Extraocular vision in a brittle star is mediated by chromatophore movement in response to ambient light

Lauren Sumner-Rooney[1,2]*, John D. Kirwan[3], Elijah Lowe[4], Esther Ullrich-Lüter[2]

[1]Oxford University Museum of Natural History, Parks Road, University of Oxford, Oxford, UK OX1 3PW.

[2]Museum für Naturkunde, Leibniz Institute for Evolution and Biodiversity Science, Invalidenstrasse, 10115 Berlin, Germany.

[3]Lund Vision Group, Department of Biology, Sölvegatan, Lund University, 223 62 Lund, Sweden.

[4]School of Biological Sciences, Ferst Drive, Georgia Institute of Technology, Atlanta, GA 30318, USA.

*Correspondence and lead contact: lauren.sumner-rooney@oum.ox.ac.uk

Summary

Almost all animals can sense light, but only those with spatial vision can 'see'. Conventionally this was restricted to animals possessing discrete visual organs (eyes), but extraocular vision could facilitate vision without eyes. Echinoderms form the focus of extraocular vision research [1–7], and the brittle star *Ophiocoma wendtii*, which exhibits light-responsive colour-change and shelter-seeking, became a key species of interest [4,8,9]. Both *O. wendtii* and an apparently light-indifferent congeneric, *O. pumila*, possess an extensive network of r-opsin-reactive cells, but its function remains unclear [4]. We show that although both species are strongly light-averse, *O. wendtii* orients to stimuli necessitating spatial vision for detection, but *O. pumila* does not. However, *O. wendtii*'s response disappears when chromatophores are contracted within the skeleton. Combining immunohistochemistry, histology and synchrotron microtomography, we reconstructed models of photoreceptors *in situ* and extracted estimated angular apertures for *O. wendtii* and *O. pumila*. Angular sensitivity estimates, derived from these models, support the hypothesis that chromatophores constitute a screening mechanism in *O. wendtii,* providing sufficient resolving power to detect the stimuli. RNA-Seq identified opsin

candidates in both species, including multiple r-opsins and transduction pathway constituents, congruent with immunohistochemistry and studies of other echinoderms [10,11]. Finally, we note that differing body postures between the two species during experiments may reflect aspect of signal integration. This represents one of the most detailed mechanisms for extraocular vision yet proposed, and draws interesting parallels with the only other confirmed extraocular visual system, that of some sea urchins, which also possess chromatophores [1].

Keywords: Vision; echinoderms; extraocular vision; behaviour; sensory biology

Results

Animals have used light in navigation, foraging, and other complex tasks since at least the Cambrian, and eyes have evolved dozens of times [12,13], often through related genetic and developmental pathways [14,15]. Most bilaterian eyes are discrete, paired, anterior and cerebrally innervated, but some visual systems are more unconventional. Echinoderms (sea stars, sea urchins, and relatives) are unusual in lacking a head, anteroposterior axis, and adult bilateral symmetry. Many respond to ambient light (colour change, covering, and shelter-seeking) and some to localised stimuli (navigation and even gaze stabilisation) [1,4,9,16–19]. Some sea stars have minute compound eyes derived from terminal tube feet [16,18], and some sea cucumbers may have simple ocelli [20]; the remaining classes lack identifiable discrete visual structures. However, sea urchins and brittle stars carry thousands of photoreceptor cells spread across the body: within skeletal pores, spines, radial nerves, and tube feet [4,6,11]. These facilitate simple photobehaviours and, perhaps, extraocular vision: the ability to resolve scenes without discrete eyes [1,4,9]. Vision in sea urchins was proposed over a decade ago [2,3] but has only recently been explicitly confirmed [1], possibly mediated by these cells, but the functional mechanisms remain elusive.

The brittle star *Ophiocoma wendtii* is a focal species in extraocular vision research; it is highly light-averse, has an extensive dispersed photoreceptor network [4,9,21,22] within skeletal pores surrounding calcite structures previously interpreted as microlenses [8,21], associated with light-

responsive chromatophores. Alongside three congenerics (cf. [23]), *O. wendtii* inhabits reef rubble in the Caribbean and Gulf of Mexico, sheltering during the day and emerging at night. Like sea urchins, they seek shade [2–4,9,24,25], but this only requires directional phototaxis (discriminating darker from brighter areas) and not spatial vision (image formation). The visual ecology of *O. pumila*, which has a similar photoreceptor network but lacks chromatophores, is unclear.

