Materials and Methods

(a) Animals

Adult northern anchovies (*Engraulis mordax*) were obtained from ocean net pens kept by Westport Seafood Ltd (Westport, Washington State, USA) and transported to the University of Victoria Aquatic Facility (Victoria, British Columbia, Canada) where they remained during the experimental period. Anchovies were kept in outdoor 5000 l re-circulating saltwater tanks, fed frozen mysid, and experienced the natural daylight cycle throughout the study. The mean weight and total length (± SD) of the animals used were 24.1 ± 1.61 g and 15.9 ± 0.23 cm (n=52). All experiments were approved by the Animal Care Committees of Simon Fraser University (protocol # 1126B-10) and the University of Victoria (protocol # 2013-005), which abide by regulations set by the Canadian Council for Animal Care.

(b) Microspectrophotometry

Absorbance and linear dichroism measurements were obtained from individual photoreceptor outer segments using a dichroic microspectrophotometer, as described previously [21].

(c) Electrophysiology

The optical and electrophysiological set-up to acquire optic nerve responses has been documented in detail before [33] and will only be summarized here, emphasizing additions for this study. The optical system for stimulus delivery consisted of a 150W Xenon light source
coupled to a monochromator whose output traversed a neutral density wheel and shutter before entering one end of a bifurcated liquid light guide. The other end of the light guide received input from a halogen source (Fiber-lite MI-150, Dolan-Jenner Industries) whose output traversed a combination of barrier and neutral density filters; this was the background channel. The outputs of both channels overlapped, converging onto the common end of the liquid light guide. The latter fitted into a holder with ball-joint base that permitted its positioning at any angle with respect to the centre of the fish’s pupil [33]. Experiments involved stimulation of the mid ventro-temporal (VT) and mid ventro-nasal (VN) retina by adjusting the angle of the fibre with respect to vertical and horizontal markers.

To generate a spectral sensitivity function, the fish was adapted to a diffuse, unpolarized light background (figure S1a,b) through the background channel of the bifurcated light guide. The fish adapted to this background for 1 hr following which compound action potential recordings from the optic nerve were acquired to a 500 ms flash stimulus. For a given wavelength, the stimulus intensity was increased incrementally and the response at threshold plotted against log (stimulus intensity) and fitted with a third degree polynomial (figure S2). The threshold value (20 µV) was chosen to lie in the lower linear part of the fit, characterized by a single major peak contribution to the optic nerve response. Sensitivity (i.e., the reciprocal of intensity at threshold) was calculated for 14 wavelengths across the spectrum 360-650 nm to generate a spectral sensitivity function. All light measurements (i.e., irradiance, in photons cm\(^{-2}\) s\(^{-1}\)) were performed with a UV-visible liquid light guide (600 µm diameter input, 0.22 NA) connected to a USB 2000 spectroradiometer (Ocean Optics) and used rotational mounts to hold the light guide at the desired position. In this manuscript, intensity and irradiance are used interchangeably.
To generate polarization sensitivity functions, the stimulus channel was fitted with a wax paper stack diffuser-linear polarizer (HNP*B Polaroid) combination, in that order, such that the stimulus was 100% linearly polarized light of a given E-vector orientation. As per other studies [29,34], the intensity at each E-vector angle and ND wheel rotation was measured with the liquid light guide of the spectroradiometer positioned at the location of the fish pupil during experiments. For a given position of the ND wheel, rotation of the polarizer resulted in a maximum intensity variation of less than 0.8 % (Table S1). The 100% linearly polarized light field was confirmed with additional measurements involving a second polarizer (i.e., an analyzer) oriented with its transmission axis perpendicular to that of the stimulus polarizer, resulting in complete extinction. This was as expected from the action of the diffuser that preceded the stimulus polarizer.

In these experiments, the unpolarized background was achieved by two additional Fiber-lite MI-150 light sources whose outputs traversed a stack of four layers of wax paper before converging onto the eye of the fish. The wax layer stack surrounded the output of the stimulus fiber and extended 10 cm from it in the vertical plane providing uniform illumination on the eye of the fish without interfering with the stimulus. Recordings were obtained for 5 positions of the polarizer transmission axis (90° - vertical, 45°, 0°-horizontal, -45°, and -90°-vertical) with the wavelength of light kept constant at 500 nm. At each position (E-vector angle), the stimulus intensity was increased progressively and the responses acquired and analyzed as per in the spectral sensitivity experiments to generate a polarization sensitivity function under the unpolarized light background described previously (figure S1a). This was possible because all background sources were identical and their intensities tunable.
In the case of polarization responses under a polarized light background (polarization adaptation experiments), the background was the output of the background channel linked to the bifurcated light guide. Rotation of the polarizer to the adapting orientation (vertical or horizontal) between recordings permitted preferential adaption of the axially dichroic short or long cones in the VT retina.

