VISION IN CEPHALOPODS

EDITED BY: Frederike Diana Hanke and Daniel Colaco Osorio
PUBLISHED IN: Frontiers in Physiology

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88945-430-3
DOI 10.3389/978-2-88945-430-3

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VISION IN CEPHALOPODS

Topic Editors:
Frederike Diana Hanke, University of Rostock, Germany
Daniel Colaco Osorio, University of Sussex, United Kingdom

Cephalopods are often very attentive experimental animals scanning their surrounding with their prominent eyes. This octopus is paying full attention to all the steps taken by the experimenter as preparation for the experiment.

Image: Frederike Hanke.

Cephalopods usually have large and mobile eyes with which they constantly scan their environment. The eyes of cephalopods are single-chamber eyes which show resemblance to vertebrate eyes. However there are marked differences such as the cephalopod eye having an everted retina instead of an inverted retina found in vertebrates. Their visual system allows the cephalopods, depending on species, to discriminate objects on the basis of their shapes or sizes, images from mirror images or to learn from the observation of others. The cephalopod visual system is also polarization sensitive and controls camouflage, an extraordinary ability almost exclusive to all

cephalopods; they are capable of rapidly adapting their body coloration as well as altering their body shape to any background, in almost any condition and even during self-motion. Visual scene analysis ultimately leads to motor outputs that cause an appropriate change in skin coloration or texture by acting directly on chromatophores or papillae in the skin. Mirroring these numerous functions of the visual system, large parts of the cephalopod brain are devoted to the processing of visual information.

This research topic focuses on current advances in the knowledge of cephalopod vision. It is designed to facilitate merging questions, approaches and data available through the work of different researchers working on different aspects of cephalopod vision. Thus the research topic creates mutual awareness, and facilitates the growth of a field of research with a long tradition - cephalopod vision, visual perception and cognition as well as the mechanisms of camouflage.

This research topic emerged from a workshop on "Vision in cephalopods" as part of the COST Action FA1301.

Citation: Hanke, F. D., Osorio, D. C., eds. (2018). Vision in Cephalopods. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-430-3

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frontiers in Physiology

EDITORIAL
published: 22 January 2018
doi: 10.3389/fphys.2018.00018

Editorial: Vision in Cephalopods

Frederike D. Hanke[1]* *and Daniel C. Osorio*[2]

[1] *Institute for Biosciences, Sensory and Cognitive Ecology, University of Rostock, Rostock, Germany,* [2] *School of Life Sciences, University of Sussex, Brighton, United Kingdom*

Keywords: eye, cephalopods, visual system, camouflage, cognition, development, movement, genetics

Editorial on the Research Topic

Vision in Cephalopods

Cephalopods' large eyes constantly scanning their environment give these fascinating animals a curious and attentive appearance; often human visitors to aquaria or divers feel that they are being watched (Darmaillacq et al.). There is a good literature on cephalopod vision, especially anatomy, learning and motor control in octopus, and on cuttlefish camouflage (see for example Young, 1960, 1962; Wells, 1978; Mather and Anderson, 1995; Kelman et al., 2008; Hanlon et al., 2011; Chiao et al., 2015; How et al.). The collection in this research topic of *Frontiers in Physiology* highlights innovative work in the field. Often methodological developments underpin advances in physiology. Here we find magnetic resonance imaging (MRI) is proving to be a valuable anatomical tool. Chung and Marshall used high resolution MRI and histology to link eye anatomy of various squid species to species-specific habitats. MRI also served to reveal changes in the three dimensional structure of cuttlefish optic lobes, first during ontogenesis, and then with the maturation of body patterning and other visuomotor behavior (Liu et al.). Innovative methods are also reported by Hadjisolomou and El-Haddad who developed a software plugin to measure size and color of multiple chromatophores mediating the cephalopods extraordinary abilities to camouflage, and by Bublitz et al. who describe a new experimental procedure to conduct behavioral experiments with octopus including the establishment of a secondary reinforcer.

Contributions united in this research topic cover a broad thematic range. Following the developmental theme explored by Liu et al., Imarazene et al. describe genes that are involved in cuttlefish eye development, while Darmaillacq et al. review literature on the development of visual function and visual learning in embryonic cuttlefish. In behavior of adult cuttlefish, Schnell et al. demonstrate lateralization of eye use during predatory and antipredatory behavior. It is fascinating to learn of similarities to vertebrate and arthropod lateralization, which support the idea that lateralization evolves to allow the animals to perform diverse tasks efficiently by allowing neural specialization.