Spatial resolution

Individual *Ophiocoma wendtii* and *O. pumila* ($40 \geq n \geq 52$) were placed centrally in a circular arena, presented with one of three stimulus patterns on the arena wall under natural daylight, and observed until they reached the perimeter, when their bearing was recorded (Figure 1A). One stimulus (hereafter, the phototactic stimulus) comprised a solid black bar against a white background, resulting in changing light intensity around the arena, detectable without spatial vision. The second (discrete visual stimulus) comprised a black bar centered on a white bar of twice the width, against an isoreflectant grey background. This produced no overall difference in light intensity and necessitates vision for detection. The third (continuous visual stimulus) comprised a continuous difference of Gaussians wavelet [1]: a black midpoint grading into lighter flanks and then the grey background. The dark centre is undetectable to photoreceptors with an acceptance angle over a given width. Stimulus signal (the entire bar or zero-crossing of the difference of Gaussians wavelet) occupied 50° of the perimeter (Figure 1A). A plain grey control stimulus was also presented to *O. wendtii*, with 'stimulus' position rotating between trials.

Bayesian modelling indicated that true population values for *O. wendtii* were more oriented than simulated disoriented data for all three stimuli (Figure S1). Headings were modelled according to a von Mises distribution and also discretized into success (individuals located the stimulus) or failure, modelled according to a Bernoulli distribution. Both were contrasted with simulated data. *Ophiocoma wendtii* oriented to all three stimuli (overlap between observed and simulated success rates <5%, Table

S1). Weakly informative priors were used and convergence was assessed by visual inspection of Markov chains and $\hat{R}$ and N_{eff} statistics.

Maximum likelihood estimates compared datasets to established animal orientation models [26] in the R package Circ_MLE [27] using AIC*c* [28]. Estimates fitted unimodal (phototactic stimulus) and axial bimodal (visual stimuli) models to *O. wendtii,* with means of 355° (phototactic) and 358°-3° and 178-183° (visual) relative to stimulus centre (0°). For bimodal distributions, kappa (concentration) values were substantially higher for mean vectors corresponding to stimulus position than their axial counterparts (Table 1). V-tests (μ=0°) rejected uniform distributions for all three stimuli, with mean orientations within 30° of, and one standard deviation encompassing, the stimulus (Figure 1B, S3).

Combined analyses support *Ophiocoma wendtii* orienting towards all three stimuli. Animals did not orient to a smaller (25°) phototactic stimulus (Figure S2, Table 1, S2). At night, in dark-adapted form, *O. wendtii* no longer oriented to 50° phototactic (n=44) or discrete visual (n=38) stimuli (Figure S2, Table 1, S2). To explore possible reasons (decreased motivation, ambient light intensity, or other circadian factors), we performed three additional experiments and assayed orientation via Bayesian modelling of these and the control according to the von Mises (angular) and binomial (discretised counts) distributions. Modelling was as above, except experimental treatments were contrasted against a control with no stimulus (n=37). In light-dark forced choice experiments performed at night, all but one animal (n=28) chose shade, indicating they remain motivated. Naturally dark-adapted animals (n=50), with contracted chromatophores, were offered the discrete visual stimulus at night, artificially lit by two bright fluorescent tubes. They were disoriented (Figure S2, Table 1, S2), suggesting that ambient light intensity is not solely responsible for disorientation. Artificially dark-adapted animals (n=39), with contracted chromatophores, were presented with the discrete visual stimulus in daylight and were also disoriented (Figure S2, Table 1, S2), negating a role for other circadian physiological processes. Maximum likelihood estimates fitted uniform distributions to both datasets and V tests did not support departures from uniform distributions for all treatments ($p>0.05$, Table S2).

In contrast, *Ophiocoma pumila* did not orient towards any presented stimuli (Figure 1B, S1, Table 1, S1-2). Bayesian modelling gave lower estimates of true population orientedness than *O. wendtii*, but higher than simulations (Figure S1). Discretized analysis indicated success rates were no different from a random distribution (overlap 8.3-29.7%, Table S1). Maximum likelihood estimates fitted uniform distributions to the phototactic and continuous visual stimuli, but an axial bimodal distribution to the discrete visual stimulus (Table 1). However, absolute orientations, before correcting for stimulus position, also returned support for non-uniform distributions in this case (unimodal, $\Delta AICc \leq 0.12$). V-tests did not reject uniform orientation for any stimulus ($p>0.05$, Table S2). Nevertheless, *O. pumila* always chose shade in light-dark forced choice experiments (n=40).

