

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.**

5

6 Tom Iwanicki<sup>1,#a\*</sup>, Cliff Haman<sup>2</sup>, Amy Liu<sup>1,#b</sup>, and John S. Taylor<sup>1\*</sup>

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8 <sup>1</sup> Department of Biology, University of Victoria, Victoria, British Columbia, Canada

9 <sup>2</sup> Department of Fine Arts, University of Victoria, Victoria, British Columbia, Canada

10 <sup>#a</sup> Current address: School of Life Sciences, Department of Biology, University of Hawai'i at  
11 Mānoa, Honolulu, Hawai'i, USA

12 <sup>#b</sup> Current address: Department of Zoology, University of British Columbia, Vancouver, British  
13 Columbia, Canada

14 \*Authors for correspondence ([iwanicki.t@gmail.com](mailto:iwanicki.t@gmail.com) and [taylorjs@uvic.ca](mailto:taylorjs@uvic.ca))

15

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17 original draft preparation, CH contributed to methodology and resources, AL contributed to  
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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 (*OPNSWI*), middle-wavelength sensitive (*OPNIMW*), and  
51 long-wavelength sensitive (*OPNILW*) 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 (*Rhomboidichthys 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 checkerboards (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 (ThermoFisher Scientific, EN0521). Total RNA was quantified using Qubit® RNA Broad Range  
 197 Assay Kit (ThermoFisher Scientific, Q10210). 1 µg of RNA from each sample was reverse-  
 198 transcribed in 40 µl 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•µl<sup>-1</sup>). cDNA template varied from 0.1 to 100 ng per  
 210 chip. Opsin 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 α = 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')
<b><i>Lws</i></b>	<i>Forward</i>	AACTCCGTCACCCACTGAAC
	<i>Reverse</i>	TCTCCAGGAGATGATGGAC
	<i>Probe</i>	<b>FAM-TTCTGGGACACCCGATGTGCA-QSY</b>
<b><i>Sws1</i></b>	<i>Forward</i>	TGTTCTCAGTGAGCCAGGTG
	<i>Reverse</i>	GGCTCCGAATGGTTTACAGA
	<i>Probe</i>	<b>FAM-TGGAATCTGCCATGGGCTCGA-QSY</b>
<b><i>Sws2B</i></b>	<i>Forward</i>	GCTCTTTCACCTGCTTCTACTG
	<i>Reverse</i>	CTATGGCATGGCTGGATTTG
	<i>Probe</i>	<b>FAM-TACAGCGACTGTTGGTGGGAATGGTCAG-QSY</b>

<b>Rh1</b>	<i>Forward</i>	CTTGGCTGCAACCTAGAAGG
	<i>Reverse</i>	CCCTCAGGGATGTAACGAGA
	<i>Probe</i>	<b>FAM</b> -TTTGCAGCCTCTGCTTGCGC-QSY
<b>Rh2A-1</b>	<i>Forward</i>	CGTCCACTTCTTCCTTCCAG
	<i>Reverse</i>	AAGACCATCAGGACGCACAT
	<i>Probe</i>	<b>VIC</b> -GGTGCTGACAGTCAAAGCTGCTGC-QSY
<b>Rh2A-2</b>	<i>Forward</i>	ACGGCTCCTGTCTTACAAT
	<i>Reverse</i>	AGCTACCAGGAAGCCAATGA
	<i>Probe</i>	<b>VIC</b> -CATTCTGACAGTCAAAGCCGCTGC-QSY
<b>Sws2A-1</b>	<i>Forward</i>	GTGACACTTGGTGGGATGGT
	<i>Reverse</i>	CATCCGAACAGAGGTGGAGT
	<i>Probe</i>	<b>VIC</b> -GGCTTGTCATCTGCAAGCCATTAGGT-QSY
<b>Sws2A-2</b>	<i>Forward</i>	GCATCAACACCCTGACCATT
	<i>Reverse</i>	ACCATACCTCCGAGTGTTGC
	<i>Probe</i>	<b>VIC</b> -TGGTGAATTTGGCTGTGGCGA-QSY
<b>Gnat2</b>	<i>Forward</i>	AGCCAGATTACCTCCCCACT
	<i>Reverse</i>	GGTCACACCCTCGAAACAGT
	<i>Probe</i>	<b>VIC</b> -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:  $\Delta TL =$   
 220  $7.5 \pm 5.9$  mm; green-filtered:  $\Delta TL = 4 \pm 7.2$  mm) or mass (broad spectrum:  $\Delta mass = 1.2 \pm 9.3$  g;  
 221 green-filtered:  $\Delta mass = 0.9 \pm 9.2$  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:  $TL = 165.20 \pm 24.20$  mm and  $mass = 61.60 \pm 29$  g; time 3 hours:  $TL = 190.80 \pm 29.42$   
 225 mm and  $mass = 89.97 \pm 36.20$  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 (*Ancylopsetta 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 rationale 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