Another group of papers within our research topic deals with vision and locomotion. Levy and Hochner elegantly describe a simple mechanism that allows *Octopus vulgaris* to control and coordinate its eight arms. It seems that octopus decides from moment to moment which arm to recruit, and usually uses the arm that is most likely to move the animal in the desired direction. For cuttlefish, Helmer et al. document a saccadic movement strategy, which might indicate that they use optic flow for distance estimation. Cuttlefish are generally bottom-dwelling animals, Scatà et al. show that they prefer to move horizontally over the ground, making detours around obstacles, moving vertically over obstacles only when this is essential, a behavioral choice which may miminize the risk of detection by predators.

And, of course, there is work on cephalopod camouflage. Cephalopods vary their appearance with unparalleled subtlety and speed by controlling the dilation of many thousands of individual chromatophores, which are innervated motoneurons that run directly from the brain. Besides

Edited and reviewed by:
Graziano Fiorito,
Stazione Zoologica Anton Dohrn, Italy

***Correspondence:**
Frederike D. Hanke
frederike.hanke@uni-rostock.de

Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *04 December 2017*
Accepted: *08 January 2018*
Published: *22 January 2018*

Citation:
Hanke FD and Osorio DC (2018) Editorial: Vision in Cephalopods. Front. Physiol. 9:18. doi: 10.3389/fphys.2018.00018

the already bespoken chromatophore fine scale monitoring software plugin (Hadjisolomou and El-Haddad), Josef et al. combine two of the poorly studied questions in an ingenious study: how cuttlefish integrate information across heterogeneous environments, and how they conceal themselves when moving. They report that moving cuttlefish match a subsample of the substrate slightly larger than the body in the direction of their movement. Continuing the theme of movement, but this time in the body patterns themselves, How et al. provide a comprehensive review of the diversity of dynamic skin patterns in cephalopods, and discuss the possible function of these remarkable and enigmatic displays.

Cephalopods are often noted for their cognitive abilities. In the first of two papers on visual cognition, Lin and Chiao show that cuttlefish can classify diverse objects as visually equivalent—resembling categorical perception—and that they can recognize objects when they are partially occluded. Learning theory continues to offer an influential framework of understanding animal cognition. Bublitz et al. work in the tradition of learning experiments of the 1960s, testing reversal learning in octopus, but with methodological innovations as already mentioned. They document a high degree of individuality: some animals do not learn the reversal, whereas others learn to reverse multiple times.

The Stazione Zoologica Anton Dohrn has for nearly 150 years offered to science the pleasures of Naples and the wealth of the sea, nurturing ground-breaking discoveries in neuroscience, behavior and evolution of cephalopods, and beyond. A fascinating contribution to the theme by Dröscher offers a historical perspective on vision research at the Stazione Zoologica. Cephalopod vision research in general and on the retinal ultrastructure in particular was initiated only a few years after the foundation of the Stazione Zoologica (Grenacher, 1884) and remained in focus throughout the twentieth century nourished by the famous work of for example Boycott (Boycott et al., 1965), Young (Young, 1971), Moody, Robertson, and Pariss (Moody and Robertson, 1960; Moody and Parriss, 1961), Sutherland, Muntz, and Mackintosh (Sutherland, 1954; Sutherland and Muntz, 1959; Sutherland and Mackintoshh, 1971) as summarized by Dröscher. Dröscher's manuscript also includes a previously unpublished early twentieth century debate on color vision between the skeptical Carl von Hess and the innovative young Karl von Frisch, future winner of the Nobel Prize in Physiology.

