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Changes in Red Color Sensitivity over the Spawning Cycle of Female Three-spined Stickleback (*Gasterosteus aculeatus*)

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Male red nuptial coloration is a primary mating signal for three-spined sticklebacks (*Gasterosteus aculeatus*), and the retinae of both sexes are especially sensitive to red during the breeding season. Red sensitivity is an important aspect of female mate choice in this species, but only when they are ready to spawn and not over the entire breeding period. Here, we aimed to determine if the red sensitivity of female sticklebacks change over their repeat spawning cycle. To this end, we assessed retinal opsin mRNA levels and behavioral red sensitivity in females over this cycle. Both methods indicated that females were more sensitive to red during spawning than in the inter-spawning intervals. Relative expression levels of red color opsin genes (*lws*) and optical motor sensitivity were high during spawning, decreased after the spawning period, and then increased again 72–96 h later when they were ready to spawn again. Thus, female sticklebacks altered their color sensitivity according to need, but the underlying mechanism remains unclear.

Key words: Red color sensitivity, Optical motor, Projector, Opsin, *lws*, Androgen, Stickleback.

BACKGROUND

In his theory of sexual selection, Darwin (1871) postulated that most dimorphic sexual characteristics are selected based on mating success rather than survival. Those dimorphic characteristics are important for attracting potential mates and deterring rivals, which together increase reproductive success (Tooke and Camire 1991). Sexual selection often depends on the strength of sexual signals, as well as sensitivity in receiving them (Andersson and Lwasa 1996). Sexual selection theory predicts a tight coupling between the evolution of sexual displays and the sensory capabilities of the receiver (Fisher 1915; Kirkpatrick 1982).

Sexual signals not only depend on sexual maturity, but often also season (Legett et al. 2019). Such seasonal rhythms are reflected in shifts in sensory sensitivity or capability (Sullivan-Beckers and Cocroft 2010). For instance, changes in pheromone detectability and olfactory sensitivity have been observed over the reproductive cycles of mammals, birds, and teleosts (Alekseyenko et al. 2006; Clark and Smeraski 1990; Moore and Scott 1991).

During the breeding season, mature male *G. aculeatus* display breeding coloration with blue eyes and a red belly. Many studies have demonstrated that the red coloration of males is an important signal for sexual selection (Semler 1971; Bakker and Milinski 1993; Künzler and Bakker 2001). Moreover, both male and female sticklebacks display higher visual sensitivity

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for red light under mating conditions than under non-breeding conditions (Boulcott and Braithwaite 2007; Shao et al. 2014).

Sexual signaling, receiver sensitivity, and endocrine systems may all play roles in how synchronized maturation is intrinsically regulated (Cowan et al. 2017). During breeding, male sticklebacks display elevated androgen levels, which induce the appearance of mating coloration (Borg 1994; Hellqvist et al. 2006) and increase male sensitivity to red coloration (Shao et al. 2014). Similarly, levels of both estradiol and the androgen testosterone are higher in female sticklebacks during the breeding period than at other times of the year (Hellqvist et al. 2006), and they also change over the spawning cycle in this multi-spawning species (*i.e.*, multiple spawning events within a given spawning season) (Roufidou et al. 2018). Although it is not known if sex steroids regulate female stickleback color vision, as androgens do in males, it has been demonstrated that waterborne exogenous estrogen can increase the expression of opsin genes (*rh1*, *rh2* and *sws2a*) in female western mosquitofish (*Gambusia affinis*) (Friesen et al. 2017).

The increased sensitivity to red in both sexes during the breeding season can be explained evolutionarily by the need to detect and evaluate sexual partners or potential rivals. However, for females, doing so is only important when they are ready to spawn and not during the intervals between spawning events. In the present study, we aimed to investigate possible changes in red color sensitivity over the female spawning cycle. We determined relative mRNA levels of the opsin genes *lws* (red), *rh2* (green), *sws2* (blue) and *sws1* (UV) in retinae of females over the spawning cycle. Furthermore, we assessed behavioral red sensitivity in females throughout the spawning cycle using an optomotor apparatus.

MATERIALS AND METHODS

This study comprised two sets of experiments. In the first, opsin mRNA levels in fish were measured over the spawning cycle. This experiment was approved by the Stockholm Northern Animal Experiment Ethical Board, Sweden (No. 44/14). In the second, behavioral red light sensitivity was measured by means of an optical motor experiment. This experiment was approved by the Institutional Animal Care and Use Committee of National Taiwan Ocean University, Taiwan (No. 107035).