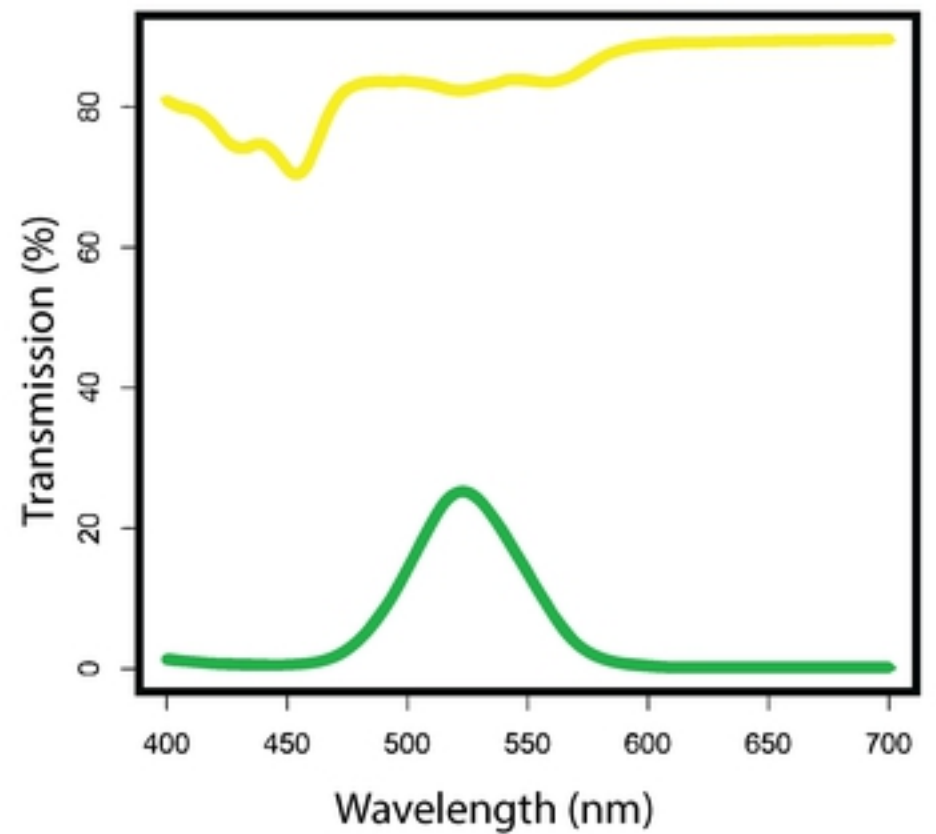
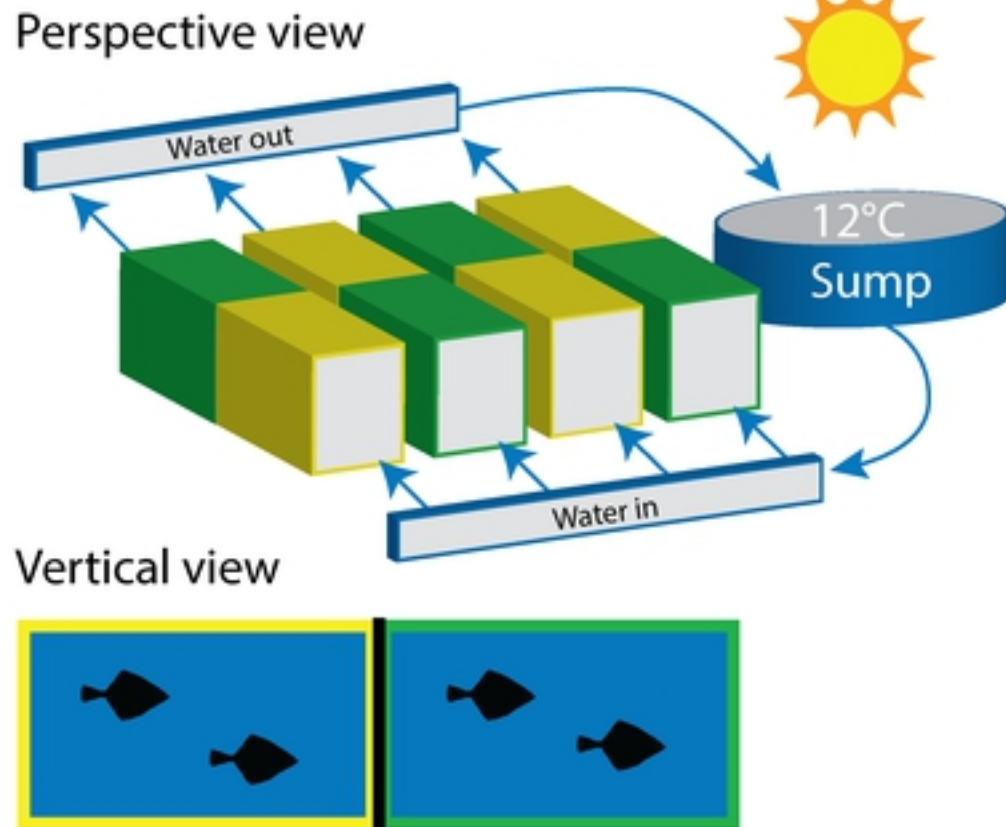
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552