This Research Topic emerged from a Workshop organized in Naples as satellite to the 2014 Annual Meeting of the COST Action FA1301 (http://www.cephsinaction.org/), led by Dr. Giovanna Ponte. We were appointed as Guest Editors following the meeting at the suggestion of Prof. Graziano Fiorito, coordinator of the CephsInAction Task-Force for scientific dissemination, who initially proposed the Research Topic to Frontiers in Physiology. We believe that the COST Action FA1301 has made a wonderful contribution to cephalopod science through meetings, research exchanges and education and will benefit our subject for many years to come. We are delighted to thank Dr. Ponte and Prof Fiorito for their support, and hospitality.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 24 February 2017
doi: 10.3389/fphys.2017.00105

Complex Visual Adaptations in Squid for Specific Tasks in Different Environments

Wen-Sung Chung and N. Justin Marshall*

Sensory Neurobiology Group, Queensland Brain Institute, The University of Queensland, St Lucia, QLD, Australia

In common with their major competitors, the fish, squid are fast moving visual predators that live over a great range of depths in the ocean. Both squid and fish show a variety of adaptations with respect to optical properties, receptors and their underlying neural circuits, and these adaptations are often linked to the light conditions of their specific niche. In contrast to the extensive investigations of adaptive strategies in fish, vision in response to the varying quantity and quality of available light, our knowledge of visual adaptations in squid remains sparse. This study therefore undertook a comparative study of visual adaptations and capabilities in a number of squid species collected between 0 and 1,200 m. Histology, magnetic resonance imagery (MRI), and depth distributions were used to compare brains, eyes, and visual capabilities, revealing that the squid eye designs reflect the lifestyle and the versatility of neural architecture in its visual system. Tubular eyes and two types of regional retinal deformation were identified and these eye modifications are strongly associated with specific directional visual tasks. In addition, a combination of conventional and immuno-histology demonstrated a new form of a complex retina possessing two inner segment layers in two mid-water squid species which they rhythmically move across a broad range of depths (50–1,000 m). In contrast to their relatives with the regular single-layered inner segment retina live in the upper mesopelagic layer (50–400 m), the new form of retinal interneuronal layers suggests that the visual sensitivity of these two long distance vertical migrants may increase in response to dimmer environments.

Keywords: magnetic resonance imagery, retinal deformation, dual-layered inner segment, complex squid retina, mid-water, optic lobe, signal convergence

OPEN ACCESS

Edited by:
Frederike Diana Hanke, University of Rostock, Germany

Reviewed by:
Jens Herberholz, University of Maryland, College Park, USA
Angelique Christine Paulk, Massachusetts General Hospital, USA

****Correspondence:***
Wen-Sung Chung
w.chung1@uq.edu.au

Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *15 September 2016*
Accepted: *08 February 2017*
Published: *24 February 2017*

Citation:
Chung W-S and Marshall NJ (2017) Complex Visual Adaptations in Squid for Specific Tasks in Different Environments. Front. Physiol. 8:105. doi: 10.3389/fphys.2017.00105

INTRODUCTION

Fish and squid are both successful visual predators. Having high sensitivity is one requirement for visual predators in foraging under the low light conditions and for detecting fast-moving objects. The light intensity in the aquatic world is largely determined by two factors, time of day (availability of sunlight) and depth (scattered and absorbed by waters) (Denton, 1990; Johnsen, 2012). After dusk, the light level at the surface drops by 8 log units compared to mid-day. Another important feature of underwater light condition is that the intensity of the downwelling sunlight is depth-dependent, with a 10-fold drop in brightness with every 75 m depth increase, even in clear open ocean (Denton, 1990). In addition, the spectral range is gradually tuned to nearly constant blue spectra over increasing depths and in clear ocean to around 475 nm (**Figure 1**). In more

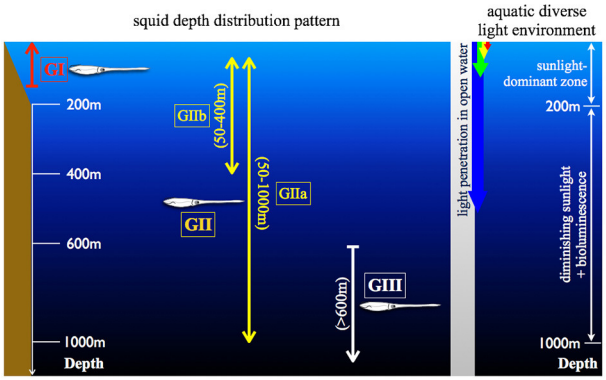

FIGURE 1 | Illustration of the diverse aquatic light condition and the squid depth distribution. (GI) indicates the coastal squid, including *Idiosepius notoides* and *Sepioteuthis lessoniana*. (GII) as the squid possess daily vertical migration and can be further divided into two subgroups (GIIa and GIIb) depending on the migration depth range. GIIa includes *Abraliopsis falco, Liocranchia reinhartdi* and *Spirula spirula*; GIIb: *Pyrotuethis margaritifera.* (GIII) as the squid constantly inhabit in the scotopic environment, including *Bathyteuthis abyssicola*. These categorized groups are consistently used in **Figures 4**, **7**, **8**.