**d) Behavioural experiments with LEDs of different spectral emission**

Free swimming anchovies (n=9) were individually trained to swim toward the unpolarized (0% polarized) light emission from two blue LEDs over that of two red LEDs (figure S1c) placed on opposite halves of a 500 l aquarium (dimensions: 112 cm long x 70 cm wide x 65 cm deep) with the sides covered in black mat plastic. This was achieved by pairing swimming toward the blue LEDs with a food reward (mysid) in trials where the intensity of the blue LEDs was matched to that of the red LEDs by modulating the driving currents. After 15-20 trials of such operant conditioning, all anchovies swam on the side of the aquarium with the blue LEDs in anticipation of the food reward. Testing involved the same set-up but with modification of the lighting conditions. Following a 10 minute acclimation period during which the anchovy swam randomly (i.e., without preference for either aquarium side) under the room lighting (figure S1d), the LEDs were turned on and the room light off. Recording was then started remotely using a Nikon video camera positioned over the aquarium. Initially, the intensity ratio of blue LED to red LED light was 0.54. Three to 3.5 minutes later, the blue LED intensity was changed to 1.91 times that of the red LED. Approximately 1.5 minutes afterwards, the room light was turned on though the blue LED emission continued on one side of the aquarium and the red LED on the other. The
room light intensity was 3.98 times that of the LEDs. Approximately 2.5 minutes afterwards the LEDs were turned off and only the room light remained (i.e., the initial acclimation conditions). Recording was then stopped and the positions of the two LED pairs reversed between opposite sides of the aquarium. The entire recording sequence then began anew. Thus, the effects of light intensity between LED sources and their position in the aquarium (set-up geometry) were accounted for in the swimming behaviour of each anchovy.

To ensure that the differences in intensity between blue and red LEDs correlated with those that the fish would theoretically perceive, I multiplied the spectral sensitivity function of the VN retina (see figure 2e) by the emission spectrum of each LED type for the various intensities tested. Comparisons of the photon catch sums showed that, at the start of the videos, the photon catch associated with the blue LEDs was 0.68 that associated with the red LEDs. Later on in the videos, when the blue LED emission was increased, its associated photon catch became 2.86 that of the red LED. These results thus illustrate the same dominant LED source as that from comparison of intensities alone. In these calculations, I did not take into account light absorption by the dioptric apparatus (cornea and lens) as preliminary microspectrophotometric measurements revealed < 5 % absorbance of these structures for λ > 400 nm; thus, they were of no consequence to the computations since spectral sensitivity below 400 nm was minimal. The measured linear dichroism of ~0 and dichroic ratio of ~1 regardless of microspectrophotometer stage rotation further indicated that there was no preferential absorption with E-vector orientation. Additional rotations of a microscope stage containing the cornea and lens between crossed polarizers showed negligible birefringence. Therefore, this intrinsic optical anisotropy could also not have contributed to the polarization sensitivity results obtained by electrophysiology. The one peak polarization sensitivity functions found are, in any case,
inconsistent with any significant structural dichroism, or ellipticity of the stimulus induced by the
dioptric apparatus as this would have led to multiple peak functions or no polarization sensitivity
at all.

(e) Behavioural experiments with polarization backgrounds

Following the termination of colour choice experiments, operant conditioning training was
performed on the same anchovies but, in this case, the animals were trained to favour swimming
under a 100% polarized over an unpolarized (0% polarized) downwelling light field. Training
involved coupling a mysid food reward with swimming under the polarized light field. As per in
the colour discrimination experiments, anchovies learnt to swim toward the polarized light field
expecting the food reward within 20 trials. Each trial lasted 20 minutes during which the fish
experienced the polarized light field on either side of the aquarium once, at random, and
following an initial 10 minutes of acclimation to unpolarized downwelling light. Untrained
anchovies showed no preference for either side of the aquarium when one half became polarized.
Progressively, this behaviour changed to swimming within the polarized half of the aquarium as
the fish became trained. An anchovy was considered trained when it swam within the polarized
half of the aquarium, regardless of side, in three consecutive trials.