Experimental animals

Methodological details of the first experiment have been described previously in the study by Roufidou et al. (2018), in which plasma steroid hormones were measured. In brief, adult non-breeding three-spined stickleback females were caught at Skåre, southern Sweden, in December 2014. The fish were maintained in 20°C brackish water (0.5% salinity) at Stockholm University. Exposure to a long-day photoperiod (16h:8h light:dark) was used to induce sexual maturation. Nesting males were used to test if females were ready for spawning, as determined by mating behavior, and also to allow females to spawn naturally. Females were sampled 0 (n = 13), 6 (n = 16), 24 (n = 13), 48 (n = 13) and 72 h (n = 13) after spawning. All but one of the females sampled after 72 h had ovulated again. After anesthesia (0.025% buffered MS-222) and plasma sampling, retinae were removed from the eyes and immersed in 500 µl RNAlater® (Ambion) on ice for 2 h before being stored at -80°C until further analysis (Shao et al. 2014). All fish were dissected during daylight hours.

Fish of the F2 strain were used for the second experiment conducted in a laboratory at National Taiwan Ocean University. This strain was sourced from Skanör, southern Sweden, and originated from the same general population as the fish employed in the first experiment. Before beginning this behavioral experiment, the fish were housed (density ≤ 0.4 fish/L) in 50 or 100 L aquaria containing clear, aerated and filtered freshwater. The aquaria were illuminated by white LED light, with a light intensity at the water surface of ~200 lux. The bottom of each aquarium was covered with sand, and plastic tubes were provided as refuges. All fish were mainly fed *ad lib* on frozen bloodworms and *Artemia*, and copepods (*Cyclops* sp.) were occassionally given as a nutritional supplement. Prior to experimentation, fish were kept at 20°C under a non-stimulatory short-day photoperiod (8h:16h light:dark), which was switched to a long-day photoperiod (16h:8h) to induce maturation. Mature females with a swollen belly were transferred to another 50 L aquarium harboring a nesting male for spawning. The female was then subjected to optomotor response testing at 0, 24, 48, 72, and 96 h after spawning. A swollen belly was observed in females 72 or 96 h after spawning, so 24 and 48 h after spawning were considered the inter-spawning phase, with the remaining time-points representing the spawning phase.

RNA extraction, reverse transcription and q-PCR

Total RNA was extracted from retinal tissue using RNeasy® Plus Universal Tissue Mini Kit (cat. 73404, Qiagen, Hilden, North Rhine-Westphalia, Germany) according to the manufacturer's protocols. Except for the time point Retinal samples of two individuals from the same time-point were pooled randomly to obtain total RNA of sufficient quantity and quality, as determined using a NanoDrop 1000 system (Thermo Scientific, Waltham, MA, USA). In the study, six mRNA samples (from 12 randomly taken females) were used at each time point. For all samples, 4 µg of total RNA was reverse-transcribed with a High-Capacity cDNA Reverse Transcription Kit (cat. 4368814, Applied Biosystems, Foster City, CA, USA) and RNAse Inhibitor (Y9240L, Enzymatics, Beverly, MA, USA).

Specific primers designed for *q*-PCR have been described previously (Shao et al. 2014) (Table 1). Before quantifications, the melting curve was tested by 10-fold serial dilutions of the templates (1/1 to 1/1000), with three replicates for each gene. R-squared values for all standard curves were > 0.995 for all experimental replicates. Expression of target gene mRNA was determined by *q*-PCR using a Roche LightCycler® 480 System (Roche Applied Science, Mannheim, Baden-Württemberg, Germany). PCR reactions contained 40 ng cDNA, 50 nM of each specific primer (Table 1), and the LightCycler® 480 SYBR Green I Mastermix (Roche, cat no. 4887352001) was used to generate a final volume of 10 µl. Reactions were performed in a white LightCycler® 480 Multiwell Plate 384 (Roche, cat no. 04729749001) with sealing foil (Roche, cat. no. 04729757001). All q-PCR reactions were performed as follows: one cycle of 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. PCR products were subjected to a melting-curve analysis, and representative samples were electrophoresed to verify that only a single product was present. Control reactions were conducted with RNA-free water to determine background content. Three replicates were used for each gene/sample.