coastal waters, this is green-shifted (Jerlov, 1976; Lythgoe, 1979). These diverse aquatic photonic environments have driven a variety of visual adaptations across different fauna (Lythgoe, 1979; Warrant and Locket, 2004; Yokoyama, 2008; Cronin et al., 2014; Chung and Marshall, 2016).

Biodiversity and fishery surveys show that squid occur over a great range of depths similar to fish (Marshall, 1979; Jereb and Roper, 2005, 2010). Their depth distribution patterns can be categorized into three major groups (**Figure 1**): (1) Coastal group (GI) inhabiting between 0 and 200 m depth. (2) Pelagic group (GII) inhabiting the water column with diurnal vertical migration between surface and hundreds of meters. (3) Deep pelagic group (GIII) inhabiting permanently scotopic depths (Clarke and Lu, 1974, 1975; Lu and Clarke, 1975a,b). Accumulated videography has confirmed that many mid-water squid are capable of reacting to point-like light as well as prey-predator interactions under similar levels of brightness as other inhabitants (Kubodera et al., 2007; Bush et al., 2009; Gilly et al., 2012). Squid are attractive for studying the evolution of vision as they have camera-like eyes sharing optical, anatomical and functional characteristics with fish, while having evolved these parallels through convergence (Packard, 1972). It is perhaps not surprising that most of these comparative studies focus on easy-to-access coastal squid, with the visual adaptation of deep-sea squid remaining poorly studied (Sivak, 1991; Sweeney et al., 2007; Makino and Miyazaki, 2010). Our goal in this study was therefore to show the various and complex adaptations in the morphology and the underlying circuitry of the squid visual system using a number of squid collected between 0 and 1,200 m.

In the mesopelagic environment (200–1,000 m depth), food and mates are not abundant and decreasing visibility through light attenuation results in strong selection pressures for remarkable visual adaptation (Warrant and Locket, 2004; Nilsson et al., 2012). In order to live in dim environments, fishes have developed many adaptations to improve sensitivity using optical improvements (i.e., spectral tuning, multi-banked rod retina, tapetum, diverticular, and tubular eyes) and neural summation or convergence (Lythgoe, 1979; Wagner et al., 1998; Warrant and Locket, 2004; Yokoyama, 2008; Partridge et al., 2014). Squid have successfully adapted to diverse aquatic visual environments, though different species might have adapted in different ways depending on the habitat light conditions. Deep-sea squid show a remarkable diversity of eye design as first noted by Chun (1910), however this has rarely been linked to photonic condition (Land, 1981). Aside from occasional reports of retinal adaptation (i.e., the fovea of *Bathyteuthis*; the dimorphic eyes of *Histioteuthis*; the elongated banked photoreceptors of *Watasenia*), the main body of knowledge in deep-sea squid visual performance is restricted to optical properties using comparisons of gross anatomy of eyes and optical qualities of the lens (Young, 1972, 1975a; Sivak, 1991; Land, 1992; Michinomae et al., 1994; Sweeney et al., 2007).

In contrast to remarkable adaptations of squid eye in morphology and optics, squid visual adaptations, particularly at the cellular and neural level, are rarely explored (Chun, 1910; Young, 1963; Sweeney et al., 2007; Makino and Miyazaki, 2010). Many previous studies revealed that squid possess a structurally simple retina, comprised of a single receptor layer and a single retinal plexus layer (Cajal, 1917; Cohen, 1973; Daw and Pearlman, 1974). The main function of the photosensitive rhabdomeric layer of the retina was thought to be photon absorption alone and thus, investigations of squid visual adaptations inside the retina have also been largely ignored (Cajal, 1917; Cohen, 1973). To date, a large portion of studies of squid visual system and the associating neural network relies on classical serial histological sectioning (Maddock and Young, 1987; Wild et al., 2015). The methodological constraints of classical histology to a single angle per specimen is clearly a limiting factor, particularly in rare deep-sea species. In order to overcome this, firstly, a contrast-enhanced magnetic resonance imagery (MRI) protocol was developed to explore the gross morphology of eyes and brains, and retinal topography in seven squid species from different habitats. With the reconstruction of three-dimension MR imagery, we discovered that variable enlargement of eyes and optic lobes, and three newly described types of retinal deformation are associated with different habitats and habits.