The photoreceptor system

Arm segments from dark- and light-adapted *Ophiocoma wendtii* and *O. pumila* were incubated with primary antibodies raised against sea urchin r-opsin Sp-Op4 [11] and acetylated α-tubulin and fluorescent secondary antibodies [4,11] for confocal microscopy. Sp-Op4 reactivity was located within and distal to pores in the arm plates of both species, consistent with putative photoreceptor cells bearing distal expansions [4] (Figure S3). Pores formed regular polygonal arrays (Figure 2), and each contained a single Sp-Op4-reactive cell, which was usually offset, oval in cross-section, and associated with acetylated α-tubulin reactivity projecting proximally (Figure S3). DAPI staining revealed multiple non-Sp-Op4-reactive cells in each pore, consistent with histology [4,21]. Similar reactivity was observed in dark- and light-adapted tissue; chromatophore retraction allowed clearer signal in dark-adapted *O. wendtii*. Arm tips frequently extend from shelters and carry the derived compound eye in asteroids, sister to brittle stars [16,18]. We observed no concentration of opsin reactivity in terminal arm plates or tube feet, indicating that *Ophiocoma* lacks similar structures (Figure S3).

We constructed composite models of the photoreceptor system in *O. wendtii* and *O. pumila* to estimate fields of view (Figure 2A-C). Using stacked confocal images of Sp-Op4-reactivity, we reconstructed eight putative photoreceptors, which were digitally positioned within arm plates

reconstructed from synchrotron scans [4], in line with immunohistochemistry and histology [4]. Pigment granules were not visible using these methods, so were manually added to *O. wendtii* in Blender, as consistently as possible with histological sections of light-adapted tissue [4]. Composite models were digitally cut along a vertical (proximal-distal/oral-aboral) plane, which was rotated through 180° in 10° increments around the base of the photoreceptor distal expansion, the likely primary sensory surface (Figure 2D-F, Table S3). Angular aperture –the widest angle of exposure to incident light at the base of this expansion, to include the entire photosensitive surface – was measured at each increment in ImageJ [29] and plotted using sphereplot in R [30,31] (Figure 2G-I). *Ophiocoma wendtii* was measured both with pigment granules and without. Pigment identity is unknown, but light-responsive chromatophores in *Diadema antillarum* and pigment cells in *Ophiocomina nigra* contain melanin [32,33]. Melanin has a broad-spectrum absorption profile and absorbs strongly in shorter wavelengths, including in *O. nigra* [33]. We measured only direct incident light, excluding possible transmission through the densely packed pigment.

Fields of view were round to elliptic (Figure 2G-I). That of *Ophiocoma wendtii* (mean angular aperture 67.9°; SD ±3.19; Table S3) was substantially smaller than *O. pumila*, (105.6°; SD ±10.55; Table S3). The pigment is likely responsible: when granules were removed, angular aperture in *O. wendtii* increased (118.4°; SD ±6.45; Table S3). Measurements assumed skeletal structures fully occluded light, so without pigmentation, apertures could be wider. We can approximate the angular sensitivity function from aperture width, if we assume it to be Gaussian and that 99% of detectable light enters through the aperture. This gives estimated acceptance angles of 31° (SD ±4.88) for light-adapted *O. wendtii*, 48° (SD ±7.11) for *O. pumila,* and 54° (SD ±5.74) for dark-adapted *O. wendtii* (Table S3).

Transcriptomic analysis

To identify molecular components potentially mediating vision, transcriptomes were sequenced from light-adapted *O. wendtii* and *O. pumila*, producing 1,901,954 and 2,166,115 transcripts, respectively. Non-coding transcripts were filtered out (TransDecoder v5.5.0) [34], leaving 105,757 and 106,403

transcripts. Mapping rates were low (15-20%). BLAST and phylogenetic methods identified potential opsins (Figure 3, Table S4). Thirteen sequences were recovered in *O. wendtii*, spanning six classes of opsins [35]: three rhabdomeric, one ciliary, four Go, one RGR, two neuropsins and two bathyopsins. In *O. pumila* six candidates were identified: two rhabdomeric, two Go, one neuropsin, and one chaopsin. Preliminary searches identified major components of the Gq-mediated transduction pathway in both species [36], except InaD and G-γ SU (e-value threshold 10^{-6}, Table S4). Some were indistinguishable using BLAST and shared the same transcript as a top hit (e.g. TRP and TRPL; PDEα SU and PDEβ SU).

