ORGANISMAL BIOLOGY

Pushing the limits of photoreception in twilight conditions: The rod-like cone retina of the deep-sea pearlsides

Fanny de Busserolles,1,2* Fabio Cortesi,1 Jon Vidar Helvik,3 Wayne I. L. Davies,4,5,6 Rachel M. Templin,1 Robert K. P. Sullivan,1 Craig T. Michell,2,7 Jessica K. Mountford,4,5,6 Shaun P. Collin,4,5,6 Xabier Irigoien,2,8,9 Stein Kaartvedt,2,10 Justin Marshall1

Most vertebrates have a duplex retina comprising two photoreceptor types, rods for dim-light (scotopic) vision and cones for bright-light (photopic) and color vision. However, deep-sea fishes are only active in dim-light conditions; hence, most species have lost their cones in favor of a simplex retina composed exclusively of rods. Although the pearlsides, *Maurolicus* spp., have such a pure rod retina, their behavior is at odds with this simplex visual system. Contrary to other deep-sea fishes, pearlsides are mostly active during dusk and dawn close to the surface, where light levels are intermediate (twilight or mesopic) and require the use of both rod and cone photoreceptors. This study elucidates this paradox by demonstrating that the pearlside retina does not have rod photoreceptors only; instead, it is composed almost exclusively of transmuted cone photoreceptors. These transmuted cells combine the morphological characteristics of a rod photoreceptor with a cone opsins and a cone phototransduction cascade to form a unique photoreceptor type, a rod-like cone, specifically tuned to the light conditions of the pearlsides’ habitat (blue-shifted light at mesopic intensities). Combining properties of both rods and cones into a single cell type, instead of using two photoreceptor types that do not function at their full potential under mesopic conditions, is likely to be the most efficient and economical solution to optimize visual performance. These results challenge the standing paradigm of the function and evolution of the vertebrate duplex retina and emphasize the need for a more comprehensive evaluation of visual systems in general.

INTRODUCTION

The retina of most vertebrates is categorized as being duplex, comprising both rod and cone photoreceptors. Each photoreceptor type differs in morphology and function, allowing animals to switch between the two systems and thus maintain vision during most of the daily (24-hour) period. Rods express the highly sensitive rod opsin gene (*rh1*), which mediates vision in dim-light (scotopic) conditions, whereas cones express up to four classes of visual pigment genes ([short-wavelength sensitive (*sws1* and *sws2*), medium-wavelength sensitive (*rh2*), and long-wavelength sensitive (*lws*), which mediates vision and color discrimination in bright-light (photopic) environments (1)]. In mesopic conditions, such as during twilight, both rods and cones are active and contribute to vision, but neither work at optimal levels (2, 3). Depending on whether a species is diurnal, nocturnal, or crepuscular, the proportion of each photoreceptor type may vary to maximize visual performance (2). In a few extreme cases, an entire photoreceptor type and function is lost, resulting in a simplex retina, such as the pure cone or pure rod retinas of diurnal lizards and deep-sea fishes, respectively (4).

The classification of photoreceptors into rods and cones is, however, not always straightforward, and some intermediate forms of photoreceptors exist and exhibit morphological, electrophysiological, and/or molecular characteristics of both cell types. This is most notable in squamate reptiles (5–7), amphibians (8), lampreys (9), and skates (10) (Table 1). These intermediate forms were first observed in snakes and geckos by Walls (4), which led to the “transmutation” theory that suggested that rods and cones could evolve or “transmute” from one type to another, via a series of intermediate states, as a result of a major ecological shift in activity pattern. For instance, the gecko ancestor was diurnal and accordingly had a pure cone retina, but cones in modern nocturnal geckos have transmuted into rod-like receptors to regain vision under scotopic light conditions (6). To our knowledge, the study presented here is the first case of photoreceptor transmutation in teleost fishes, specifically the pearlsides, *Maurolicus* spp. The results suggest that photoreceptor transmutation is not always an evolutionary transition to regain a lost function but also an adaptation to optimize vision in a specific ecological niche, in this case, the twilight environment.

Pearlsides are deep-sea teleost fishes that live in the upper part of the mesopelagic zone (~200 m) and vertically migrate to feed on zooplankton present in the upper layers of the ocean (0 to 100 m). Contrary to other vertically migrating fishes that ascend to feed throughout the night, pearlsides mostly migrate to the surface at dusk and dawn (twilight) and spend the night at greater depths without foraging (11). This behavior is best explained by the “antipredation window” theory, which suggests that it is advantageous to spend short periods of time foraging at twilight when light levels are sufficient for prey detection but low enough to hide from predators (12). Whereas animals that are active during this time period usually use a combination of rods and cones for vision (2), pearlsides, like most deep-sea teleost fishes, have a morphologically pure rod retina implying an *rh1*-based scotopic visual system (13), which is at odds with their daily behavior. To explore this paradox, the visual system of two species of pearlsides, *Maurolicus*

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*Corresponding author. Email: f.debusserolles@uq.edu.au

1Queensland Brain Institute, The University of Queensland, Brisbane, Queensland 4072, Australia. 2Red Sea Research Center, Biological and Environmental Sciences and Engineering Division, King Abdullah University of Science and Technology, Thuwal 23955-6900, Saudi Arabia. 3Department of Biology, University of Bergen, Bergen 5020, Norway. 4The Oceanes Institute, The University of Western Australia, Crawley, Western Australia 6009, Australia. 5School of Biological Science, The University of Western Australia, Crawley, Western Australia 6009, Australia. 6Department of Environmental and Biological Sciences, University of Eastern Finland, Yliopistokatu 7, FI-80101 Joensuu, Finland. 7Institute for Marine Research, AZTI-Tecnalia, Herrera Kaia, Portugaledea z/g, 20110 Pasaia (Gipuzkoa), Spain. 8IKERBASQUE, Basque Foundation for Science, Bilbao, Spain. 9Department of Biosciences, University of Oslo, Oslo 0316, Norway. 10Department of Biological and Environmental Sciences, The University of Western Australia, Crawley, Western Australia 6009, Australia. 