Expression of each opsin gene was normalized as a fraction of the total cone opsin gene expression in each sample (as individual rather refers to a fish) following modifications by Fuller et al. 2004 and Spady et al. 2006 to the Carleton and Kocher 2001 equation:

$$T_i / T_{all} = (1 / (1 + E_i)\text{^} C_{ti}) / \sum (1 / (1 + E_i)\text{^} C_{ti})$$

where T_i / T_{all} is the relative gene expression ratio for a given gene normalized by total cone opsin gene expression, E_i is the PCR efficiency for each pair of primers (Shao et al. 2014), and C_{ti} is the critical cycle number for each gene. Proportional opsin mRNA levels are given as percentages of total cone opsin gene expression.

Optomotor responses

An optomotor apparatus, as described previously (Corral-López et al. 2017) but with some modifications, was used to determine the behavioral sensitivity of fish to red light. The response test chamber (121 × 60.5 × 69 cm) was covered with two layers of light-proof black fabric to block lateral ambient light, and a commercial projector (EB-U42, Epson) was placed on top. Fish were individually placed in a matte black bucket (2.5 L) placed ~75 cm from the projector (Fig. S1). During the test, fish behavior was recorded using a webcam (Microsoft LifeCam Studio) installed on the side of the bucket, and all fish were later scored by the same person (LL) using the software RECentral 4 (AVermedia Inc. version 4.3.1.74). Peak photon fluxes at each wavelength of the stimulus were determined using a

Table 1. Sequences of the primers used for PCR and q-PCR

Gene	Forward primer	Reverse primer
lws E_i = 95.4	5'-CCTGGGAGAGATGGATAGTTGTGT-3'	5'-TCCAGCCGTTGCCCATT-3'
rh2 E_i = 93.1	5'-ACCATCACGTCGGCTGTCA-3'	5'-TGGCCATGAATCCCTCAAG-3'
sws1 E_i = 94.2	5'-CTCGTCACAGCCAAATACAAGAA-3'	5'-AATCCTGCCAAGGTGATGTTG-3'
sws2 E_i = 94.7	5'-GCGGTCCCACCTCAACTACA-3'	5'-CGGACACGAGAAGGTTTGACA-3'
rh1 E_i = 99.1	5'-CGCCGCCCAGCAGGAGT-3'	5'-GCGTAGGGCACCCAGCACAC-3'

Efficiency (E_i) (%) for q-PCR primers.

spectrometer (QE65 Pro, Ocean Optics, USA) with an optic fiber (1065 μm in diameter; QP1000-2-UV-VIS, Ocean Optics, USA) and the software SpectraSuite (Ocean Optics, USA), which had been calibrated together by the manufacturer. To measure responses to the stimulus, we measured the spectrum and light intensity of the red strips projected on the inner side of the bucket. To this end, the end of the optic fiber was placed in the center of the bucket, at 5 cm below the surface and oriented towards the wall of the bucket vertically at the distance of 7 cm.

As a stimulus, a circular digital animation of 16 alternating stripes rotating at a speed of ~3 rpm was generated using Powerpoint (Microsoft). Corral-López et al. (2017), used a similar animation comprising complementary colors (*i.e.*, red and green) at varying contrast levels was used to study the color perception of guppy (*Poecilia reticulata*). However, our study focused on red color sensitivity as opposed to color discrimination. The presence of green stripes (or green wavelengths in grayscale) could have biased our results, especially as the three-spined stickleback is more sensitive to green than red light (Shao et al. 2014). Hence, we used red (#FF0000) and black (#000000) stripes defined by the RGB color model (Fig. S1). The radiant spectrum of the red stripe was 605 nm (λ_{max}) which is within the absorption range of stickleback's LWS opsin (λ_{max} 566–618 nm) (Novales Flamarique 2013), and it was approximated to the spectrum that had been known to cause distinct optical motor (594 nm: Cronly-Dillon and Sharma 1968; 620 nm: Boulcott and Braithwaite 2007) or ERG (600 nm: Shao et al. 2014) responses between immature and mature sticklebacks. A 50% neutral density filter (Veego Corporation, Taiwan; Fig. S2) was fitted to the lens of the projector to reduce stray light, which ensured that black stripes remained dark. The maximum peak transmittance of the red stripes was measured to be 605 nm (Fig. S3).

