

# Evidence for distributed light sensing in the skin of cuttlefish, *Sepia officinalis*

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We report that the skin of cuttlefish, *Sepia officinalis*, contains opsin transcripts suggesting a possible role of distributed light sensing for dynamic camouflage and signalling. The mRNA coding for opsin from various body regions was amplified and sequenced, and gene expression was detected in fin and ventral skin samples. The amino acid sequence of the opsin polypeptide that these transcripts would produce was identical in retina and fin tissue samples, but the ventral skin opsin transcripts differed by a single amino acid. The diverse camouflage and signalling body patterns of cephalopods are visually controlled, and these findings suggest a possible additional mechanism of light sensing and subsequent skin patterning. Cuttlefish, along with a number of other cephalopod species, have been shown to be colour-blind. Since the opsin in the fin is identical to that of the retina ( $\lambda_{\max} = 492$  nm), and the ventral transcripts are also unlikely to be spectrally different, colour discrimination by the skin opsins is unlikely. However, spectral discrimination could be provided by involving other skin structures (chromatophores and iridophores), which produce changeable colours and patterns. This 'distributed sensing' could supplement the otherwise visually driven dynamic camouflage system by assisting with colour or brightness matching to adjacent substrates.

**Keywords:** cephalopod; opsin; extra-ocular; camouflage; signalling

## 1. INTRODUCTION

Visual pigments are found in animal retinæ, where they enable light to be absorbed for the process of vision. They have also been found in the skin of some reptiles, amphibians and fishes, e.g. *Anolis*, *Xenopus* and some cichlid chromatophores, as well as the structurally reflecting iridophores of neon tetras, *Paracheirodon innesi* (Lythgoe *et al.* 1984; Oshima 2001; Ban *et al.* 2006; Kasai & Oshima 2006), and a number of invertebrates (Steven 1963; Yerramilli & Johnsen 2010). Rhodopsin and opsin-like genes are even expressed in human skin, where they may have some physiological function (Tsutsumi *et al.* 2009). Ban *et al.* (2006) showed that there are multiple types of visual pigments in the skin of the freshwater fish *Tilapia*, which introduces the possibility that dermal colour and pattern

changes for camouflage and communication are regulated *in situ*, i.e. at the level of the dermal structures that produce the colours and patterns. In other words, the dermal structures could act as receptors and effectors, independent from, or, in addition to, visual input to the eyes.

Here, we show evidence for opsin expression in certain skin areas of cuttlefish, *Sepia officinalis* (figure 1). The skin of cuttlefish has structures that provide changeable and almost instantaneous camouflage and signalling patterns. Cuttlefish skin contains thousands of pigmented chromatophores (black, red or yellow pigment), which are organs that consist of an elastic pigment sac attached to which are numerous single muscle fibre cells that are innervated directly from the brain (Florey 1969). In addition, there are two types of structural reflectors (iridophores and leucophores) that provide iridescent, polarized light reflectance as well as broadband (white) diffuse scattering, respectively (Mäthger *et al.* 2009). Despite the impressive range of camouflage and signalling body patterns that cuttlefish can employ (Hanlon & Messenger 1988), all evidence to date suggests that cuttlefish, along with a number of other cephalopods, are colour-blind (Brown & Brown 1958; Marshall & Messenger 1996; Mäthger *et al.* 2006); but see e.g. a cephalopod with colour vision (Michinomae *et al.* 1994).

It was therefore exciting to find opsin gene expression in the skin of cuttlefish because this may augment the otherwise visually driven colour change system, and/or possibly provide an explanation for how cuttlefish can achieve such impressive camouflage and signalling body patterns in the absence of colour perception.

## 2. MATERIAL AND METHODS

### (a) Tissue collection

Cuttlefish, *S. officinalis*, were hatched and reared at the Marine Biological Laboratory in Woods Hole, USA. Adult cuttlefish (1+ years old) were used for PCR analysis. Animals were euthanized by over-anaesthetizing them in a solution of ethanol in sea water (ethanol concentration was gradually raised to approximately 5%; cessation of breathing movements and reflexes indicated death had occurred). The following tissues were dissected for analysis: (i) *Retina*. The eyes were dissected away from the head and a dorsoventral slit was made to remove the outer, distal, surface of the eye, leaving the proximal part containing the retina exposed so that the retina could be clearly identified and removed. (ii) *Ventral skin*. Skin samples were dissected from the most central part and the lateral parts of the ventral mantle. (iii) *Dorsal skin*. Skin samples were dissected from the central and lateral parts of the dorsal mantle. (iv) *Fourth arm and fin*. The skin protrusions of the fourth arm and samples of the mantle fin were dissected. (v) *Gills*. Gills were presumed not to express opsin. All samples were stored at  $-80^{\circ}\text{C}$  for later RNA isolation.