*Corresponding author. Email: f.debusserolles@uq.edu.au
RESULTS AND DISCUSSION

Pearlside retinal transcriptomes

Retinal transcriptome sequencing of both species revealed the expression of three distinct opsin genes: rh1 (usually restricted to rods) and, surprisingly, two cone opsins (rh2-1 and rh2-2; Fig. 1A and fig. S1). The rh2 genes were expressed at similar levels and comprised ~99% of the total opsin expression in the retina (Fig. 1B and table S1), suggesting that pearlsides rely almost exclusively on their cone photopigments for vision, a strategy expected in animals devoid of rods. This is further supported at the amino acid level. In general, amino acid sites 122 and 189 confer photoreceptor specificity in vertebrates, with Gln122 and Pro189 sites being cone-specific and Glu122 (except Gln122 in deep-sea fish rods) and Ile189 sites being rod-specific (1). Whereas the pearlside rh1 showed typical deep-sea rod specificity (that is, Gln122 and Ile189), pearlside rh2s showed the cone typical Gln122 and Pro189 combination (fig. S2).

At the level of the phototransduction cascade, the Ga subunit of transducin (critical for signal transduction; Fig. 2A and fig. S3) and arrestin (involved in pigment recovery; Fig. 2B and fig. S4) were also found to be mainly cone-like (1) and were expressed at a ratio of around 99% cone to 1% rod (tables S2 and S3), strongly supporting the use of a predominantly cone-based visual system, a first in adult teleost fishes. A detailed analysis of the transcriptomes revealed that pearlsides express three Ga transducins: one rod transducin (gnat1) and two cone transducins (gnat2-1 and gnat2-2; Fig. 2A). Although with low support values, the pearlside cone transducins form their own clade, indicating a lineagespecific duplication that might have facilitated photoreceptor transmutation (fig. S3). Alternatively, the gnat-2 duplication might have a much deeper phylogenetic origin that has so far been missed (1, 14) and, as for the opsins (15), would imply that the evolutionary history of these genes is more complex than previously thought. The transcriptomes also contained four arrestin genes: two rod arrestins (saga and sagb) and two cone arrestins (arr3a and arr3b; Fig. 2B). In the medaka (Oryzias latipes), rod arrestin orthologs, saga and sagb (fig. S4), are expressed in different parts of the photoreceptor. Saga is expressed in the outer segments and mediates phototransduction, whereas sagb is expressed in the synapses (function unknown) (16). It is therefore possible that only saga is involved in phototransduction in pearlsides, explaining the presence of two rod arrestin genes but only one rod opsin.

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**Table 1. Characteristics summary (morphology, opsin, phototransduction cascade, and electrophysiology) of the transmuted photoreceptors of different species compared to true rods and true cones.** Lizard data are for the genus Anolis. Lamprey is the sea lamprey Petromyzon marinus. Salmonander is the tiger salamander Ambystoma tigrinum. Pearlside is the Mueller’s pearlside M. muelleri. The snake with the cone-like rods is the diurnal garter snake Thamnophis proximus. The snake with the rod-like cones data are for the nocturnal genus Hypsiglena. The gecko is the nocturnal Tokay gecko Gekko gekko. The skate is the genus Raja. R, true rod; C, true cone; n.a., not available; poly, polysynaptic.

<table>
<thead>
<tr>
<th>Photoreceptor characteristics</th>
<th>True rod (1, 19)</th>
<th>True cone (1, 19)</th>
<th>Cone-like rod</th>
<th>Rod-like cone</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Outer segment shape</strong></td>
<td>Long, rod-shaped (cylindrical)</td>
<td>Short, cone-shaped (distally tapering)</td>
<td>C</td>
<td>R</td>
</tr>
<tr>
<td><strong>Outer segment discs</strong></td>
<td>Individual sealed disc, separated from the plasma membrane</td>
<td>Discs continuous with the plasma membrane (open)</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td><strong>Incisure</strong></td>
<td>Present</td>
<td>Absent</td>
<td>n.a.</td>
<td>C</td>
</tr>
<tr>
<td><strong>Paraboloid</strong></td>
<td>Absent</td>
<td>Present</td>
<td>C</td>
<td>R</td>
</tr>
<tr>
<td><strong>Oil droplet</strong></td>
<td>Absent</td>
<td>Sometimes present</td>
<td>R</td>
<td>n.a.</td>
</tr>
<tr>
<td><strong>Synaptic ending</strong></td>
<td>Small, spherical, oligosynaptic</td>
<td>Large, conical, flat-end base, polysynaptic</td>
<td>n.a.</td>
<td>C</td>
</tr>
<tr>
<td><strong>Opsin</strong></td>
<td>rh1</td>
<td>sws1, sws2, lws, rh2</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td><strong>Spectral sensitivity (nm)</strong></td>
<td>480–510</td>
<td>rh2, 450–530 sws1, 360–440 sws2, 400–450 m/lws, 510–560</td>
<td>482</td>
<td>500</td>
</tr>
<tr>
<td><strong>Phototransduction cascade</strong></td>
<td>Cone-like</td>
<td>Rod-like</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td><strong>Cell physiology</strong></td>
<td>Rod properties (high sensitivity)</td>
<td>Cone properties (fast, never saturate)</td>
<td>n.a.</td>
<td>R</td>
</tr>
</tbody>
</table>

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muelleri from the Norwegian fjords and Maurolicus mucronatus from the Red Sea, was investigated.
shows the almost exclusive use of cone opsins in pearlside vision. Table S4. (numbers are shown in fig. S1. Pearlside-specific accession numbers are given in opsins gene class was not present in the pearlside transcriptome. The ancestral rh2 function may be partitioned between the two rh2 wavelength sensitive; Fig. 1. Vertebrate opsin gene phylogeny and pearlside opsin gene expres-
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diated genes (\(\text{rh1-1} \quad \text{rh1-2} \quad \text{rh2-1} \quad \text{rh2-2}\)) contained three
feature of the pearlside visual system. Instead, as was suggested for rh1-
duplicated genes (\(\text{rh1-1} \quad \text{rh1-2} \)).

Pearlside spectral sensitivities

Vertebrates distinguish colors by comparing the visual input between two photoreceptors that are sensitive to different parts of the light spectrum and are active at the same time (17). Accordingly, the expression of two rh2 opsins, presumably with different peak spectral sensitivities (\(\lambda_{\text{max}}\)) and expressed in different photoreceptors, could confer color vision to pearlside. However, the rh2 genes in both species showed very few amino acid differences, with none occurring at key spectral tuning sites (fig. S2) (18–20). Therefore, the two rh2 opsins are likely to have similar \(\lambda_{\text{max}}\) values, making color vision an unlikely feature of the pearlside visual system. Instead, as was suggested for rh1-duplicated genes (\(\text{rh1-1} \quad \text{rh1-2}\)) in the zebrasfish (\textit{Danio rerio}) (21), the ancestral rh2 function may be partitioned between the two rh2 copies in pearlside. After comparing the synonymous substitution rate in the coding region [coding DNA sequence (CDS)] of the \(\text{rh2}\) paralog to that in the 3’ untranslated regions (3’UTRs), it seemed plausible that gene conversion might have acted to conserve the rh2 spectral sensitivities (15, 22). The occurrence of gene conversion was confirmed by reconstructing a phylogeny using only the information contained in the 3’UTR and the last 50 base pairs (bp) of exon 5, which revealed that the rh2 duplication occurred in the common ancestor of pearlside (fig. S5).