We observed several stereotypical behaviors, similar to those previously described for guppy under similar experimental conditions (Corral-López et al. 2017), when fish were subjected to the rotating stimulus. When brightness was high, the fish tended to follow the stripes by swimming in a circular pattern (optomotor circling) or by rotating their body to follow the stripes (compass reaction). As the brightness decreased, fish turned their head toward the rotating stripes (nystagmic reaction). When the fish was not able to see the intensity of the stimulus, the fish swam without a clear circular pattern (zero motion) or remained static (frozen). The optomotor test was initiated at 50% brightness and sequentially reduced to the finest scale of 0.5% according to binary search of optomotor responses. The red light intensity of the behavioral threshold was then spectrometrically determined in each set of tests. When the fish display "zero motion" or "frozen" behaviours under rotating image, the light intensity of the test was considered below the optical motor threshold.

Each round of experimental testing began with 1 min of adaption to allow the fish to settle. Then the image of colored stripes was projected statically for 1 min before initiating clockwise rotation for a further 1 min. Then, after stalling rotation of the projection for another 1 min, it was switched to counterclockwise rotation for a further 1 min (Fig. S4). Upon completing that protocol, the brightness of the stripes was adjusted for the next round of testing. The entire optomotor test took less than 2 h. All optomotor response testing was done during daylight hours of a long-day photoperiod.

To compare behavioral responses of fish, behavioral red sensitivity was calculated as the reciprocal of the threshold light intensity. Additionally, behavioral red sensitivity at different time-points for each fish was normalized against the time-point for its lowest threshold measurement (Shao et al. 2014). Data for fish that did not finish the entire experiment or that did not finish the entire test series were excluded. A total of seven females completed the test.

Statistics

As some of the data from both mRNA and optomotor experiments were not normally distributed according to the Shapiro-Wilk normality test ($p < 0.05$), we employed non-parametric statistical analyses. Multiple group comparisons were performed using the one-way Kruskal-Wallis test, followed by post-hoc analysis with a Dunn test (Olsvik et al. 2005). Data from just two groups, *i.e.*, opsin mRNA levels from the inter-spawning and spawning periods (see above), were compared using non-parametric Mann-Whitney *U*-tests. For the optical motor experiment, one-way Kruskal-Wallis tests with Dunn's post-hoc test were used for multiple group comparisons. A non-parametric pairwise Wilcoxon signed-rank test (Mobley et al. 2020) was used to compare the optical motor data for different time-points and to compare mean red sensitivities between spawning and inter-spawning periods (SPSS v. 20).

RESULTS

Opsin mRNA levels

Relative mRNA levels for cone pigments over the spawning cycle are illustrated in figure 1. Female fish displayed lower *lws* and higher *rh2* mRNA levels during

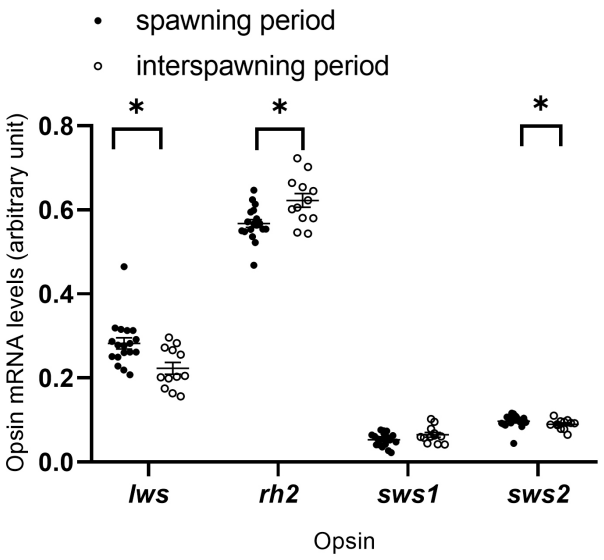

Fig. 1. Relative mRNA levels of retinal double cone opsins (*lws* and *rh2*) and single cone opsins (*sws1* and *sws2*) in the spawning phase (0, 6 and 72 h after spawning; n = 18, note n refers to samples, each of which is pooled from two fish) and inter-spawning phase (24 and 48 h after spawning; n = 12) (means ± SE; *p < 0.05 Mann-Whitney U test; the detail statistic results were shown in the text).

the inter-spawning phase (24 and 48 h) compared to during the spawning phase (0, 6 and 72 h) (Fig. 1) (*lws* p = 0.012; U = 171, W = 342 and *rh2* p = 0.007, U = 49, W = 220, Mann-Whitney U tests), whereas the difference was only just significant for *sws2* (p = 0.048; U = 155, W = 326) (Fig. 1) and no difference between spawning and inter-spawning phases was observed for *sws1* (p = 0.15; U = 73, W = 244).