### (b) RNA isolation

Tissue samples (50 mg) were transferred to TriReagent (Molecular Research Center) and total RNA was isolated following manufacturer's instruction. Total RNA was resuspended in 20  $\mu\text{l}$  of DEPC-treated water (0.1% DEPC) and quantified using a NanoDrop1000 (Thermo Scientific). RNA samples were DNase treated using the Turbo DNA-free DNase Kit (Ambion) according to the manufacturer's 'rigorous' protocol. Treated RNA was subjected to PCR as described below to verify removal of residual genomic DNA carryover. *Polymerase chain reaction and DNA sequencing* Total RNA (0.2  $\mu\text{g}$ ) was reverse transcribed using 0.1  $\mu\text{g}$  oligo dT (Promega) and M-MLV reverse transcriptase (Promega). PCR was performed using primers SepRhodF (AAAGAATCAGGTC-CACTGG) and SepRhodR (GGCGGCATCTACAAAATAAG). These primers were designed using the previously described *S. officinalis* rhodopsin mRNA sequence, GenBank accession number (AF000947; Bellingham *et al.* 1998). This primer pair amplifies a fragment

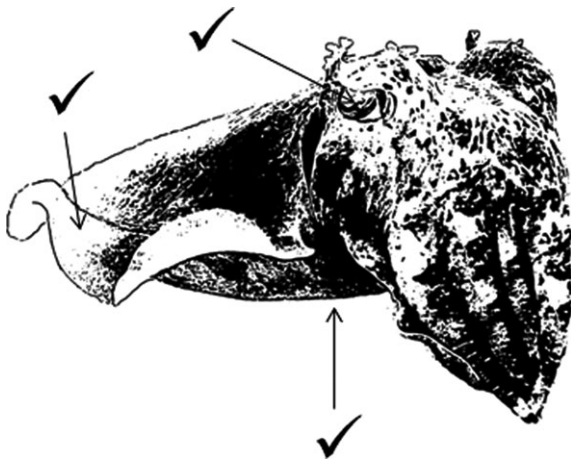


Figure 1. Locations of dermal opsin transcript in cuttlefish skin (indicated by tick mark). The amino acid sequence was identical for retina and fin. In ventral skin, opsin transcripts differed by a single amino acid.

corresponding to bp 101–1640. PCR was carried out using Apex 2× TaqRed Master Mix (Genesee Scientific), primers (0.2 μM), Mg (3.0 mM) and nuclease free water. Cycling parameters included initial denaturation at 95°C for 10 min followed by 40 cycles of 95°C 15 s, 55°C 15 s, 72°C 2 min. A final PCR extension step was performed at 72°C for 10 min. PCR products were visualized using agarose gel electrophoresis. Resulting bands were excised and purified with Ultrafree-DA spin columns (Millipore). Purified DNAs were cloned in TOPO/pCR 2.1 (Invitrogen) and positive colonies were grown for plasmid DNA. Templates were prepared using QIAprep Spin Miniprep Kit (Qiagen) and sequenced using a modified dideoxy chain termination method with Big Dye Terminator (Applied Biosystems). Sequencing reactions were precipitated and pellets resuspended in Hi-Di Formamide with EDTA (Applied Biosystems) and analysed using a 3730 Sequencer (Applied Biosystems). Two positive clones were selected from each template and sequenced in both directions, in duplicate. Sequence analysis was performed using CLC GENOMICS WORKBENCH 3.7 (CLC Bio).

### 3. RESULTS

PCR products of appropriate size were present in cDNA samples from retina, dorsal fin and mantle tissue isolated ventrally centre (mvc) from two individuals. PCR amplification was absent in samples from fourth arm, mantle (dorsal, centre), mantle (dorsal, side), mantle (ventral, side), as well as no template PCR controls. PCR products from one individual were sequenced as described. Nucleotide sequences from retina and fin were identical and were consistent with the *S. officinalis* rhodopsin mRNA sequence (GenBank accession number AF000 947) except for base pair substitutions at bp 1203 (T/A), 1269 (G/A) and 1291 (C/G). These nucleotide differences resulted in amino acid difference at amino acid 335 (S/T), 357 (A/T) and 364 (T/S) (GenBank accession number GU906262). The two isolated clones sequenced from mantle (ventral centre) were each different from the retina and fin nucleotide sequence. One isolate (mvc1, GenBank accession number GU906 264) had two nucleotide substitutions and the second (mvc2, GenBank accession number GU906 263) had four nucleotide substitutions. At the amino acid level, each sequence differed at one location, mvc1 at amino acid 455 (R/Q) and mvc2 at amino acid 413 (N/Y) (figure 2).