In vitro expression was subsequently attempted to determine the spectral sensitivities of the \textit{M. muelleri} opsins using a vitamin A\(_1\) chromophore (that is, 11-cis-retinal chromophore). Although some deep-sea fishes have been found to use both A\(_1\) and A\(_2\)-based chromophores for vision (23), this seems not to be the case for pearlside based on a lack of cytochrome P450 family 27 subfamily \textit{c} member 1 (\textit{cyp27c1}) expression in their transcriptomes. The Cyp27c1 enzyme has been shown to convert vitamin A\(_1\) to vitamin A\(_2\) in zebrafish, leading to the replacement of 11-cis-retinal with 11-cis-3,4-didehydroretinal: This change results in a long-wavelength shift of the spectral peak for visual photopigments (24). In vitro ultraviolet (UV)–visible spectrophotometry was successfully performed for the rod opsin pigments expressed in \textit{M. muelleri} (rh1) and human (RH1; control), with peak spectral sensitivity (\(\lambda_{\text{max}}\)) values of 494 and 495 nm, respectively (Fig. 3A), closely matching the predicted \(\lambda_{\text{max}}\) values of 496 nm for \textit{M. muelleri} and a previous spectral study of the human RH1 photopigment (25). The predicted value of \textit{M. muelleri} rh1 (\(\lambda_{\text{max}} = 496\) nm) was calculated on the basis of two other deep-sea teleost fishes from the same family, namely, \textit{Argyropelecus aculeatus} and \textit{Argyropelecus gigas}, where their spectral peaks were determined to be 477 nm using microspectrophotometry (26). In comparison to these two fishes, the spectral peak of the \textit{M. muelleri} rh1 is long-wavelength–shifted by 19 nm, which corresponds to Phe\(_{261}\)Tyr (+10 nm) and Ser\(_{292}\)Ala (+9 nm) substitutions (20, 26). Unfortunately, both \textit{M. muelleri} rh2-1 and rh2-2 pigments did not re-
generate or reconstitute with 11-cis-retinal in vitro, despite numerous attempts. This is not uncommon with photopigments studied in vitro, where technical artefacts, such as misfolding of overexpressed proteins, may cause experiments to be unsuccessful (27–29). Nonetheless, logical predictions may be made on the basis of published data, as well as those present in this study (Fig. 3B). Although most rh2 pigments yield spectral peaks between 470 and 510 nm (20), those found in deep-sea species are typically short-wavelength–shifted. For example, the deep-sea teleost \textit{Scopelarchus analis} has an rh2 photopigment with a \(\lambda_{\text{max}}\) value of 444 nm (30, 31), and the elephant shark (\textit{Callorhinichus milii}) has an rh2 photopigment with a \(\lambda_{\text{max}}\) value of 442 nm (32); the former was measured by in vivo microspectrophotometry, and the latter was measured by in vitro UV-visible spectrophotometry. When comparing the known tuning sites for rh2 photopigments (18, 20) identified in \textit{M. muelleri} (this study) and \textit{S. analis} (30), the only difference found was at site 300 with Ile and Leu in \textit{M. muelleri} (for both rh2-1 and rh2-2) and \textit{S. analis}, respectively (fig. S2). From an extensive comparative study of spectral tuning in deep-sea fish visual pigments (26), a Leu\(_{300}\)Ile substitution results in a short-wavelength shift of 3 nm. Thus, for \textit{M. muelleri}, the rh2 pigments are predicted to have spectral peaks at 441 nm (Fig. 3B). When comparing known rh1 tuning sites between pearlside species, no differences were observed; hence, rh1 in \textit{M. mucronatus} is predicted to have a \(\lambda_{\text{max}}\) value of 494 to 496 nm (that is, measured and predicted). When rh2 pigments were compared between \textit{Maurolucic} species, only a single difference between known tuning sites was found at site 299, with Ala in \textit{M. mucronatus} and Ser in \textit{M. muelleri}. Analyses
of spectral tuning in deep-sea fish visual pigments indicate that a Ser<sup>390</sup>Ala substitution results in a short-wavelength shift of 6 nm (26); therefore, both rh2 pigments of <i>M. mucronatus</i> are predicted to exhibit a spectral peak at 435 nm (Fig. 3B).

**Distribution and morphology of <i>M. muelleri</i> photoreceptors**

Given the high levels of cone-specific pigment expression, it was expected that at least a few cone photoreceptors (based on morphological criteria) would occur in the pearlside retina. However, similar to previous findings (13), no cone-like cells were found, and RNA in situ hybridization (R-ISH) using opsin-specific probes confirmed that both <i>rh1</i> and <i>rh2</i> genes were expressed only in rod-like photoreceptors in <i>M. muelleri</i> (Fig. 4). Moreover, R-ISH and anti-rhodopsin (rod pigment) immunohistochemistry (IHC) corroborated the transcriptomic data, with both <i>rh2</i> genes being expressed collectively in about 99% of photoreceptor cells and <i>rh1</i> in the remaining 1% of photoreceptors (Fig. 4).

The density of the <i>rh2</i>-expressing photoreceptors varied across the retina with an increase in cell density in the ventral region (that is, elongated area ventralis; Fig. 4D and fig. S6). This pattern matched the retinal ganglion cell topography (fig. S7), and assuming a horizontal position of the fish in the water column, this high photoreceptor density zone is likely to facilitate the detection of silhouettes situated above the fish against the lighter background of the upper mesopelagic zone (33). The small population of <i>rh1</i>-expressing photoreceptors was confined to the peripheral margins of the retina (Fig. 4B and figs. S8 and S9) and might, therefore, be used to detect motion, although their extremely low level of expression questions their ecological significance.