Discussion

Visually guided behaviour

We present the first unequivocal evidence that *O. wendtii* has extraocular vision, likely mediated by photoreceptors expressing (at least) r-opsin. *Ophiocoma wendtii* orients toward both discrete and continuous visual stimuli, constituting low-resolution vision [37]. Although strongly light-averse, *O. pumila* was disoriented. Both express at least two r-opsins and requisite phototransductive components [36], though *O. wendtii* returned one additional r-opsin. A candidate c-opsin was also recovered in *O. wendtii*, but reactivity to c-opsin Sp-Op1 antibodies (previously applied to brittle stars [38]) was sparse in arm plates, spines and tube feet [4]. Other opsin classes of opsin differ between the two species, but are rarely linked to vision (e.g. Go-opsins [39]; cnidopsins [40]). Rhabdomeric photoreceptors are implicated in vision in other echinoderms [10,11] and r-opsin-reactive cells are very numerous in *Ophiocoma* (though opsins may also have non-visual functions [41]). Overall, we suggest that vision in *O. wendtii* is likely mediated by r-opsin.

Spatial resolution of a dark stimulus of 30–50° would be coarse relative to suggestions in some other echinoderms [2,3,16,18], but consistent with preliminary investigations in *Ophiocoma wendtii* [4] and recent results in *Diadema africanum* [1]. The reef habitats of *Ophiocoma* are bright, crowded, and complex, and the nearest contrast-providing object is often within one metre. *Ophiocoma wendtii* is also generally larger, found in denser habitats and under larger rocks than *O. pumila* [42; pers. obs.].

When exposed during daylight, the two behave differently: *O. wendtii* moves rapidly across the substrate to shelter beneath nearby objects, whereas *O. pumila* buries itself or retreats into small crevices *in situ* (pers. obs.). Coarse resolution in *O. wendtii* may therefore meet its ecological needs (Figure 1D).

Factors besides resolution may affect orientation in *Ophiocoma wendtii*. These stimuli differ in several properties, including local contrast. Following transformation in AcuityView [43], the 25° phototactic stimulus appears more prominent than the 50° continuous visual stimulus (Figure S4). Other optical and neural factors may also alter detection. Conversely, *O. wendtii* may detect, but ignore, smaller stimuli. Sympatric *Ophiocoma* maintain separate niches partly through shelter selection, with *O. wendtii* occupying larger crevices than *O. pumila* [42]. Thus, *O. wendtii* may only target larger objects.

Changing responsiveness: a role for chromatophores?

Loss of orientation in *Ophiocoma wendtii* at night is intriguing and cannot be conclusively attributed to light intensity, motivation or circadian processes, and r-opsin reactivity persists in dark-adapted arms. The chromatophores may facilitate vision in *O. wendtii* by providing screening pigment; the substantial difference in estimated fields of view between *O. wendtii* including and excluding pigment could at least partially explain the behavioural shift if these estimates are accurate. *Ophiocoma pumila* also possesses thousands of putative rhabdomeric photoreceptors [4], expresses multiple opsins, and avoids light, but lacks chromatophores and did not orient to presented stimuli. While *O. pumila* may respond to other, untested, stimuli, a screening mechanism is required for directional photoreception and vision [37]. We suggest that chromatophores are an exaptation to a pre-existing photoreceptor network, enabling spatial resolution. Within Ophiocomidae, chromatophores seem to be derived and are only reported in a few species [9,44] (at least one, *O. scolopendrina*, orients to distant shadows when light-adapted [Michiels and Anthes, pers. comm.]). The photoreceptors may ancestrally fulfill a non-visual, but nonetheless vital, task such as exposure avoidance as in *O. pumila*. Adding a screening pigment could unlock the potential for spatial resolution. Chromatophores may serve an unrelated

primary purpose and indirectly impact photoreception; losing vision at night seems maladaptive, perhaps indicating a different principal function. Chromatophore contraction probably increases light availability to photoreceptors at night [21]: dark-adapted animals are still negatively phototactic, and are more sensitive [9,22]. Pigmentation also increases contrast against sandy substrate, possibly impairing crypsis during the day. Chromatophores may, directly or indirectly, enhance diurnal predator avoidance without compromising sensitivity, crypsis and light-avoidance at night. This is the first example of colour-change controlling vision, raising interesting possibilities for other presumed non-visual taxa both within echinoderms and beyond. There are clear parallels with light-responsive pigment migration in arthropod eyes and cephalopod chromatophores, for example.