### 4. DISCUSSION

It remains to be established whether opsin gene expression in the skin of *S. officinalis* is correlated with a ‘visual distributed sensing’ capability because we have not yet demonstrated protein expression that could link the opsin gene with a physiological or behavioural function. Certainly, these dermal opsins may not have any functional significance. Nevertheless, some oceanic squid have well-developed extra-ocular photoreceptors that measure downwelling light and adjust ventral counterillumination (Young *et al.* 1979; Young & Mencher 1980), so it would not be completely surprising to find a similar system in shallow-water, benthic cephalopods such as cuttlefish that rely heavily on camouflage for protection in diverse habitats.

The skin opsins may provide an explanation for how cuttlefish can achieve their impressive camouflage and signalling body patterns in the absence of colour perception. Currently, we have found one opsin type in the retina and fin (which is transparent and could sense light above and below), suggesting that any skin photoreceptive abilities would be ‘monochromatic’, just like the cephalopod eye (Brown & Brown 1958; Marshall & Messenger 1996; Mäthger *et al.* 2006). Even though the opsins found in the ventral skin are slightly different, they are most probably functionally the same as those of the retina and fin, so that spectral discrimination at the level of the skin opsin is unlikely. However, we do not want to rule out the possibility that future studies may reveal additional dermal opsins tuned to different wavelengths.

Nevertheless, even a single skin opsin could help regulate dynamic colour and body patterning and we mention three possibilities to stimulate future research. (i) Opsins may not convey any wavelength information but may detect reflectance properties of the environment, which in turn may influence the relative expansion of chromatophores for brightness matching of the habitat. (ii) Opsins may be closely associated with chromatophores that act as spectral tuning filters and convey wavelength information to the dermal opsins. Chromatophores expand and retract, and occur in different colour classes, and they could function much the same way as oil droplets function in colour vision of many animals such as turtles (Liebman & Grandt 1975). In fact, the butterfly *Heliconius erato* has photoreceptors with one opsin pigment that provides colour discrimination using different perirhabdomal filter pigments (Zaccardi *et al.* 2006). (iii) Iridophores, structural reflectors some of which are also actively controlled, could perform similar roles as spectral tuning filters. Another more distant, but exciting, possibility is that iridophores, which not only reflect narrow waveband light but also polarize reflected light at certain incident angles (Mäthger *et al.* 2009), could pass polarization information from the ambient light field to the skin opsins, so that the skin may even function to analyse linear polarization in the animal’s environment.

One further intriguing possibility is that the dermal opsins work in conjunction with the statocysts to drive the countershading reflex in cuttlefish. This reflex causes cuttlefish to expand whichever chromatophores