Subsequent immunofluorescence, transmission electron microscopy (TEM) and 3View-based three-dimensional (3D) reconstruction revealed two types of rod photoreceptors at the morphological level: a rod-like cone (the main cell type expressing <i>rh2</i>) and a “true” rod (labeled with anti-rhodopsin antibodies and expressing <i>rh1</i>) (Fig. 5 and movie S1). Both cell types showed the classic anatomical characteristics of a rod (Table 1): a long cylindrical outer segment, individual sealed discs surrounded by a plasma membrane, incisures (Fig. 5F), and the absence of both a paraboloid and an oil droplet. Structurally, the main...
to determine the expressed in rod-like photoreceptor cells: (i) Pearlside cone opsins found in the two pearlside species, *M. muelleri* (black) and *M. mucronatus* (gray).

differences between the two photoreceptor types were restricted to the vitreated region of the receptors (the nucleus and synaptic terminal). In the true rod, the nucleus was displaced toward the inner retina, whereas its vitreated region of the receptors (the nucleus and synaptic terminal). In *M. muelleri*, the true rod, the nucleus was displaced toward the inner retina, whereas its vitreated region of the receptors (the nucleus and synaptic terminal).

**Likely evolutionary scenario leading to photoreceptor transmutation in pearlside**

Two possible evolutionary scenarios could lead to cone opsins being expressed in rod-like photoreceptor cells: (i) Pearlside cone photo-receptors transmuted to morphologically resemble rod cells or (ii) cone opsin(s) and visual phototransduction genes co-adapted and replaced the conventional rod-based machinery in 99% of all rod cells. The occurrence of about 1% of the true rod photoreceptors expressing a rod opsin, the cone-like synaptic ending of the rod-like cones, and cone-specific amino acid residues of rh2 opsins strongly support the first scenario. However, to demonstrate either scenario, a complete ontogenetic analysis of the pearlside retina, at both molecular and morphological levels, is required (34).

Although photoreceptor transmutation normally occurs following the loss of a photoreceptor type or a specific opsin gene (4, 35, 36), this is unlikely to be the case for pearlside fishes. First, they express both rod and cone opsins and associated phototransduction genes. Second, teleosts, including deep-sea species with pure rod retinas as adults, have both cones and rods at some stage during their development (37). Finally, adult individuals from species within the same family (Sternop-tychidae) have both photoreceptor types (38). Hence, the transmuted rod-like cones of the pearlside are likely to be an ecological adaptation rather than an evolutionary regain of function.

**Ecological significance**

The likely explanation for the peculiar visual system of the pearlside is an adaptation to the surrounding light environment. Pearlsides inhabit...
the upper mesopelagic zone of the ocean where two main sources of light are found: the downwelling light produced by the sun and stars, and bioluminescence produced and emitted by the organisms themselves. However, adaptation to bioluminescent light seems unlikely for the following reasons: (i) Pearlsides are only active in light intensities between 0.2 cd m\(^{-2}\) (full moon) and 127 cd m\(^{-2}\) (sunset/sunrise) (39), that is, in mesopic conditions and/or the lowest intensities of photopic illumination (Fig. 6A), which would severely limit visualization of bioluminescent signals (40). (ii) The predicted peak spectral sensitivity for both rh2 opsins is between \(\lambda_{\text{max}} = 435 \text{ and } 441 \text{ nm in both species (Fig. 3B), which is offset from most bioluminescent emissions at 475 nm (41) but instead matches the blue-shifted twilight spectrum of downwelling light at 450 nm (Fig. 6B) (42). (iii) Although pearlsides are bioluminescent, the unique ventral placement of their photophores (43) suggests that they are used for camouflage via countershading rather than for communication, implying a dire need to visualize downwelling light (44). (iv) Increased photoreceptor and ganglion cell densities in the ventral part of the retina (figs. S6 and S7) indicate that their visual system is optimized to detect shadows of objects situated above the eye rather than perceive bioluminescent signals directly (33). Therefore, the visual system of pearlsides appears to be specifically adapted to the ambient blue-shifted mesopic light conditions in which they live (Fig. 6).

Instead of having two photoreceptor types that do not function at their full potential under mesopic conditions, pearlsides have evolved a more efficient and economical solution by combining properties of both rods and cones in a single cell type to optimize visual performance. Under this premise, transmuting cones to morphologically resemble rods, while retaining the cone pigment and the cone phototransduction cascade rather than the reverse situation, would result in the greatest gain of sensitivity. Specifically, whereas cone photopigments have a higher tolerance to light intensities before reaching saturation and can be very stable, a rod morphology with a larger outer segment, which allows a greater packing of photopigment molecules, would result in a higher photon catch and hence greater sensitivity (1). Furthermore, whereas rh1 peak spectral sensitivities across vertebrates seem to have a lower limit of around 470 nm (26), rh2 peak spectral sensitivities appear more variable, where a comparable short-wavelength-shifted pigment with a \(\lambda_{\text{max}}\) value of 442 nm has previously been described in the deep-sea elephant shark (32). Further studies on the pearlside rod-like cones are, however, needed to verify their predicted spectral sensitivities.

Fig. 5. Morphology of the two photoreceptor types in *M. muelleri*. (A) Schematic of the rod-like cone (yellow; left) and rod (blue; right) drawn from the 3D reconstruction using 3View. OS, outer segment; IS, inner segment; SE, synaptic ending; Di, discs; Mt, mitochondria; ILM, inner limiting membrane; Nc, nucleus. Note the displaced nucleus and synaptic ending in the rod. (B) Immunofluorescence labeling of transverse retinal cryosections. Rod outer segments were labeled with anti-rhodopsin antibodies (red), inner segments with NeuN (white), cell nuclei with 4’6-diamidino-2-phenylindole (DAPI) (blue), and synaptic connections with synapsin (green). Note that NeuN does not usually stain photoreceptor inner segments, but in *M. muelleri*, the inner segments of the rods were strongly labeled compared to the rod-like cones. PRL, photoreceptor layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; c, rod-like cone; r, rod; cse, rod-like cone synaptic ending; rce, rod synaptic ending. Scale bar, 5 \(\mu m\). (C to F) TEM of transverse retinal sections showing the two photoreceptor types (C), the polysynaptic ending of the rod-like cone (D), the oligosynaptic ending of the rod (E), and the sealed discs and incisures of the outer segments (F). The white arrowheads in (D) and (E) show the synaptic ribbons, and the black arrowheads in (F) show the incisures present in the rod-like cone. Scale bars, 2 \(\mu m\) (C) and 1 \(\mu m\) (D to F).
dicted (horizon) at the surface (f, light gray dashed line). Note how the spectral sensitivity of each light at 500 m (e, dark gray dashed line), and moonlight (full moon at 70° elevation above skate). Twilight (w), dawn/sunset/sunrise, and sunlight (a, red line; pre-dicted λ_max = 441 nm; fig. 57), the deep-sea myctophid Symbiophorus evermanni (b, dark gray line; λ_max = 476 nm), and the nocturnal squilfish Neopon samara (c, light gray line; λ_max = 502 nm) along with the relative down-welling vector irradiance spectra (courtesy of S. Johnsons) of their respective light environments: twilight (~6.5° solar elevation) at the surface (d, black dashed line), downwelling light at 500 m (e, dark gray dashed line), and moonlight (full moon at 70° elevation above horizon) at the surface (f, light gray dashed line). Note how the spectral sensitivity of each species is tuned to the light spectra of their respective habitat.