Light exposure conditioning (7 weeks)



Camouflage assay under white light (3 hours)

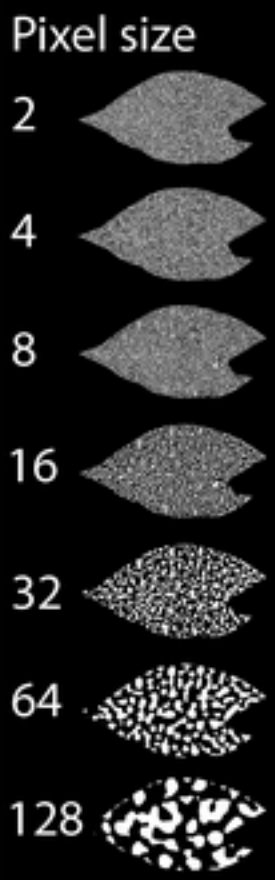
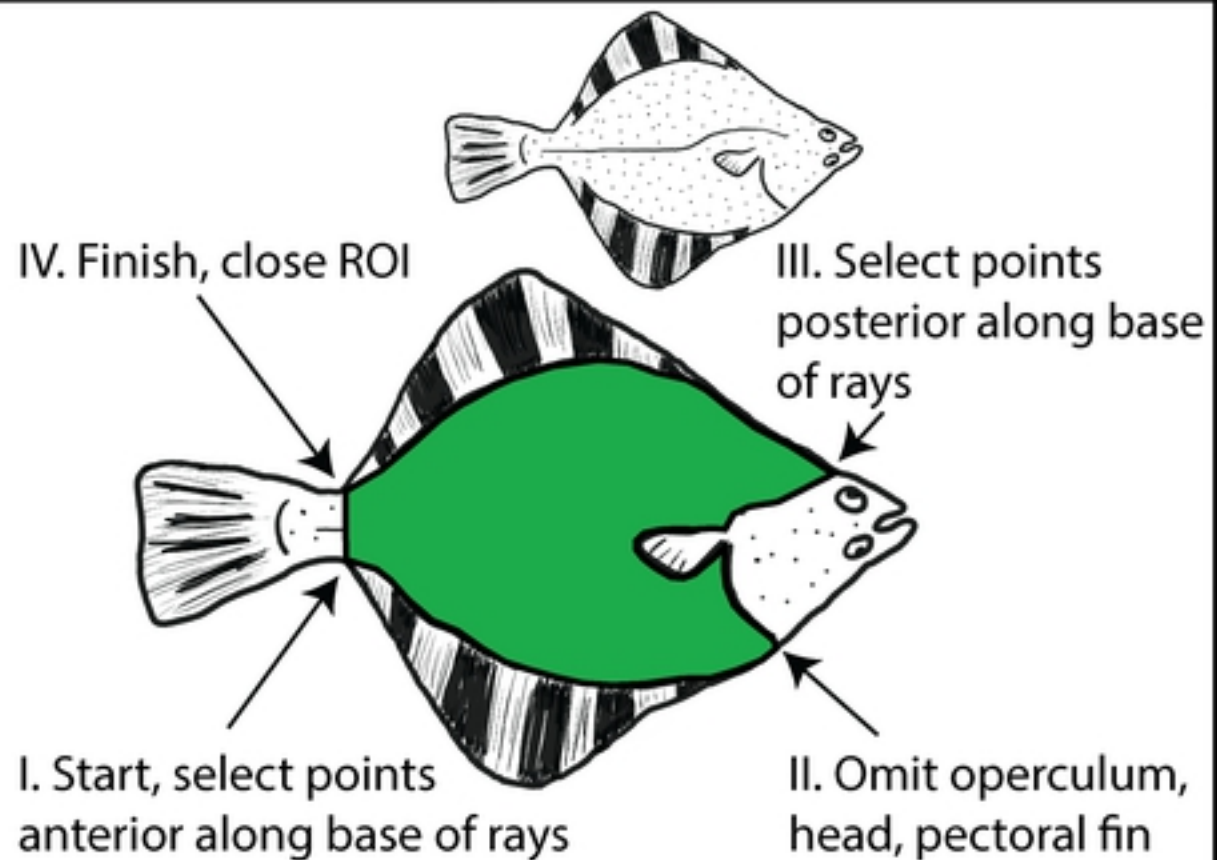
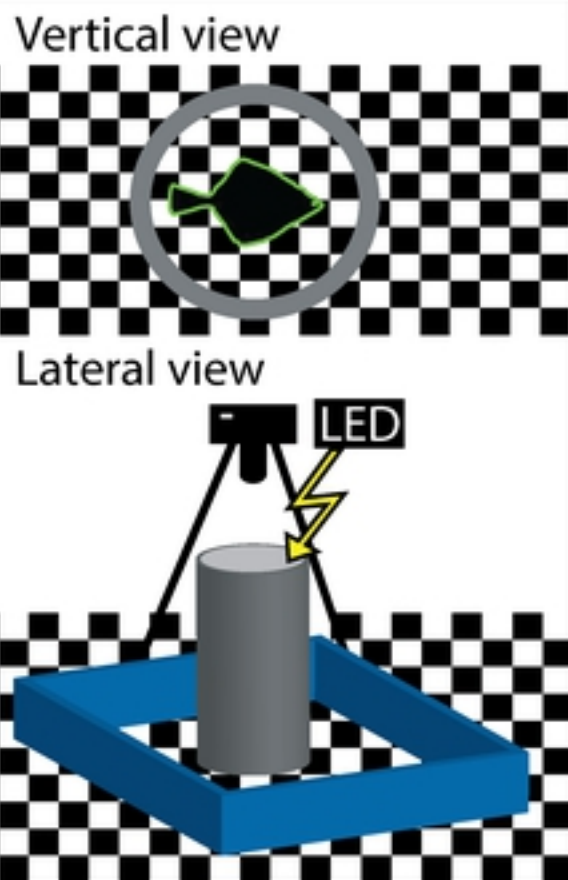