|              |                   |                   |                   |                    |                   |                    |     |     |
|--------------|-------------------|-------------------|-------------------|--------------------|-------------------|--------------------|-----|-----|
|              |                   |                   | 20                |                    | 40                |                    | 60  |     |
| retina & fin | MGRDIPDNET        | WWYNPTMEVH        | PHWKQFNQVP        | DAVYYSLGIF         | IGICGIIIGCT       | GNGIIVIYLF         | FT  | 60  |
| mantle vc 1  | MGRDIPDNET        | WWYNPTMEVH        | PHWKQFNQVP        | DAVYYSLGIF         | IGICGIIIGCT       | GNGIIVIYLF         | FT  | 60  |
| mantle vc 2  | MGRDIPDNET        | WWYNPTMEVH        | PHWKQFNQVP        | DAVYYSLGIF         | IGICGIIIGCT       | GNGIIVIYLF         | FT  | 60  |
|              |                   |                   | 80                |                    | 100               |                    | 120 |     |
| retina & fin | KTKSLQTPAN        | MFIINLAFSD        | FTFSLVNGFP        | LMTISCFIKK         | WVFGMAACKV        | YGFIGGIFGL         |     | 120 |
| mantle vc 1  | KTKSLQTPAN        | MFIINLAFSD        | FTFSLVNGFP        | LMTISCFIKK         | WVFGMAACKV        | YGFIGGIFGL         |     | 120 |
| mantle vc 2  | <u>KTKSLQTPAN</u> | <u>MFIINLAFSD</u> | <u>FTFSLVNGFP</u> | <u>LMTISCFIKK</u>  | <u>WVFGMAACKV</u> | <u>YGFIGGIFGL</u>  |     | 120 |
|              |                   |                   | 140               |                    | 160               |                    | 180 |     |
| retina & fin | MSIMTMSMIS        | IDRYNVIGRP        | MAASKKMSHR        | RAFLMIIFVW         | MWSTLWSIGP        | IFGWGAYVLE         |     | 180 |
| mantle vc 1  | MSIMTMSMIS        | IDRYNVIGRP        | MAASKKMSHR        | RAFLMIIFVW         | MWSTLWSIGP        | IFGWGAYVLE         |     | 180 |
| mantle vc 2  | <u>MSIMTMSMIS</u> | <u>IDRYNVIGRP</u> | <u>MAASKKMSHR</u> | <u>RAFLMIIFVW</u>  | <u>MWSTLWSIGP</u> | <u>IFGWGAYVLE</u>  |     | 180 |
|              |                   |                   | 200               |                    | 220               |                    | 240 |     |
| retina & fin | GVLCNCSFDY        | ITRDSATRSN        | IVCMYIFAF         | FPILIIFFCY         | FNIYMAVSNH        | EKEMAAMAKR         |     | 240 |
| mantle vc 1  | GVLCNCSFDY        | ITRDSATRSN        | IVCMYIFAF         | FPILIIFFCY         | FNIYMAVSNH        | EKEMAAMAKR         |     | 240 |
| mantle vc 2  | <u>GVLCNCSFDY</u> | <u>ITRDSATRSN</u> | <u>IVCMYIFAF</u>  | <u>FPILIIFFCY</u>  | <u>FNIYMAVSNH</u> | <u>EKEMAAMAKR</u>  |     | 240 |
|              |                   |                   | 260               |                    | 280               |                    | 300 |     |
| retina & fin | LNAKELRKAQ        | AGASAEMKLA        | KISIVIVTQF        | LLSWSPYAVV         | ALLAQFGPIE        | WVTPYAAQLP         |     | 300 |
| mantle vc 1  | LNAKELRKAQ        | AGASAEMKLA        | KISIVIVTQF        | LLSWSPYAVV         | ALLAQFGPIE        | WVTPYAAQLP         |     | 300 |
| mantle vc 2  | <u>LNAKELRKAQ</u> | <u>AGASAEMKLA</u> | <u>KISIVIVTQF</u> | <u>LLSWSPYAVV</u>  | <u>ALLAQFGPIE</u> | <u>WVTPYAAQLP</u>  |     | 300 |
|              |                   |                   | 320               |                    | 340               |                    | 360 |     |
| retina & fin | VMFAKASAIH        | NPLIYSVSH         | KFREIAIENF        | PWII SCCQFD        | EKEVEDDKDA        | ETEI PAAEQS        |     | 360 |
| mantle vc 1  | VMFAKASAIH        | NPLIYSVSH         | KFREIAIENF        | PWII SCCQFD        | EKEVEDDKDA        | ETEI PAAEQS        |     | 360 |
| mantle vc 2  | <u>VMFAKASAIH</u> | <u>NPLIYSVSH</u>  | <u>KFREIAIENF</u> | <u>PWII SCCQFD</u> | <u>EKEVEDDKDA</u> | <u>ETEI PAAEQS</u> |     | 360 |
|              |                   |                   | 380               |                    | 400               |                    | 420 |     |
| retina & fin | GGETADAAQM        | KEMMAMMQKM        | QQQQAAYPPQ        | GAYPPQGGYP         | PQGYPPPPAQ        | GGYPPQGYPP         |     | 420 |
| mantle vc 1  | GGETADAAQM        | KEMMAMMQKM        | QQQQAAYPPQ        | GAYPPQGGYP         | PQGYPPPPAQ        | GGYPPQGYPP         |     | 420 |
| mantle vc 2  | <u>GGETADAAQM</u> | <u>KEMMAMMQKM</u> | <u>QQQQAAYPPQ</u> | <u>GAYPPQGGYP</u>  | <u>PQGYPPPPAQ</u> | <u>GGYPPQGYPP</u>  |     | 420 |
|              |                   |                   | 440               |                    | 460               |                    |     |     |
| retina & fin | PPQGYPPAQG        | YPPQGYPPPQ        | GAPPQGAPPQ        | AAPPQGVNDQ         | AYQA*             |                    | 465 |     |
| mantle vc 1  | PPQGYPPAQG        | YPPQGYPPPQ        | GAPPQGAPPQ        | AAPPQGVNDQ         | AYQA*             |                    | 465 |     |
| mantle vc 2  | <u>PPQGYPPAQG</u> | <u>YPPQGYPPPQ</u> | <u>GAPPQGAPPQ</u> | <u>AAPPQGVNDQ</u>  | <u>AYQA*</u>      |                    | 465 |     |

Figure 2. Deduced amino acid alignment of opsin transcripts isolated from retina, dorsal fin and mantle (ventral-centre). Two transcripts were isolated from mantle tissue. Differences in amino acid indicated with square. Transmembrane regions are underlined. Complete open reading frames are shown.

face upwards, irrespective of the animal's body orientation (Ferguson *et al.* 1994). Countershading is a widespread camouflage method in the animal kingdom and may be particularly effective in aquatic habitats (Ruxton *et al.* 2004).

The exact physiological and/or behavioural functions of cuttlefish dermal opsin genes remain to be established. We are developing an *S. officinalis* opsin antibody for immunohistochemical studies so that protein expression can be investigated, which will allow us to begin addressing questions of functionality of this remarkable finding.

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