Females displayed the lowest relative *lws* mRNA levels 24 h after ovulation, with levels increasing thereafter and peaking at 72 h after ovulation (Fig. 2). Indeed, *lws* mRNA levels were significantly higher at 72 h than at 24 h (*d.f.* = 4, p = 0.012; one-way Kruskal-Wallis). In contrast, relative *rh2* mRNA levels increased after spawning, were highest at 24 h, and then decreased (Fig. 2). Relative *rh2* mRNA levels were significantly higher at 24 h than at 72 h (*d.f.* = 4, p = 0.037; one-way Kruskal-Wallis). However, we did not detect any significant differences between time-points for the other two opsin genes *i.e.*, *sws1* and *sws2* (*d.f.* = 4, p = 0.21 and 0.19, respectively; one-way Kruskal-Wallis) (Fig. 2).

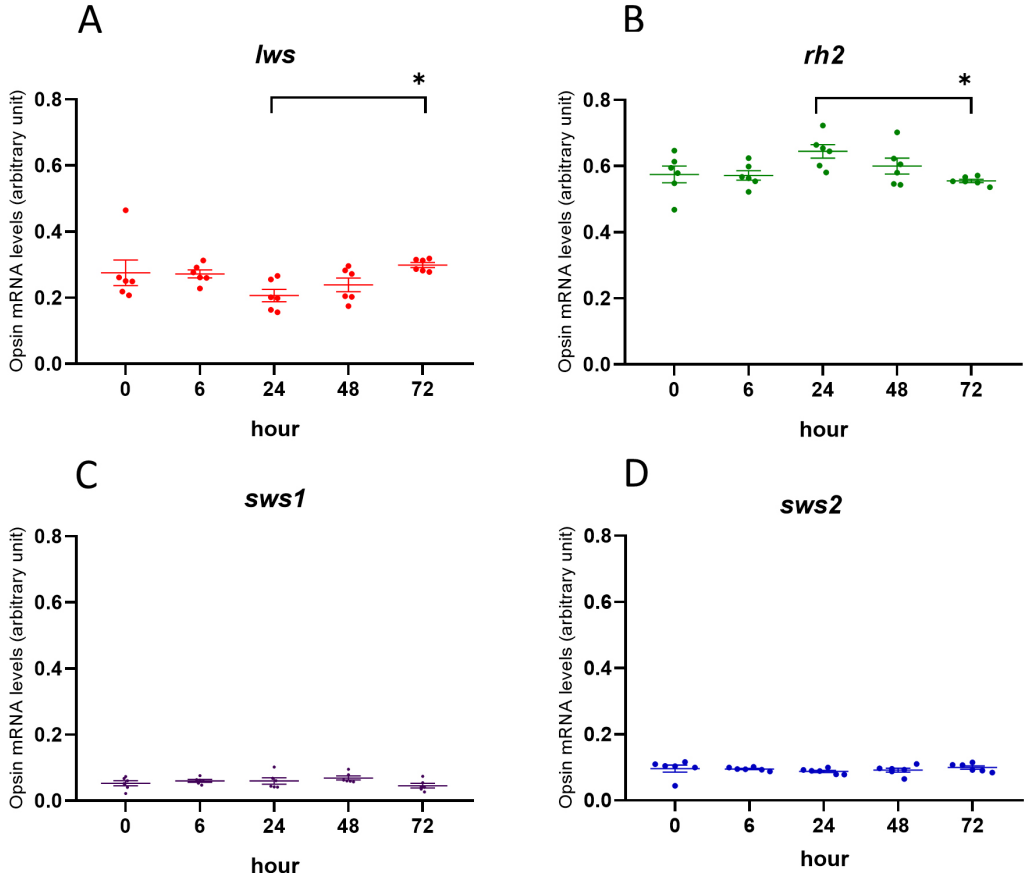

Fig. 2. Relative mRNA levels of retinal *lws* (A), *rh2* (B), *sws1* (C) and *sws2* (D) (means ± SE; n = 6; *p < 0.05 one-way Kruskal-Wallis test). Statistically significant differences in plasma testosterone and estradiol levels (at p < 0.05 one-way Kruskal-Wallis test) are indicated by letters.