Figure 1



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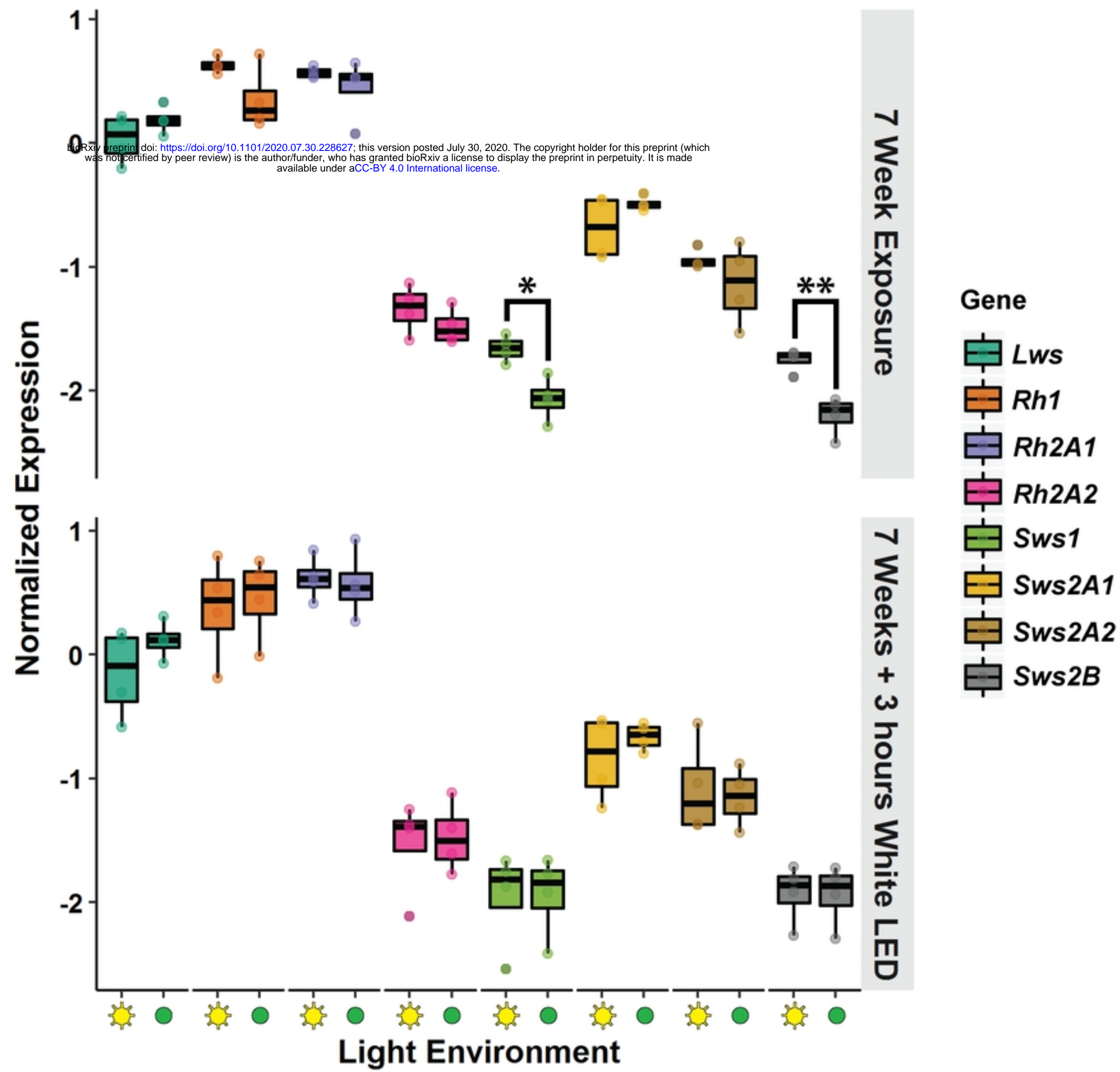