Integration and processing

Integrating information from thousands of dispersed photoreceptors seems a considerable challenge. Other animals with radial symmetry or dispersed photoreceptor systems, such as cnidarians, cephalopods or arthropod larvae, may face similar obstacles [45,46]. Given the complex and highly flexible body shape of brittle stars, integration must differ from the proposed whole-body "compound eye" in spherical echinoids [47]. Axons converge into bundles before joining a network ultimately innervated by the radial nerve cord [4]. Adjacent cells could spatially or temporally summate to amplify signal, or act antagonistically to facilitate edge detection. The brittle star nervous system is not centralized; each arm appears to act and react autonomously, with the nerve ring coordinating them, rather than centralising processing [48]. Signal integration may occur within individual arms, with locomotory responses coordinated centrally.

Reviewing recorded experiments showed differing locomotion between species. *Ophiocoma wendtii* (n=62) usually moved with two leading arms ('reverse rowing' [49]), whereas *O. pumila* (n=37) moved with a single outstretched leading arm (χ^2=22.8, p<0.0001; Figure 1C). Reverse rowing was more common among *O. wendtii* that oriented to the stimulus, but this remains equivocal (χ^2=3.3, p=0.067). Nevertheless, it suggests signal comparison between adjacent leading arms could facilitate orientation.

Similar analysis on test orientation in sighted sea urchins would provide further insight. Alternatively, animals may respond directly to habitat heterogeneity using temporal firing patterns. High photoreceptor frequency coupled with rapid movement (up to 0.25 ms^{-1}) could result in individuals detecting 'flicker' in regions of contrast (indicating structural heterogeneity) and targeting these. However, animals lacked obvious rhythmic movements typical of horizontal scanning phototaxis [50].

Evolution of echinoderm vision

Vision exists in at least three of the five extant echinoderm classes [1,16,20,current study]. Rhabdomeric opsins are implicated in sea urchins, sea stars and brittle stars [1,11, current study,16]. Vision in *Ophiocoma* is starkly different to sea stars, which sport discrete eyes and actively hunt prey, perhaps exerting greater selective pressure for sharper vision. However, similarities with sea urchins emerge: rhabdomeric photoreceptors within skeletal pores, and light-induced chromatophore movement , shared by diadematid sea urchins (the only group with vision explicitly demonstrated [1]). These prompt questions of homology: is a (non-visual) extraocular photoreceptor system present throughout Eleutherozoa, and have sea urchins and brittle stars evolved similar visual systems independently by simply adding chromatophores? Holothurians, sister to echinoids, and crinoids, the most plesiomorphic living echinoderms, could be key to solving this evolutionary puzzle.

Conclusions

We present the first experimental evidence for spatial vision in brittle stars and propose the most detailed mechanism yet for extraocular vision. Dispersed photoreceptors appear to mediate orientation to visual stimuli in *Ophiocoma wendtii*, but *O. pumila* did not respond. One key functional difference may be chromatophores in *O. wendtii*, potentially facilitating spatial resolution via exaptation by screening pre-existing photoreceptors. This bears striking resemblance to diadematid sea urchins, which may also exploit chromatophore movement around a dispersed photoreceptor network.

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Author contributions

Conceptualization: LSR, EUL; Methodology: All authors; Software: JDK, LSR; Validation: All authors; Formal Analysis: LSR, JDK, EKL; Investigation: LSR, EKL; Resources: All authors; Writing – Original Draft: LSR; Writing – Review and Editing: All authors; Visualisation: LSR, EKL; Funding Acquisition: LSR, EUL.

Declaration of Interests

The authors declare no competing interests.

Figure 1 – Light-responsive behaviour in *Ophiocoma*. A, Orientation experiments and stimuli. Animals were placed at the centre of an area and presented with one of three stimuli, providing phototactic (P), discrete visual (DV) or continuous visual (CV) cues. **B**, Orientation of animals at first contact with the arena wall, divided by species and stimulus type. Stimulus period is 50° (zero crossing for the continuous visual stimulus). While *Ophiocoma pumila* did not clearly orient to any of the stimuli, *O. wendtii* oriented to all three (see Results and Supplemental Files S1-4 for all statistical analyses). Central arrows show mean vector direction and length; blue rings show circular standard deviation for mean vector direction. See Figure S2 for additional stimuli. **C**, Locomotory mode during orientation experiments by species and success/failure to orient to the stimulus (all stimulus types). *Ophiocoma pumila* overwhelmingly used a single leading arm, and *O. wendtii* used two. **D**, Scene from the collection site at Isla Solarte, Panama, transformed in AcuityView [43] at the finest (30°) and coarsest (50°) spatial resolutions supported by experimental results. See also Table S1-2 and Figure S1-4.