Follow-up histological examination found that the enlarged eyes combined with loss of light filtering screening pigments also link to dim light conditions. Furthermore, histological and immunohistological evidence showed that two mid-water squid species known to show regular migratory behavior between 50 and 1,000 m possess a new type of retinal feature in the inner segment layer. Here instead of a single cell layer, two types of retinal cells are found with complex neural interconnections. This new form of the dual-layered inner segment squid retina is suggested to be equivalent to the neural summation mechanism of the vertebrate's retina, improving visual sensitivity and dynamic range of light reception.

TABLE 1 | List of specimens and their living depth range.

Habitats	Species	Specimens and the associating collection depth range in this study		MRI [samples and mantle length (mm)]	Histology [samples and mantle length (mm)]	Immuno-histology [samples and mantle length (mm)]	Notes (sampling methods and locations, and known distribution depths)
		Day	Night				
Coastal waters	*Idiosepius notoides*	10 (1–3 m)	5 (1 m)	8 (ML8-14)	6 (ML8-14)	3 (ML10-14)	I^{A}, 0–10 m^{α}
	Sepioteuthis lessoniana	5 (1–3 m)	5 (1 m)	4 (ML13-30)	4 (ML15-45)	3 (ML20-25)	I^{A}, 0–100 m^{β}
Mid-waters	*Abraliopsis falco*	15 (400–1000 m)	n/a	1 (ML16)	4 (ML16-31)	3 (ML16-24)	III^{C}, 400–1,000 m^{γ}
	Pyroteuthis margaritifera	8 (300–400 m)	26 (50–100 m)	1 (ML12)	4 (ML12-24)	2 (ML14-16)	$II^{B,D,E}$, 50–500 m^{β}
	Spirula spirula	n/a	5 (150–600 m)	2 (ML13, 42)	4 (ML13-42)	–	$II^{B,D,E}$, 300–1,750 m^{β}
	Liocranchia reinhardti	7 (400–1,000 m)	18 (50–100 m)	2 (ML12, 32)	4 (ML25-95)	1 (ML115)	II,III^{B-E}, 0–1,200 m^{β}
	Bathyteuthis abyssicola	2 (600–1,200 m)	1 (800 m)	2 (ML15, 64)	2 (ML15, 64)	–	II^{B}, 700–2,500 m^{β}

Sampling methods: I, Seine net; II, RMT8; III, RMT16.
Sampling locations and Vessels: A, Moreton Bay, Queensland 2010); B, Coral Sea (RV Cape Ferguson, December 2009); C, Peru-Chilean Waters (RV Sonne, August 2010); D, Coral Sea (RV Cape Ferguson, December 2010); E, Coral Sea (RV Cape Ferguson, May 2011).
References of squid living depths: α as Jereb and Roper (2005); β Jereb and Roper (2010); γ as the current study.

MATERIALS AND METHODS

Animals

Cephalopods used in this study were collected from surface to 1,200 m depth. Two coastal squid species were collected using a seine net (water depth 1–3 m) close to Moreton Bay Research Station, Stradbroke Island, Queensland. Pelagic cephalopods were sampled using a Rectangular Midwater Trawl Net (RMT) with the trawling speed 0.8–2 knots from four deep-sea cruises. Collecting location and depth range of selected animals are listed in **Table 1** and Supplementary Figure 1.