Conclusions
Teleost fishes are remarkably adaptive, as revealed by their diverse forms and varied visual specializations. This study represents the first description of a teleost fish visual system that combines the characteristics of both rods and cones into a single photoreceptor type to presumably optimize vision in twilight conditions. This exceptional visual solution challenges the current paradigm of the evolution of the vertebrate duplex retina and the limits of visual adaptation. It also highlights the need for more comprehensive evaluations of visual systems in general and a more cautious approach in classifying photoreceptors into rods and cones.

MATERIALS AND METHODS
Sample collection and ocular tissue preservation
Adult individuals of similar sizes of the Mueller’s pearlside (M. muelleri) were collected in Mafjorden, Norway, aboard the research vessel G.O. Sars in October 2014. For each individual, the standard length and rostro-caudal eye diameter were measured with digital calipers to an accuracy of 0.1 mm before dissection. Eyes were enucleated, the cornea and lens were dissected free of the posterior chamber, and the lens diameter was measured as above. The eyecups were fixed either in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4) or in RNAlater (Thermo Fisher). RNAlater samples were stored at ~80°C until further processing. The whole heads of three specimens of M. muelleri were also fixed in 4% PFA at 4°C for 48 hours.

Adult individuals of the pearlside, M. mucronatus, were collected in the Red Sea, Saudi Arabia, aboard the research vessel Thuwal in November 2014. Each individual was measured, and the eyes were enucleated in a similar fashion as for M. muelleri and preserved in RNAlater only.

The research undertaken in Saudi Arabia was in accordance with the policies and procedures of the King Abdullah University of Science and Technology. Permissions relevant for the research have been obtained from the applicable governmental agencies in the Kingdom of Saudi Arabia. The research undertaken in Norway followed the local animal care guidelines approval by the Norwegian Veterinary Authorities.

Transcriptome sequencing, quality filtering, and de novo assembly
Total RNA of five individuals of M. muelleri and five individuals of M. mucronatus were processed from RNAlater-fixed eyes and sequenced separately. Briefly, each eyecup was homogenized in TRIzol reagent, and total RNA was extracted using the PureLink RNA Mini Kit (Thermo Fisher) following the manufacturer’s protocol. Total RNA quality was checked using a Eukaryotic Total RNA chip on the Bioanalyzer 2100 (Agilent Technologies). mRNA was subsequently extracted from 2 µg of total RNA using a Dynabeads mRNA Purification kit (Thermo Fisher), and complementary DNA (cDNA) libraries were prepared from 50 ng of mRNA following a NEBNext Ultra Directional RNA Library Prep Kit protocol (item: E7420, New England Biolabs). The quality of libraries was checked using a Bioanalyzer DNA 1000 chip. Library concentrations were measured using a Qubit dsDNA BR Assay kit (Thermo Fisher). Samples were pooled in equimolar ratios, and 100-bp paired-end sequence reads were obtained using Illumina HiSeq 2000.

Transcriptomes were uploaded to the Genomics Virtual Laboratory (GVL 4.0.0) (46) on the Queensland Galaxy Server (http://galaxy-qld.genome.edu.au/galaxy/), and data were converted using FASTQ Groomer (Galaxy v.1.0.4) (47). Sequences were quality-checked with FastQC (Galaxy v.0.53) and trimmed with Trimmomatic (Galaxy v.0.32.2) (48) using customized settings [ILLUMINA CLIPPER (TrueSeq3); HEADCROP (10 bp); LEADING (Q20), TRAILING (Q20), SLIDING-WINDOW (average of 4 bp with Q20); and MINLEN (80 bp)]. Trimming and quality filtering removed overrepresented sequences, retaining only those sequences with a minimum length of 80 bp and a quality score of ≥20. Two transcriptomes per species were subsequently assembled de novo using Trinity (Galaxy v.0.0.2) (49) with default options.
settings, a k-mer coverage of at least four for initial contig construction, and a minimum contig length of 200 bp. Transcriptomes are available on the short-read archive database (www.ncbi.nlm.nih.gov/sra/) (tables S1 to S3) and assemblies on Dryad (http://datadryad.org/).

Gene extraction, phylogenetic reconstruction, and expression analyses

Putative opsin sequences of the two *Maurolicus* spp. were identified by initially mapping assembled contigs to publicly available opsin coding sequences (CDS) of the dusky dottback, *Pseudochoeram fuscus* (50), in Geneious v9.1.5 (www.geneious.com) using customized sensitivity settings (fine tuning, none; maximum gap per read, 30%; word length, 14; maximum mismatches per read, 40%; maximum gap size, 1000 bp; and index word length, 12). Similarly, publicly available zebrafish (*D. rerio*) CDS was used to identify rod- and cone-specific signal transduction genes (*gnat1* and *gnat2*) and pigment recovery genes (*sag* and *arr3*) (I). Mapped contigs were extracted and scored for similarity to publicly available gene sequences using BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi). However, de novo assembly of highly similar genes, in this case, opsin gene paralogs, using short-read libraries is prone to errors, often causing misassemblies (chimeric genes) or missing lowly expressed “rare” paralogs/isoforms/alleles (51, 52). Therefore, a second approach was used to corroborate the initial results by manually extracting pearside opsin genes from back-mapped reads [similar to the study of Cortesi et al. (15)].