Figure 2 – The photoreceptor system and role of chromatophores in *Ophiocoma*. A-C, Composite models of photoreceptors (in colour), skeletal elements (grey) and pigment granules (brown) in live position were reconstructed from confocal and tomographic image stacks and histological sections (**D**, inset), respectively. **D-F**, Composite models were cut in the vertical plane and angular aperture (in white) was measured at 10° increments around the base of the distal expansion of each photoreceptor (yellow). **G-I**, Apertures were used to plot estimated fields of view for *Ophiocoma wendtii* (**A,D,G**), *O. pumila* (**B,E,H**) and dark-adapted *O. wendtii* (**C,F,I**). The presence of pigmentation granules (brown) in *O. wendtii* appears to substantially limit angular apertures (**G**). For full results and estimated acceptance angles, see Table S3. Scale bars: A-C, 25 µm; D-F, 10 µm. See also Figure S3.

Figure 3 – Candidate opsins recovered from transcriptomes of *Ophiocoma wendtii* and *O. pumila* arm segments. Opsins belonging to six and four classes were recovered from *O. wendtii* and *O. pumila* respectively, including multiple r-opsins in both. In most cases echinoderm, asterozoan and ophiuroid opsins formed clades, with the exception of two neuropsins. A full interactive tree can be found at https://itol.embl.de/tree/12861118213118011547676392#. See also Table S4. Silhouettes: Phylopic.

Table 1 Results of orientation experiments – Maximum likelihood analyses on relative exit bearings. φ, mean direction; κ, concentration from circ_MLE; P, phototactic stimulus; DV, discrete visual stimulus; CV, continuous visual stimulus.

*These experiments were conducted in natural darkness at night, with dark-adapted animals.

[¥]These experiments were conducted under artificial light at night, with dark-adapted animals.

[§]These experiments were conducted during the day, with dark-adapted animals.

**1 animal that did not complete the task was excluded from analyses.

Species	**Stimulus (size)**	**n**	**Best model (ΔAIC*c* = 0)**	**Model type (φ_1, φ_2)**	**Concentration (κ_1, κ_2)**
O. wendtii	P (30°)	40	M1	Uniform	0
O. wendtii	P (50°)	40	**M2B**	**Unimodal (355°)**	**43.5**
O. wendtii	DV (50°)	52	**M4B**	**Axial bimodal (3°, 183°)**	**45.844, 0.173**
O. wendtii	CV (50°)	44	**M3B**	**Axial bimodal (178°, 358°)**	**0.983, 4.457**
O. wendtii	P (50°)*	44	M1	Uniform	0
O. wendtii	DV (50°)*	38	M1	Uniform	0
O. wendtii	DV (50°)[¥]	50	M1	Uniform	0
O. wendtii	DV (50°)[§]	39	M1	Uniform	0
O. pumila	P (50°)	40**	M1	Uniform	0
O. pumila	DV (50°)	48	**M4B**	**Axial bimodal (176°, 356°)**	**0.705, 18.235**
O. pumila	CV (50°)	44	M1	Uniform	0

STAR Methods

Lead contact and materials availability

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Lauren Sumner-Rooney (lauren.sumner-rooney@oum.ox.ac.uk). Transcripts are available at https://osf.io/9fz3b/files/ and on NCBI (XXXXXXX). Code for analysing behavioural and morphological data is available as R markdown files at https://github.com/JohnKirwan/Ophiocoma_orientation .

Experimental model and subject details

Specimens

Wild adult *Ophiocoma wendtii* and *O. pumila* were collected from shallow reef rubble at Punta Hospital, Isla Solarte, Bocas del Toro, Panama (9°19'44.4"N, 82°12'21.6"W, 0–3 m) in May-June 2017 (main behavioural experiments and tissue sampling) and June-July 2019 (dark-adaptation experiments on *O. wendtii*), and housed in outdoor flow-through unfiltered seawater aquaria under a natural 12:12 hr light:dark cycle at the Smithsonian Tropical Research Institute Bocas del Toro research station on Isla Colón, Panama. Sex could not be determined. Animals ranged from disc diameter 2 cm to 3 cm. Animals that autotomized arms during or following collection were excluded from behavioural experiments. Specimens were collected under MiAMBIENTE research permits SE/A-10-17, SE/A-35-17 (main behavioural experiments and tissue sampling) and SE/A-48-19 (dark-adaptation experiments on *O. wendtii*) and preserved material was exported for further study under MiAMBIENTE export permit SEX/A-42-17.

