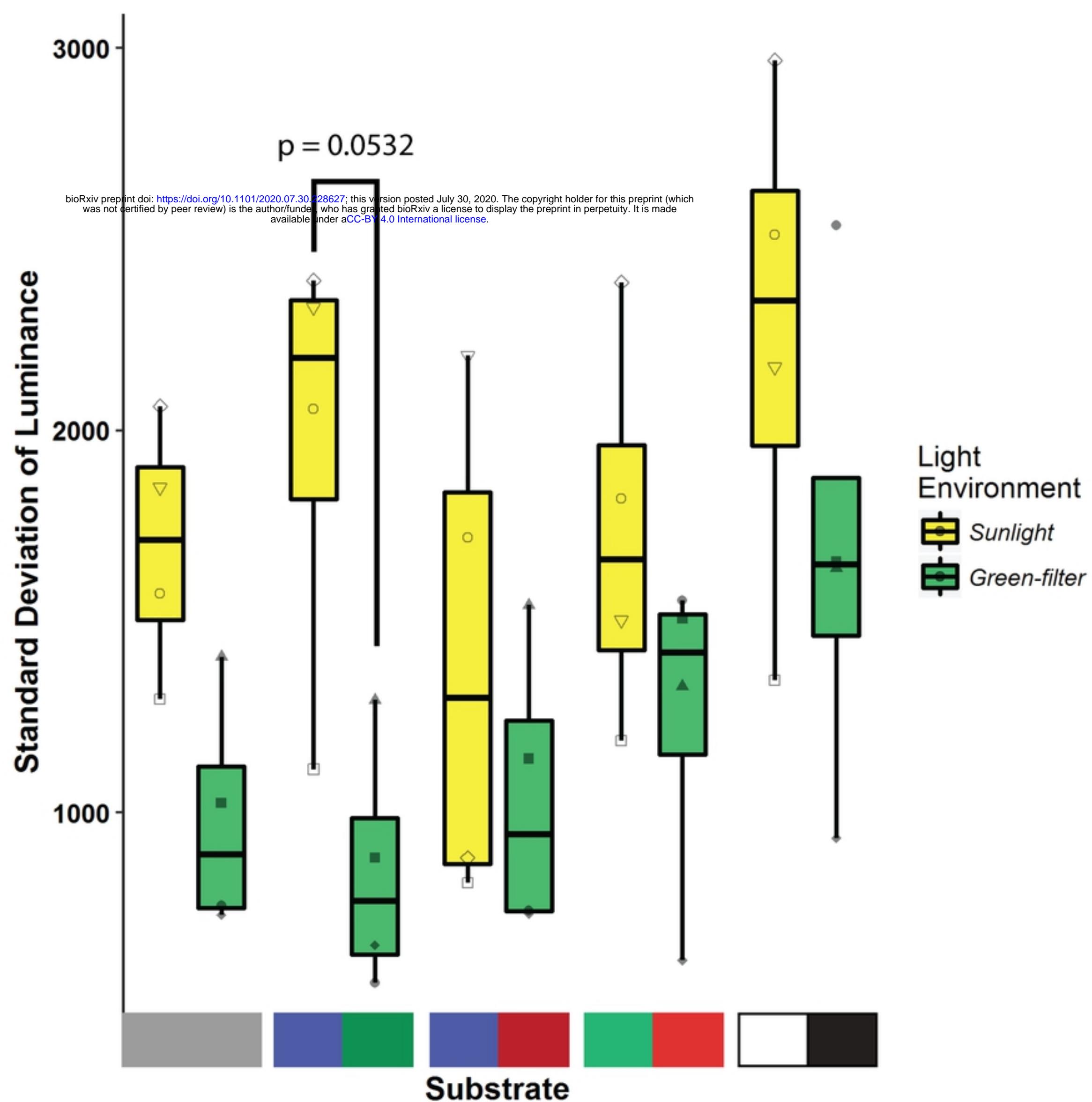


Figure 2