In this manual approach, unassembled reads were first mapped against the dusky dottback opsin CDS using medium-sensitivity settings (70% identity threshold for mapping) in Geneious v9.1.5. Matching reads were manually split and extracted by copy-moving from single polynucleotide polymorphism (SNP) to SNP and taking advantage of paired-end matching to cover gaps between SNPs (fig. S10). Extracted reads were assembled de novo, and their consensus was used as a species-specific reference for low-sensitivity (highly accurate) mapping using customized identity parameters (100% identity threshold for mapping). During this cyclic mapping, unassembled reads were mapped and assembled repeatedly against an extending reference until the assembled gene included >300 bp of the 3′ UTR. Alignments were continuously inspected visually to ensure unambiguous mapping of genes. Both approaches were also used to search for the expression of *cypr27c1*.

Pearside opsin and phototransduction gene sequences were confirmed and assigned to a particular class based on phylogenetic relationships with gene sequences from a number of species obtained from GenBank (www.ncbi.nlm.nih.gov/genbank/) or Ensembl (www.ensembl.org/) (Figs. 1A and 2, A and B, and figs. S1 and S3 to S5). The CDS of gene-specific data sets (that is, opsin, transducin, and arrestin) was aligned using the L-INS-I algorithm in MAFFT v.7.222 (53), and the most appropriate model of sequence evolution was estimated in jModeltest v.2.1.6 (54), using the Akaike information criterion for model selection. Bayesian inference phylogenetic hypothesis was calculated on the CIPRES platform (55), using GTR+I+Γ models and Markov chain Monte Carlo searches with two independent runs and four chains each in MrBayes v.3.2.6 (56). Each run was set to 10 million generations, with trees sampled every 1000 generations (that is, 10,000 trees per run) and a burn-in of 25%. A similar approach was used to test for gene conversion between rh2 paralogs, only in this case the phylogeny was reconstructed using the information contained in the 3′ UTR and the last 50 bp of exon 5 alone (fig. S5). To corroborate the phylogenetic hypotheses, we reran our data sets using Bayesian inference with a GTR+Γ model (using parameters as above) as well as maximum likelihood using RAxML v.8.2.9 (57) and 1000 iterations for bootstrapping. However, no substantial differences in tree structure or node support could be found, and the resulting trees and data alignments have been deposited in Dryad. GenBank accession numbers for genes used in different phylogenies are depicted after the species names in figs. 1A and S1 to S5.

To analyze differences in gene expression of pearside opsins and phototransduction genes, unassembled reads were mapped against pearside genes (CDS and 3′UTRs for duplicated rh2 genes and only CDS for all other genes) using customized low-sensitivity settings in Geneious v9.1.5 (a minimum overlap between reads of 80 bp and 98% and 95% identity thresholds for opsin and phototransduction gene mapping, respectively). A higher identity threshold was used for opsin genes to assure the unambiguous mapping of reads against highly similar rh2 copies, but allowing for 2% differences to enable the mapping of reads containing heterozygous positions. Proportional gene expression (opsin, transducin, or arrestin) was then calculated according to $\frac{\text{TP}}{\text{TN}} = \frac{\Sigma \text{Ni}}{\Sigma \text{Ti}}$, where $T_i$/$T_{\text{all}}$ is the gene expression ratio for a given gene $T_i$, normalized by the total gene expressions $T_{\text{all}}$, and $\text{Ni}$ is the number of mapped reads for a given gene divided by its length. First, the proportional expression of rod-specific genes was compared to the combined proportional expression of cone-specific genes (that is, $rh1$ versus $rh2$, *gnat1* versus *gnat2*, and *sag* versus *arr3*), and second, the proportional expression of cone-specific genes was compared among themselves (Figs. 1B and 2, C and D). However, visual inspection of reads showed that, despite using high identity thresholds, read mapping against the CDS of rh2 paralogs remained inaccurate. Therefore, to resolve ambiguous mapping between cone opsin paralogs, all rh2-specific reads were extracted and submapped only against the 3′ UTR of the genes. Proportional gene expression of rh2 paralogs was then recalculated using normalized read counts from this submapping approach (Fig. 1B and table S1).

Molecular cloning of visual opsins and R-I SH

Using one eyecup of *M. muelleri* fixed in RNA later, total RNA was extracted using TRI reagent (Sigma), and DNase treatment (TURBO DNA-free, Ambion) was performed. Subsequently, cDNA was synthesized using SuperScript III (Invitrogen) reverse transcriptase with oligo (dT) primers following the manufacturer’s instructions. Gene-specific primers for *M. muelleri* visual opsin genes [*rh1*: forward primer MMRH1F1, GAGGCCCCGTATGAGTACCTCAG; reverse primer MMRH1R1, CCA-CAGATGACGTTGAGAG (gives a 1004-bp product); *rh2*: forward primer MMRH2x1F3, AACGGATCTGGGCTGTGAG; reverse primer MMRH2x1R3, CTGCGGACACGAAGGACG (gives a 1000-bp product)] were used to amplify opsin sequences from cDNA derived from retinal tissue using polymerase chain reaction (PCR). Bands of the correct size were excised from the agarose gel, purified (MinElute Gel Extraction Kit, Qiagen), and cloned into StrataClone vector (Stratagene). To extract plasmids, a MiniPrep (QIAprep Spin Kit, Qiagen) was used with cultures grown overnight from positive colonies (PCR-screened with M13 TOPO primers) following the manufacturer’s instructions. All positive plasmids were sequenced using a 3730XL analyzer (Applied Biosystems) at the University of Bergen Sequencing Facility (Norway). Sequenced opsins were confirmed by comparing the sequences to annotated *M. muelleri* opsin genes (table S4) and orthologs in other species using BLASTN and TBLASTX algorithms.

Antisense digoxigenin-labeled riboprobes for *rh1* and *rh2* opsins were prepared following the manufacturer’s instructions (Roche Diagnostics). For the synthesis of the riboprobes, opsin-specific PCR products using forward and reverse primers with T3 and T7 RNA polymerase sites (un-
derlined) \((rh1: \text{MMRH1F1T3}, \text{CATTACCTCACTAAAGGGAA}-\text{GAGCGCTTATGAGTACCTCAG})\) and \(\text{MMRH1R1T7}, \text{TAATACGTCACTATAAGGCCCCACAGATCGTGGAGGAG})\); \(rh2: \text{MMRH2x1F3T3}, \text{CATTAAACCTCTAGAAGGAGAAGACC}-\text{CTTGGGCTTGTAG})\) and \(\text{MMRH2x1R3T7}, \text{TAATACGTCACTATAAGGCGTGGGACACGGAAAGAAGAG})\) were used as template, as described by Thiss and Thiss (58), and the synthesized probes were precipitated by 0.1\% volume of 4 M LiCl and 3\% volume of 100\% ethanol together with transfer RNA (Roche Diagnostics).

