Readme Document regarding data repository for Royal Society Proceedings B paper as follows.

In addition to the ***Materials and Methods*** document, find also the ***Introduction*** document that describes the outline for this paper. The final readme, ***File Descriptions***, describes how the files are arranged in various Zip files. The data within these zip files should be considered the gold standard data, although considerably more data exists than is reported in this repository. Please contact the authors directly using the contact details above for any additional data.

MATERIALS AND METHODS

(a) *Animals*

Adult Atlantic longfin squid (Doryteuthis pealei, aka Loligo pealei; figure 1a) were collected by brief trawling runs from the coastal waters near Woods Hole, Massachusetts. From large holding tank populations, individuals that showed no evidence of skin damage in at least one fin were transferred to separate holding tanks for males and females, respectively. Each squid was fed daily with small live fish (Fundulus spp.) and animals were kept for up to 2 weeks before being used for experiments.

(b) *Dissection and electrophysiology*

Set-up: The chamber was filled with Tris buffered (20 mM; pH 7.8) natural seawater (NSW), which was re-circulated and held at a constant temperature (15 °C) using a refrigerated water chiller. The dissection tray was viewed with a stereo dissection trinocular microscope with fluorescence system on a boom stand positioned above the fin to allow dissection, video imaging, and spectrometry. Light for dissection, imaging and measuring spectra was provided by a halogen fibre-optic light source (Schott).

Tissue preparation: Squid were anesthetized by immersion in seawater containing a sub-lethal concentration of ethanol (3%), and then killed by decapitation and decerebration. The ventral mantle muscle was partly cut away and the internal organs removed. Only fins that had no evidence of skin damage were used in preparations. The preparation was placed dorsal side down in a large perfusion chamber and pinned down near the periphery of the fin and remaining mantle. We readily observed nerves that ran in tracts from the large fin nerve, and branched subsequently to either terminate within iridophores or pass through them (figures S1 and 4a). One intact and three reduced preparations were developed for this research.

“Intact” fin preparation: A small window (1 x 1 cm) of ventral skin and fin muscle near the base of the fin was removed by surgical dissection, leaving the inner most layer of the dorsal skin exposed, to enable the location of nerves. Nerves were tightly interwoven with connective tissue and travelled throughout the iridophore layer, which required very precise and careful dissection to remove enough length of nerve for electrical stimulation. Once the nerve was dissected away from the connective tissue, the dorsal skin was opened fully to allow access from the dorsal surface. The preparation was inverted and pinned ventral surface down (figure S2a). Immediately prior to stimulation, nerves were cut and sucked into glass pipettes that formed a tight seal around the nerve bundle. Custom pipettes with suitable internal diameters were made by manually cutting and polishing with a Sutter ceramic tile.

Three reduced preparations: Similar to intact fin however: (i) tissue above and below iridophore layer was removed (figures 2d-e and S2b), (ii) tissue above the iridophore layer was removed (figures 3a-b and S2c). (iii) Tissue below the iridophore layer was removed (figures 3c and S2d).

To stimulate iridophores reliably and periodically over several hours, we stimulated with 5 V pulses. Each pulse was 300 µs in duration and was repeated at a frequency of 10 Hz over a period of 15 sec unless otherwise stated. Upon neural stimulation, both chromatophores and iridophores responded. The iridophore-activated field included part of the chromatophore-activated field and areas immediately anterior. By increasing the stimulation frequency (0.5-80 Hz) and/or voltage (1-50 V), a higher number of iridophores with higher reflectances could be observed, but this trend eventually saturated.

(c) *Videography and spectrometry*

Once a nerve was connected to the stimulator (Model 2100, A-M Systems), we coordinated electrical stimulation with videography or spectrometry (through the trinocular port of the stereo microscope), using a Power Lab data acquisition unit (PL3504) and Lab Chart software (AD Instruments) to trigger equipment, including a blue LED light pulse (observable in the video or spectra measurements). High definition video was collected at 30 frames per second (fps) with a Canon EOS 5D Mark II digital camera in manual mode. Prior to spectra collection, a diffuse reflectance standard (WS-1, Ocean Optics) was used to calibrate the spectrometer (Ocean Optics, Model QE65000) for the halogen light source (Schott). Light was directed from above, within 5 degrees of vertical, using a dual fibre optic light guide. Spectral and reflectance measurements were collected via a 1 mm optical fibre assembly coupled from the microscope to a spectrometer, every 0.3 seconds over 3-8 minutes and data was logged with SpectraSuite to the computer's hard disk. During each spectral measurement, video was recorded simultaneously by using a Zeiss eye piece adaptor and a HD video recorder (Sony HDR-XR520V) to collect representative images for each spectral change. Note that automatic brightness correction was applied during the Sony HDR-XR520V acquisition.

To avoid chromatophore activity affecting the quantification of iridophore responses, we selected only iridophores for spectral and reflectance measurements that were anterior to the chromatophore receptive field (where chromatophores did not respond to electrical stimulation). To analyze spectra data, collected over several minutes, we used Matlab (Mathworks) to normalize and subtract the background level of reflectance, and then smooth the data with a Savitzky-Golay filter to more easily visualize and compare responses between iridophores and preparations. We plotted the mean and standard deviation for the normalized, background subtracted, and filtered spectra from iridophores for the baseline and maximum reflectance. To determine spectral dynamics, background reflectance was subtracted and the wavelength at peak reflectance found for each measurement over time.

(d) *Whole-mount immunohistochemistry and confocal imaging*

After stimulation and simultaneous videography or spectrometry, we forward filled some of the large nerve bundles with 3% Lucifer yellow (LY). The nerve was re-cut about 1-2 mm shorter, and then sucked immediately into a tight fitting glass pipette, and the electrode rapidly backfilled with 3% LY in 200 mM Lithium Chloride using a microfil needle. After 20 minutes, the LY dye had diffused down the nerve sufficiently to be seen with a fluorescence dissection microscope. We filled the nerve for a minimum of 10 hours with the re-circulating bath in operation. The injected tissue was fixed for 12 hours in 4% paraformaldehyde in Tris-buffered NSW, then triple rinsed and stored in Tris NSW at 4 ˚C.

Tissue was processed as described by Gonzalez-Bellido and Wardill [30]. Briefly, the tissue was dehydrated and rehydrated in a series of steps to remove lipids and any trapped air, and then permeated with Collagenase (0.5 mg/ml) and Hyaluronidase (300 µg/ml) to open the nerve sheaths. An anti-Lucifer yellow antibody conjugated to NeurAvidin was used in conjunction with Biotin conjugated to Dylight 633 to shift the excitation into the red and away from tissue autofluorescence. Tissue was then cleared in thiodiethanol (TDE) and mounted inside a hole in a metal slide which was contained with #0 cover glass to increase the working distance of the objective. Automated imaging was undertaken with a Zeiss 780 confocal microscope, collecting multiple tiled z-stacks with auto-brightness correction to deal with changes in structural complexity with increasing depth. Image z-stacks were stitched with the "Grid/Collection stitching" plug-in within "Fiji" software, version 1.46p [31]. Tracing of neurons was completed with the freely available software "Vaa3D", version 2 [32].