The illumination for these experiments was room lighting (figure S1d) and the two types
of downwelling light fields were generated by placing a filter stack consisting of, from top to
bottom, 4 layers of wax paper and a linear polarizer (100% polarization background), and the
reverse stack for the unpolarized background. The two stacks, on either side of the aquarium
(figure S3), were made of the same large polarizer-wax combination of panels; these were
stacked, mounted and cut into two halves, thereby minimizing any structural differences between them. These stacks sat on mounts within 1 cm of the top of the aquarium, hence providing a large field of illumination that mimicked crepuscular conditions in local waters, when the polarization is strongest [34]. In these experiments, the polarizers were always oriented with their E-vectors parallel to the width dimension of the aquarium. During training, the two stacks were alternated and flipped on either side. Once the anchovies were trained, they readily detected the half of the aquarium with the downwelling polarized light field and swam consistently within its limits. The fish were not fed during testing.

Because intensity differences can be created by differential reflection at walls and other surfaces, especially for oblique rays of light coming into the aquarium, I performed a full characterization of light conditions on either side of the aquarium. The most oblique, direct ray of light originating from the outside that could be perceived by an anchovy at one end of the aquarium coming from the opposite end was 30° from the horizontal. Thus, in addition to downwelling light measurements at symmetrical positions in the aquarium, and sidewelling measurements near the walls, I also acquired intensity measurements at opposite ends of the aquarium with the spectroradiometer light guide oriented at 30° and 45° to the horizontal. These measurements (figure S4) were conducted with a rotatable mount that held the light guide at the desired angle 10 cm from the bottom of the aquarium. In the case of wall reflection measurements, the light guide was positioned facing the wall, 5 cm from it. In the case of oblique light measurements, the light guide was pointed toward the opposite end of the aquarium at the desired angle. These measurements revealed the following: (1) the amount of light transmitted by each stack was the same regardless of polarity and it was also the same when comparing both stacks, within the measurement capabilities of the spectroradiometer (threshold at ~ 10⁹ photons
cm<sup>-2</sup> s<sup>-1</sup>), (2) the same gradient of light intensity from centre to wall of the aquarium was present on either side, (3) reflections from the walls were the same on both sides of the aquarium, and negligible; the mean intensity value was 0.0004 that of the downwelling light at the centre of the aquarium and barely measurable with the spectroradiometer, and (4) there were no significant intensity differences for oblique rays coming to the far walls of the aquarium from the opposite side. In addition, upwelling light was not detectable by the spectroradiometer. Together, these results demonstrate that there were no intensity confounds that the anchovy could have cued into during polarization discrimination experiments.

Three anchovies died during the process of training such that testing was performed on the remaining 6 anchovies. Testing involved an initial period of recording when the two stacks, each covering one side of the aquarium, transmitted unpolarized light. This was followed by the remote flipping of one stack such that the light transmitted on that side of the aquarium was then 100% polarized. Two test trials were conducted per anchovy in which opposite stacks were flipped to account for location of the polarized light field. In these experiments, filming was conducted through one of the lateral sides of the aquarium and black mat plastic covered the remaining sides.
Tables

Table S1. Integrated irradiance for the various positions of the transmission axis of the polarizer (E-vector) tested during electrophysiological recordings, for one position of the ND wheel. The same trend persisted for other positions of the ND wheel as the latter only modulated intensity.

<table>
<thead>
<tr>
<th>E-vector (°)</th>
<th>0</th>
<th>45</th>
<th>90</th>
<th>135</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irradiance (photons cm⁻² s⁻¹)</td>
<td>1.29 x 10¹⁴</td>
<td>1.28 x 10¹⁴</td>
<td>1.28 x 10¹⁴</td>
<td>1.29 x 10¹⁴</td>
<td>1.29 x 10¹⁴</td>
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</table>
**Figure legends**

**Figure S1.** Spectral characteristics of background lights used in the study. (a) Normalized spectrum of the unpolarized background used in electrophysiology experiments; the integrated irradiance (300-750 nm) was $1.87 \times 10^{14}$ photons cm$^{-2}$ s$^{-1}$. (b) Normalized spectrum of the long wavelength (> 550 nm) unpolarized background used in electrophysiology experiments; the integrated irradiance (500-750 nm) was $1.78 \times 10^{14}$ photons cm$^{-2}$ s$^{-1}$. (c) Normalized spectrum of the blue and red LEDs used during behavioural experiments. The integrated irradiance was $6.79 \times 10^{13}$ photons cm$^{-2}$ s$^{-1}$ and $1.26 \times 10^{14}$ photons cm$^{-2}$ s$^{-1}$ for the blue (400-600 nm) and red (500-700 nm) LED pairs, respectively, at the start of the videos, and $2.41 \times 10^{14}$ photons cm$^{-2}$ s$^{-1}$ and $1.26 \times 10^{14}$ photons cm$^{-2}$ s$^{-1}$ following the enhanced blue LED irradiance, about 3.5 minutes into the videos. (d) Normalized spectrum of overhead room lighting during behavioural experiments. The integrated spectrum (350-750 nm) was $1.41 \times 10^{15}$ photons cm$^{-2}$ s$^{-1}$ for the spectral (colour) discrimination experiments and $6.41 \times 10^{13}$ photons cm$^{-2}$ s$^{-1}$ for the polarization discrimination experiments.