The head of three \(M.\ \text{muelleri}\) fixed in 4\% PFA was briefly washed in phosphate-buffered saline (PBS), and the eyes were dissected and incubated in a solution of 25\% sucrose and 25\% Tissue-Tek (Sakura Finetek) overnight at 4°C. Each eye was then transferred in a mold of Tissue-Tek and rapidly frozen on an iron block precooled in liquid nitrogen. Parallel sectioning (10\% mm) of the eyes was performed with a Leica CM3050 S cryostat. Before storage at \(-22°C,\) the tissue was air-dried for 1 hour at room temperature and for 10 min at 65°C. One parallel series of the sectioned eye was stained for the presence of \(rh1\) expression, and the other parallel series was stained for \(rh2\) transcripts by R-ISH, as described by Sandbakken et al. (59). Photographs were taken with a Leica 6000B microscope mounted with a Leica DFC digital camera.

In vitro expression and spectral sensitivity
Total RNA was extracted from a single \(M.\ \text{muelleri}\) eye previously fixed in RNA later (Thermo Fisher), using the PureLink RNA Mini Kit (Thermo Fisher) following the manufacturer’s protocol. Oligo(dT)-primed retinal mRNA (2 mg) was converted to cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche), according to the manufacturer’s instructions. Both \(rh1\) and \(rh2\) coding sequences were amplified using the following primer sets (where \(rh1\) forward and reverse oligonucleotides were designed to detect both \(rh1\)-1 and \(rh1\)-2 gene paralogs): \(rh1\): 5′-CGGCGTCGACGCTGCAGGGCCCACAGATGAC-3′ (PE_MMuelleri_RH1_F) and 5′-CGCGGTCGACGTGCGAGGGCCACAGATGAC-3′ (PE_MMuelleri_RH1_R); \(rh2\): 5′-CGGCGGTAATCCACATGAGACCAGGAGGAC-3′ (PE_MMuelleri_RH2_F) and 5′-CGGCGGACGCGTGGAGGACAGGAAAGGAGAG-3′ (PE-MMuelleri_RH2_R).

PCR reagents and conditions were used as previously described (27, 60, 61). Oligo primers comprising the full-length coding sequences were excised from a 1.2\% agarose gel and purified using a QIAquick Gel Extraction kit (Qiagen) before digestion with 1 U each of Eco RI and Sal I restriction enzymes and repurification with a QIAquick PCR Purification kit (Qiagen). These digested fragments were cloned into a pMT4 mammalian expression vector via the same restriction enzyme sites mentioned above, as previously described (60), and sequenced by the Australian Genome Research Facility to confirm the presence and fidelity of all three \(M.\ \text{muelleri}\) visual opsin sequences.

Each pearlside opsin plasmid (210 mg) of DNA in total) was separately mixed with GeneJuice Transfection Reagent at a DNA/GeneJuice ratio of 3:1, according to the manufacturer’s instructions (Novagen), and used to transfect 12 large tissue culture plates (140 mm) that were preseeded with a nearly confluent monolayer of human embryonic kidney (HEK) 293T cells. The transfected cells were harvested after 48 hours, pelleted, and washed four times in PBS to remove all dead cells and any remaining traces of cell culture medium. Visual photopigments were repeatedly regenerated with excess 11-cis-retinal (5-20 \(\mu\)M) under dark conditions. Cells were lysed with dodecylmaltoside detergent (1\%), in the presence of the protease inhibitor phenylmethyisulfonyl fluoride (20 mg/ml), to release membrane fractions containing the opsin proteins. Lysate was then subjected to affinity chromatography over a CNBr-activated Sepharose-binding column coupled to an anti-1D4 monoclonal antibody (62), as previously described (60). Triplicate absorbance spectra were recorded in complete darkness using a Shimadzu UV-visible spectrophotometer (UV-2700) before bleaching with white light for 1 hour. A difference spectrum was calculated by subtracting the bleached spectrum from that measured in the dark, which was then fitted to a modified Govardovskii rhodopsin A\(_1\) template (63) using Microsoft Excel to determine the \(\lambda_{\text{max}}\) as previously shown (18, 32, 60).

Spectral sensitivities were predicted by analyzing amino acid substitution at 13 known key spectral tuning sites [83, 122, 124, 132, 207, 208, 211, 261, 265, 292, 295, 298, and 300 (18, 20, 26)] (fig. S2). Because several studies thus far suggest that similar tuning sites are used for both \(rh1\) and \(rh2\) pigments (18–20), all 13 sites were analyzed to predict spectral sensitivities for each gene.

Preparation of retinal whole mounts
Retinal whole mounts were prepared according to standard protocols (64–66). Radial cuts were performed to flatten the eye and subsequently the retina in toto onto a glass slide, where the orientation was confirmed by making a small additional cut in the nasal or dorsal part of the eye. The sclera was gently removed, and the retina was bleached overnight in a solution of 3\% hydrogen peroxide in 0.1 M PB.

Immunohistochemistry
Anti-rhodopsin (rod opsin) antibody was used to selectively label the true rod photoreceptor population from whole mounts of three individuals of \(M.\ \text{muelleri}\). Eyes fixed in 4\% PFA were dissected and bleached as described previously. To optimize immunolabeling, two pretreatments were performed after bleaching: antigen retrieval [incubation in sodium citrate buffer (pH 6) at 60°C for 30 min] followed by endogenous peroxidase inactivation (15 min in a solution of 10\% methanol and 3\% hydrogen peroxide in 0.1 M PB) (67). Retinas were then rinsed three times (15 min each) in 0.1 M PB and incubated for 30 min at room temperature in a blocking solution (5\% goat serum and 0.3\% Triton X-100 in 0.1 M PBS) with 50 \(\mu\)M glycine. Retinal whole mounts were incubated under gentle rocking at room temperature for 48 hours in a blocking solution containing anti-rhodopsin (mouse monoclonal, 1:500; MAB5316, Millipore). Thereafter, the retinas were washed three times in 0.1 M PB (15 min each) and further incubated for 24 hours in a mixture of horseradish peroxidase–conjugated anti-mouse secondary antibody (1:500, Jackson Immunoresearch), 5\% goat serum, and 0.05\% thiomersal in 0.1 M PB. Finally, after being washed three times (15 min) in 0.1 M PB and twice (5 min) in 0.1 M sodium acetate buffer, retinal whole mounts were incubated in a solution containing 5\% of 3,3′-diaminobenzidine solution, 2\% nickel sulfate, and NH\(_4\)Cl (100 mg/ml) in 0.1 M sodium acetate sodium for 5 min before adding 30\% hydrogen peroxide and incubating for another 6 min. Finally, retinas were rinsed three times in 0.1 M sodium acetate buffer (15 min each) and mounted in 100\% glycerol on a glass slide for analysis.