Method details

Behavioural experiments

Spatial resolution

Animals were placed at the centre of a circular arena filled with unfiltered seawater (diameter 60 cm, depth 50 cm), with a target presented at one edge (Figure 1). We recorded animals' movement from the centre to the edge of the arena and measured angular bearings (both absolute and relative to the centre of the stimulus) when the disk made initial contact with the side. We used these headings (relative to the stimulus) to represent animal direction at each trial because *Ophiocoma* characteristically travel linearly from the centre, such that the headings indicate their route (Figure S2H). We rotated the position of the stimuli, cleaned the arena floor and walls, and replenished water between trials. Experiments were performed in natural light beneath a diffuser. Stimuli consisted of black vertical bars on a white background (phototactic stimulus), nested black and white vertical bars on an intermediate grey background (discrete visual stimulus), and a continuous stimulus with a central dark region grading into brighter flanks and beyond these into the intermediate background (continuous visual stimulus; Figure 1). To ensure that the printed patterns reflected the desired amount of light, the reflectance of a series of test pieces was measured using an RPS900-R spectroradiometer (International Light, MA, USA). Individuals were presented with each stimulus type only once and were tested up to twice daily.

To test whether animal tracks were straight, we tracked *O. wendtii* in 29 control trials and 23 trials with the visual stimulus, chosen at random. Paths were tracked from video recordings at 0.05 second intervals from start of locomotion until reaching the arena wall using custom software [51] within MatLab. Plots of these tracks (Figure S2H) show that tracks are quite straight, substantiating our metric.

Phototaxis

To test for negative phototaxis in dark-adapted *Ophiocoma wendtii* (n=28, at night) and light-adapted *O. pumila* (n=40, during the day), animals were placed, individually, centrally at the bottom of a seawater tank, one half of which was exposed to ambient light, and the other half shaded. Movement into either half was recorded for up to 60 seconds, or until an arm touched the tank wall, if this

occurred first. Movement towards the shaded half was considered evidence of phototaxis. Tanks were cleaned and reoriented between trials.

To test for functionality of described ventral photoreceptors in *Ophiocoma wendtii*, animals (n=19) were placed at the centre of a fully illuminated tank in which one half of the base was covered with black plastic on the outer face. Their movement into the dark or the light half was recorded as above. Tanks were cleaned, refreshed and reoriented between trials.

Light vs. tactile stimuli

To determine whether light or tactile stimuli were dominant cues in sheltering behaviour, individuals of *Ophiocoma wendtii* (n=23) were placed in a tank where one half was illuminated and had a transparent acrylic 'false bottom' suspended 1 cm above the base, providing an unshaded crevice. The other half had no false bottom but was shaded Movement was monitored for up to 60 seconds, or until an arm contacted the wall of the tank. Tanks were cleaned, replenished and reoriented between trials.

Immunohistochemistry

Light- and dark-adapted arm segments and arm tips from *Ophiocoma wendtii* and *O. pumila* were tested for reactivity to sea urchin rhabdomeric (Sp-Op4) opsin. Segments were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 30 minutes at room temperature before washing in PBS and decalcifying in 2% ascorbic acid in 0.15 M sodium chloride solution for 72 hours [adapted from 11]. Samples were rinsed in PBS and stored in 0.05% sodium azide in PBS. Tissue used for sectioning was rinsed in PBS for 20 minutes before embedding in 4% agarose gel. Thick sections (150 µm) were taken using a Leica VT 1200S vibratome. Arm segments and sections were washed in PBS and 0.1% Triton X (PBST) and blocked in PBST and 0.5% normal goat serum (NGS) for one hour before incubation with anti-acetylated tubulin (1:200) and anti-Sp-Opsin4 (*Strongylocentrotus purpuratus*, 1:50) overnight, all at room temperature. This antibody binds to discovered opsins in other brittle stars, which have high sequence similarity to homologs in *S.*

374 *purpuratus* [6,36]. Specimens were then washed in PBST and incubated with either Alexa Fluor 633
375 goat anti-mouse (1:500) or Alexa Fluor 488 goat anti-rabbit (1:500) for at least three hours at room
376 temperature, rinsed with PBST and visualized on a Leica TCS SPE confocal laser scanning microscope.
377 Images and image stacks were captured using Leica Application Suite Advanced Fluorescence v.2.6.3
378 and prepared in Fiji [29].