Figure 3

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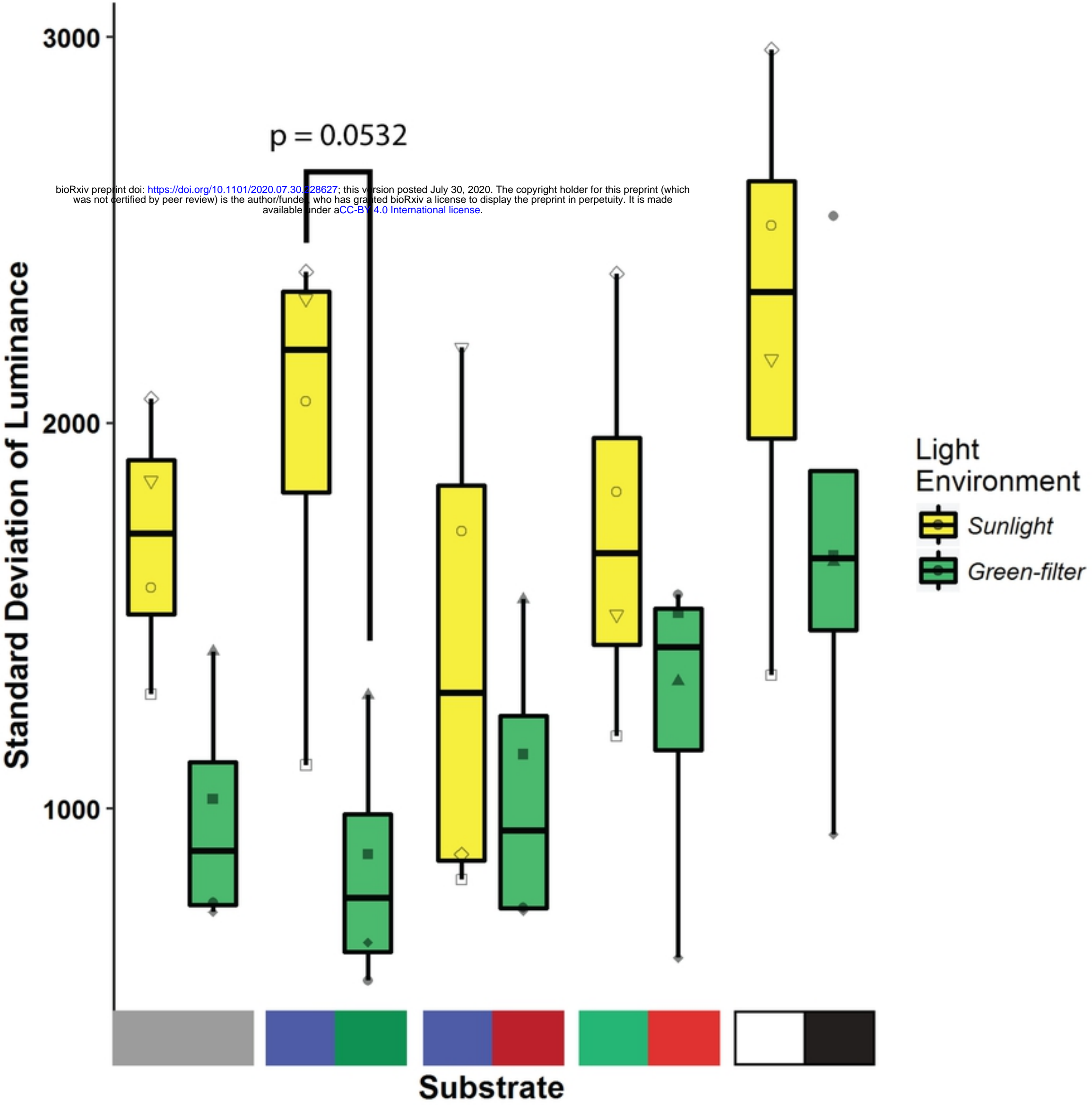


Figure 2