Behavioral sensitivity to red light

Patterns of behavioral red sensitivity over the spawning cycle are shown in figure 3. Pairwise comparison of respective mean values for red sensitity during spawning (0, 6, 72 and 96 h) or the inter-spawning period (24 and 48 h) revealed that female fish displayed lower sensitivity to red during the inter-spawning period compared to other time-points (p = 0.028, Z = -2.19; Wilcoxon signed-rank test) (Fig. 3).

Red sensitivity at 24 h was lower than that at 0 or 96 h (*d.f.* = 5, p = 0.022 and p = 0.014 respectively; one-way Kruskal-Wallis). Furthermore, pairwise analysis showed that red sensitivity at 24 h after spawning was significantly lower than at the time-point directly after spawning (0 h) and at 6 h after spawning (p = 0.018 Z = -2.37 and p = 0.043 Z = -2.03, respectively; Wilcoxon signed-rank test) (Fig. 4). Moreover, red sensitivity at 96 h was significantly higher than that at 24 or 48 h (p = 0.018 Z = -2.36 and p = 0.017 Z = -2.37, respectively; Wilcoxon signed-rank test). No significant differences were detected for other time-point comparisons (Fig. 4).

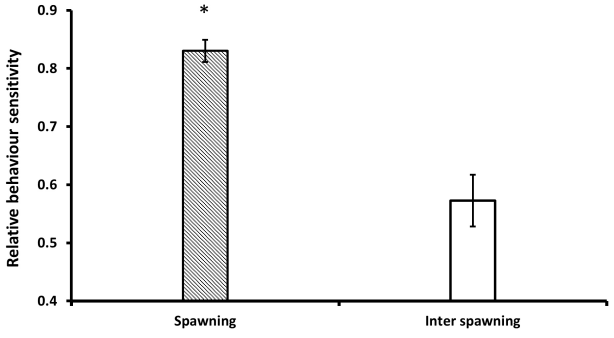

Fig. 3. Mean behavioral red sensitivity of female stickleback in the spawning phase (0, 6, 72 and 96 h) and inter-spawning phase (24 and 48 h) (means ± SE; n = 7; p < 0.028 Wilcoxon signed-rank test).

DISCUSSION

Here, we show that red color sensitivity in female sticklebacks shifts rapidly over a spawning cycle. In particular, both relative levels of retinal *lws* mRNA and behavioral red sensitivity in females were higher when they were ready to spawn compared to the inter-spawning intervals.

A previous study revealed that androgens can enhance visual sensitivity to red color in male sticklebacks. More specifically, an 11-ketoandrostenedione (11KA) implant increased retinal *lws* mRNA levels and red sensitivity in non-breeding male sticklebacks under a short-day photoperiod (Shao et al. 2014), whereas castration had the opposite effect under a stimulatory long-day photoperiod. In the present study, female stickleback displayed the lowest behavioral red sensitivity and retinal *lws* mRNA levels 24 h after spawning. However, both those parameters increased gradually from 24 to 96 h after spawning. A previous study shown that levels of testosterone and estradiol in female stickleback increased from 0 to 24 h and then declined 48 h after spawning (Roufidou et al. 2018). This is the opposite pattern one would expect if these sex hormones stimulated red sensitivity. Our results do not support the hypothesis that sex steroids directly stimulate behavioral red sensitivity in female sticklebacks, especially over such a short time scale, though it should be mentioned that opsin genes are possible subject to rapid regulation,