Immunofluorescence was performed on three retinas cut transverse-ly using a vibratome (Leica 1000S). Thick sections (50 \(\mu\)m) were collected into a multiwall chamber and incubated in a blocking solution (0.5\% bovine serum albumin, 0.05\% saponin, 0.1\% Triton X-100, and 0.05\% sodium azide in 0.1 M PBS) for 30 min at room temperature. Sections were incubated with the following primary antibodies for 48 hours at room temperature: the synaptic vesicle marker anti-SV2 (mouse monoclonal at 1:300, Developmental Studies Hybridoma Bank) and the neuronal marker NeuN (rabbit polyclonal used at 1:1000, Merck Millipore). Subsequently, sections were washed three times in
mounted (photoreceptor layer up) in 100% glycerol on a microscopic slide. For similar analysis of ganglion cells, retinas were flat-mounted on a gelatinized slide (ganglion cell layer facing up) and stained for 5 min in 0.1% cresyl violet, following the protocol of Coimbra et al. (65). Shrinkage using this technique was deemed negligible because the retinal whole mounts were attached to the slides during all steps (65). Different types of analyses were performed for high-density cell types (that is, rod-like cone photoreceptors and ganglion cells) and low-density cell types (rod photoreceptors).

Following the protocols described by de Busserolles et al. (33, 70), topographic distribution of rod-like cone photoreceptor and ganglion cell populations was assessed using the optical fractionator technique (71) modified by Coimbra et al. (67, 72) for its use in whole-mounted retinas. Briefly, the outline of the retinal whole mounts was digitized using a 4× objective (numerical aperture, 0.13) mounted on a compound microscope (Olympus BX50) equipped with a motorized stage (MAC5000, Ludl Electronic Products), a digital video camera (MicroFire, Optronics), and a computer operating Stereo Investigator software (MicroBrightField). Using a 60× oil immersion objective (numerical aperture, 1.35) for ganglion cell counts and 100× oil objective (numerical aperture, 1.40) for photoreceptor counts, cells were randomly and systematically counted using the parameters listed in table S5. The counting frame and grid size were chosen carefully to maintain the highest level of sampling (~200 sampling sites) and achieve an acceptable Schaeffer coefficient of error (CE). The CE is a measure of the accuracy of the total number of cell estimates and is considered acceptable below 0.1. Topographic maps were constructed using the statistical program R v.2.15.0 (R Foundation for Statistical Computing, 2012) with the results exported from the Stereo Investigator Software according to Garza-Gisholt et al. (73). The Gaussian kernel smoother from the Spatstat package (74) was used and adjusted the sigma value to the distance between points (that is, grid size).

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**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/11/eaa04709/DC1

fig. S1. Gene coding region (CDS) inferred vertebrate opsin gene phylogeny.

fig. S2. Reconstruction of amino acid changes at known key spectral tuning sites of pearlside opsin.

fig. S3. Vertebrate arrestin gene phylogeny.


fig. S5. A channel-specific transducin gene phylogeny.

fig. S6. Topographic distribution of rod-like cone photoreceptors in three individuals of *M. muelleri*.

fig. S7. Topographic distribution of ganglion cells (excluding amacrine cells and glial cells) in three individuals of *M. muelleri*.

fig. S8. Expression of rh2 and rh1 opsin genes from sectional RNA in situ hybridization analyses of the eye of *M. muelleri*.

### Distribution of the different neural cell types across the retina

For topographic analysis of photoreceptors, retinas were whole-mounted (photoreceptor layer up) in 100% glycerol on a microscopic slide. For similar analysis of ganglion cells, retinas were flat-mounted on a gelatinized slide (ganglion cell layer facing up) and stained for 5 min in 0.1% cresyl violet, following the protocol of Coimbra et al. (65). Shrinkage using this technique was deemed negligible because the retinal whole mounts were attached to the slides during all steps (65). Different types of analyses were performed for high-density cell types (that is, rod-like cone photoreceptors and ganglion cells) and low-density cell types (rod photoreceptors).

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photoreceptor types in movie S1. 3D reconstruction of the two photoreceptor types in using the optical fractionator methods in six retinas of (including base pair coverage), and proportional transducin gene expression.

table S2. Summary of transcriptomes, GenBank accession numbers, transducin mapping (including base pair coverage), and proportional transducin gene expression.

table S3. Summary of transcriptomes, GenBank accession numbers, arrestin mapping (including base pair coverage), and proportional arrestin gene expression.

Figure S4. Single-genie GenBank accession numbers of gene sequences produced during this study. Table S5. Summary of the stereology parameters used for the analysis of the rod-like cone photoreceptors and ganglion cell distribution along with the quantitative results obtained using the optical fractionator methods in six retinas of M. muelleri.

Figure S2. Close-up 3D reconstruction of the nucleus and synaptic terminal of the two photoreceptor types in M. muelleri.

REFERENCES AND NOTES


Submitted 27 July 2017 Accepted 17 October 2017 Published 8 November 2017 10.1126/sciadv.aaq0709

Citation: F. de Bussoleres, F. Cortesi, J. V. Helvik, W. I. L. Davies, R. M. Tempelin, R. K. P. Sullivan, C. T. Michell, J. K. Mountford, S. P. Collin, X. I., S.K., and J.M. performed the experiments. F.d.B., F.c., J.V.H., and W.I.L.D. analyzed the data. S.P.C., X.I., S.K., and J.M. supervised the project. All authors wrote the manuscript. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper. Supplementary Materials, Dryad repository, and GenBank (single gene accession no. MF805819-MF805840 and transcriptome accession no. for the project PRJNA140015 and for the samples SRX3161870, SRX3161880, SRX3161913-SRX3161915, and SRX3162900). Additional data related to this paper may be requested from the authors.
Pushing the limits of photoreception in twilight conditions: The rod-like cone retina of the deep-sea pearlside


Sci Adv 3 (11), eaao4709.
DOI: 10.1126/sciadv.aao4709