**Figure S2.** Representative plot of the amplitude of the optic nerve ON response as a function of log (irradiance) of the stimulus. In this case, the stimulus was unpolarized light of $\lambda = 560$ nm and the fish was adapted to the background shown in figure S1a. The data points were fitted with a 3$^{rd}$ order polynomial and the irradiance value corresponding to the 20 µV response criterion calculated. The inverse of this value was the sensitivity of the fish.
**Figure S3.** Schematic side views of the aquarium along its length (a) and width (b) showing major features of the set-up for polarization discrimination experiments. The drawings are not to scale. All side walls were covered with black mat plastic except for the one facing the camera. During training trials, feeding of the anchovies was carried out with a small tube that fitted between the top of the aquarium and the stacks (i.e., the mount height) and delivered a small volume of water containing mysids. The stacks were flipped remotely using a dedicated grab tool.

**Figure S4.** Characteristics of the light field in the aquarium used for behavioural experiments of polarization discrimination. (a) Downwelling spectral irradiance at 10 cm from the bottom of the aquarium along the mid-line (location marked with an arrow in c). (b) Sidewelling spectral irradiance near the wall of the aquarium (light guide pointing toward the wall, 5 cm from it; location marked with arrowhead in c). (c) Locations of measurements within a schematic bird’s eye view of the aquarium, and relative integrated irradiances (values are relative to the highest intensity measured, 1; arrowhead points to the corresponding location). The dots depict 5 locations that are equidistant from the imaginary centre line (dashed) running along the width of the aquarium. Downwelling light measurements were taken along the imaginary centre line (dashed) running along the length of the aquarium, 1/3rd toward the walls of the aquarium on either side, and at 5 cm from the walls on either side. Each of these measurements is depicted by two numbers in parenthesis; the first corresponds to the intensity for the polarizer-diffuser stack in that order, the second corresponds to the reverse stack, i.e., diffuser-polarizer. The dot locations next to the walls have an additional set of intensities of lower magnitude; these correspond to the horizontal measurements with the light guide pointing at the wall, 5 cm from it.
Locations marked with a star have three sets of measurements. These correspond to the light guide pointing toward the wall, 5 cm from it (lowest intensities), or toward the opposite side of the aquarium at a 30° angle (medium intensities) or 45° angle (highest intensities). The values are not significantly different as a function of stack configuration or between corresponding locations on either side of the aquarium.

**Movies**

**Movie S1.** Colour (wavelength) discrimination by the northern anchovy during a behavioural trial. Prior to testing, the anchovy was trained to swim toward the light from the blue LEDs over that of the red LEDs. When the room lights are turned off, the anchovy swims within the region of the aquarium that is dominated by blue LED emission, within the right side of the aquarium. This is irrespective of light intensity as irradiance from the blue LEDs changes from 0.54 to 1.91 times that of the red LEDs as the video progresses. When the room lights are turned on over the LEDs, the anchovy still favors the side of the aquarium associated with the blue LEDs. When the LEDs are turned off and the room lights remain, the anchovy shows no preference for either side of the aquarium, now characterized by homogeneous downwelling light. This movie can be found at: https://figshare.com/s/f3483560458b9132bfd3

**Movie S2.** The same anchovy as per movie S1 shows swimming preference for the left side of the aquarium when the downwelling light on this side is dominated by blue LED emission. This movie can be found at: https://figshare.com/s/6d76eb32fb99ed93ae6
**Movie S3.** Polarization discrimination by the northern anchovy during a behavioural trial. Prior to testing, the anchovy was trained to swim toward a 100% linearly polarized light field over an unpolarized light field. When presented with downwelling, unpolarized light on either side of the aquarium, the anchovy shows no swimming preference for either side. When the downwelling light on the left side becomes 100% linearly polarized, the anchovy swims on the left side of the aquarium, as per its training. The dotted line in the video denotes the middle of the aquarium.

This movie can be found at: https://figshare.com/s/693f51a91d95a3254b31

**Movie S4.** The same anchovy as per movie S3 shows swimming preference for the right side of the aquarium when the downwelling light on this side becomes 100% linearly polarized. This movie can be found at: https://figshare.com/s/754b09d17355bf1fb10d
Figure S2

\[ y = -27.102 \times 3 + 1128.5 \times 2 - 15603 \times + 71666 \quad (R^2 = 0.98) \]