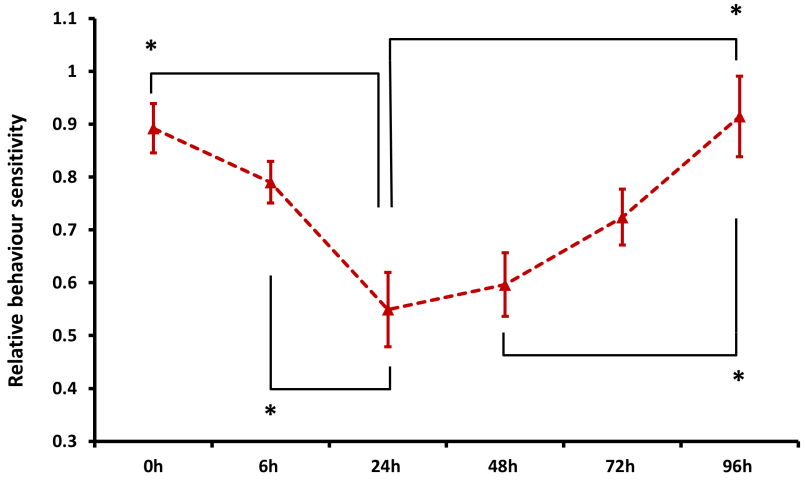

Fig. 4. Relative behavioral red sensitivity over the spawning cycle in female stickleback. Statistically significant differences (at p < 0.05 Wilcoxon signed-rank test) between pairs of time-points are indicated by * (means ± SE; n = 7). The detail statistic results are given in the text.

as evidenced by their hourly variations according to diurnal rhythms (Halstenberg et al. 2005). However, switching of expression of opsin types within a cell may take longer. Treatment with thyroid hormone can induce an ultraviolet-to-blue (SWS1-to-SWS2) switch in opsins of the signal cones of salmonid fish retina (alevin or parr developmental stages), which usually occurs during smolting, with treatment periods being as long as two or six weeks depending on developmental stage (Cheng et al. 2009; Novales Flamarique 2013).

Mating signals and sexual characteristics are more important during the breeding season than at other times (Kodric-Brown 1998). Diminished investment in mating signals outside the breeding season may provide benefits such as reducing predation risk (Zollinger and Brumm 2015) and increasing resource acquisition (Lindström et al. 2009). Likewise, the sensory ability to detect such mating signals may change synchronously to maximize receptor efficiency (Clark et al. 1990). However, such shifting patterns have primarily been attributed to yearly or seasonal rhythms and/or breeding cycles (Boulcott and Braithwaite 2007; Cronly-Dillon and Sharma 1968; Shao et al. 2014). In the present study, red color sensitivity of female sticklebacks also changed within a spawning cycle.

Greater sensitivity to mating signals could drive sexual selection. However, additional resource investments and changes in sensory systems may also contribute to reduced fitness (Gwynne and Bailey 1999). An increased rate of opsin metabolism in photoreceptors during the breeding period may compete for resources, and elevated red color sensitivity may influence responses to other colors. In vertebrates, cone cells expressing different types of opsin respond differentially to light of different wavelengths, and are thus responsible for color perception. As demonstrated previously (Shao et al. 2014), the ebb and flow of relative expression levels of *lws* and *rh2* genes reflect red color sensitivity of male stickleback, suggesting that a possible shift in double cone assembly, *i.e.*, various proportions of red/red or green/red double cones, may influence the overall color vision of fishes. Due to the physical properties of water, long wavelength light (~680 nm, red) is not transmitted as far as medium or short wavelength light (Braun and Smirnov 1993). Thus, whereas spawning female stickleback are especially sensitive to red, which is presumably optimal for courtship and mate choice, it may not be ideal for other aspects of life history, such as foraging or predator avoidance.

CONCLUSIONS

Our data indicate that the female stickleback changes color sensitivity rapidly over the spawning cycle, which could cope with changing needs at different phases of the spawning cycle.

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Authors' contributions: YTS and BB initiated the study, and LCL, CR and YTS participated in the experiments. YTS and BB were involved in the writing.

Competing interests: The authors declare that they have no competing interests.

Availability of data and materials: All data and materials are available in the paper and in the supplementary materials.

Consent for publication: Not applicable.

Ethics approval consent to participate: All animal care and experiments followed protocols approved by the Institutional Animal Care and Use Committee of National Taiwan Ocean University or the permit by Stockholm Northern Animal Experiment Ethical Board.

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Supplementary materials

Fig. S1. Setup of our optomotor response test. Lower panel represents the varying brightness of the different stripes projected into the bucket. (download)

Fig. S2. (A) Spectrum and the intensity of a full-wavelengths LED (3W, 3H0F5, EHE Corporation, Taiwan) with or without the 50% neutral density filter (Veego Corporation, Taiwan), and (B) The transmittance (%) of the neutral density filter. (download)

Fig. S3. Spectrum and intensity of projected red stripes. (download)

Fig. S4. Schematic of the optomotor response test procedure. The raw data of the figures. (download)