379 ***Photoreceptor angular apertures***

380 Dorsal (aboral) arm plates from *Ophiocoma wendtii* and *O. pumila* were scanned by [4], using
381 synchrotron X-ray tomography at the TOMCAT beamline (Swiss Light Source, Paul Scherrer Institut,
382 Villigen, Switzerland). Samples were scanned using an X-ray energy of 20 keV, 1501 projections, and
383 an exposure time of 250 ms. Voxel size was 1.75 µm (x, y and z) and models were reconstructed in
384 AMIRA (FEI Visualization Science Group). Eight photoreceptor cells were reconstructed from image
385 stacks of immunostained cells in Amira and aligned with models of arm plate structure generated from
386 synchrotron tomography according to immunohistochemistry and histological sections. For
387 *Ophiocoma wendtii*, pigment granules were modelled in Blender from histological sections. To
388 estimate the field of view of each photoreceptor, combined models in Blender were intersected by a
389 plane in the y (proximal-distal) axis, which was rotated around the centre of each photoreceptor in 10°
390 increments (SF6). The potential aperture width for the photoreceptor was measured at each increment
391 using ImageJ, totaling 18 angles per photoreceptor. These were projected onto the surface of a sphere
392 using the R package sphereplot [30] to plot estimated field of view around the photoreceptor.

393 ***Transcriptomic analysis***

394 *Transcriptome sequencing*

395 Arm segments were taken from individuals of *Ophiocoma wendtii* and *O. pumila* adapted to sunlight
396 (n=3) and dark (n=3) conditions and fixed in RNALater before freezing at -20°C. Total RNA was extracted
397 using a peqGold TriFast Kit (PEQLAB VWR Life Science) following the manufacturer's instructions.
398 Samples were individually homogenized in 1ml TriFast using a Minilys Homogenizer with the Precellys

ceramic kit 1.4/2.8 mm (PEQLAB VWR Life Science). All total RNA samples were checked for purity and quantity using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and a 2100 Bioanalyzer Instrument (Agilent Technologies) with the RNA 6000 Pico Assay.

Quantification and statistical analysis

Behavioural experiments

We used multiple approaches to analyse orientation data. The data were modelled as angles according to the von Mises distribution and counts (of successful orientations or otherwise) with the binomial distribution using the Bayesian probabilistic modelling language Stan [52] and the R package brms [53]. The animals' bearing at each treatment were divvied into those which approached a 72° sector with the target centre at its midpoint (successes) and those which were oriented to all other directions (failures). A joint model incorporated data for each species and two stimulus types (high- and low-contrast visual stimuli). Parameters were estimated over 40 000 draws in four chains using weakly informative priors. Convergence was assessed by visual inspection of the Markov chains and by $\hat{R}$ and N_{eff} statistics and posterior predictive checks were used to test model efficacy.

The likelihood of models representing differing distributions of circular data were compared using circ_MLE [27] within R. Due to relatively small samples sizes (n/K) and the representation of our target models in the tested set in circ_MLE [unimodal; axial bimodal; from 26], we compared model fit using the corrected Akaike information criterion. Absolute and relative terminal bearings were analysed using the circular and circ_MLE packages within R [31]. Raleigh and V tests (with *a priori* μ=0° for the position of the centre of the target) as well as Rao's test were used to test uniformity in the circular package [54].

Transcriptomic analysis

Transcriptome assembly

Light- and dark-adapted reads were used to assembled reference transcriptomes for each species using Trinity (v2.6.6) [34] and the following parameters: --normalize_reads --normalize_by_read_set –trimmomatic. These parameters normalize k-mers of length 25 to a coverage of 20 for each read set. Post assembly, transcripts were filtered and translated using TransDecoder (v2.1), selecting for longest open reading frames of at least 100 amino acids in length.

Opsin identification

Opsin amino acid sequences were identified using BLAST v.2.7.1 [55]. Opsins totaling 169 sequences were compiled from Lowe et al. [10] and Ramirez et al. [35],including several melatonins. The closest matching sequences (with an e-value cutoff of $1e^{-10}$) for each *Ophiocoma* species were combined with the 169 sequences from Lowe et al. [10] and aligned using Mafft v.7 [56]. Alignments were trimmed using trimal [57], homologous regions were removed where gaps were present in more than 20% of the sequences, or the similarity score was below 0.001, unless this removed more than 40% of the original alignment. Phylogenetic trees were constructed using the aligned and trimmed sequences using iqTree with a GTR+gamma substitution model [58–60] and visualized using iTOL [61].

Data and code availability

Transcripts are available at https://osf.io/9fz3b/files/.

Code for analysing behavioural and morphological data is available as R markdown files at https://github.com/JohnKirwan/Ophiocoma_orientation .

Supplemental information

Table S4 – Transcripts matching to known echinoderm opsins in BLASTn search (Excel file). Related to Figure 3.

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