Magnetic Resonance Imagery (MRI) and Anatomic Examination

Chung and Marshall (2014) developed the contrast-enhanced MRI protocol for a coastal squid, revealing advantages in examining central nervous system, rapid gross anatomical and optical analyses in this soft-bodied creature. Although some previous MRI work showed good results using live *Aplysia* and crayfish (Ziegler et al., 2011), keeping an anesthetized squid alive and still for a long MRI scan still encounters significant difficulties. In an effort to achieve high resolution MRI of squid brain (30 μm voxel resolution), with a few modifications of contrast agent treatment, we expanded the cephalopod MRI examination from the freshly-dead coastal squid to preserved deep-sea squid also. The two coastal species and five mid-water squid species were anesthetized in cold seawater mixed with 2% $MgCl_2$ and preserved in neutral formalin in the field and transported back to the laboratory. The freshly-preserved specimens (in neutral formalin less than 2 months after catching) were removed from storage and rinsed repeatedly with 0.1 M PBS to minimize the residue of the fixative. Secondly, four aged-preserved specimens (over a year in 70% EtOH after catching, including 2 *Bathyteuthis abyssicola* and 2 *Spirula spirula*) were removed from the storage and rehydrated through a series of reduced alcohols. Finally, all these preserved samples were soaked into 0.1 M PBS added with MRI contrast agent, 1% ionic Gd-DTPA (Magnevist, Bayer, Leverkusen, Germany), overnight before imaging.

The contrast-enhanced specimen was placed into the fomblin-filled (Fomblin oil, Y06/6 grade, Solvay, USA) container to prevent dehydration and then vacuumed for 15 min to remove air bubbles trapped inside animal body. The container was then placed in a custom-built surface acoustic wave coil (4–25 mm diameter) (M2M Imaging, Brisbane, Australia). Imaging was performed at temperature of $22 \pm 0.1^{\circ}C$ on a 700 MHz wide-bore microimaging system (Bruker Biospin, Karlsruhe, Germany) consisting of a 16.4 T vertical bore magnet interfaced to an AVANCE II spectrometer (Bruker Biospin, Karlsruhe, Germany) running the imaging software Paravision 4 (Bruker Biospin, Karlsruhe, Germany) in the Centre for Advanced Imaging at the University of Queensland. All scans were performed overnight (12–18 h) using a T_2^*-weighted 3D-Flash sequence (TR/TE = 50 ms/14 ms, average = 8), resulted in voxel resolution between 9 and 30 μm. The individual which obtained the highest voxel resolution in each species was selected for further morphologic and quantitative analysis of eyes and brain lobes.

A series of MR image stacks (Unix files) were imported into the image processing software OsiriX (Version 4.1.2, Pixmeo, Switzerland) for inspection of anatomical structure, post-construction of 3D virtual images and volumetric estimates of lobes and eyes. First, the retinal topography of each species was constructed by measuring the length of receptors per 100 × 100 μm^2 retinal patch across an entire eye. Identification of brain lobes was based on the published anatomical studies that also aid determining the boundaries between tissue types (Young, 1974, 1976, 1977; Messenger, 1979; Young, 1979; Nixon and Young, 2003; Wild et al., 2015; Koizumi et al., 2016). A region of interest (ROI) was manually segmented and assigned to different ROI-series files using OsiriX. The segmented structure was then used to obtain the quantitative volume using the analysis tool *ROI Volume* in OsiriX. In order to compare the enlargement of eyes and optic lobes across squid species, the volume of the ROI was expressed as a percentage of the total head volume.

Histology of the Deformed Squid Retinal Structure

When regionally differentiated eye structure and the corresponding visual axis region were confirmed by MRI, image-guided information of the deformed retinal structure was used to decide the best sectioning angle for light microscopy (as the red dash line shown in **Figure 4**). The retinal sample was repeatedly rinsed with 0.1 M PBS to remove the fomblin oil and then transferred into cryoprotectant (30% sucrose mixed with 0.1 M PBS) prior to embedding in the mounting medium, Optimal Cutting Temperature compound (OCT) (Tissue-Tek, Sakura Finetek, USA) mixed with 10% sucrose. The eyecup was cut at 12 μm thickness at −25°C using a cryostat (CM1100, Leica, Germany) and stained in Haematoxylin and Eosin.

Histology of the Light- and Dark-Adapted Squid Eyes

In an effort to study dynamic screening pigment movement, all living specimens were separated into two light-treated groups where one group was exposed to the room light and the other one was kept in a lightproof tank for 1 h dark-adaptation before fixation. The light-adapted animals were deeply anesthetized in 2% $MgCl_2$ mixed seawater and then decapitated and fixed in 4% neutral paraformaldehyde (PFA) mixed seawater. The dark-adapted specimens were anesthetized and decapitated under dim red illumination, and kept in the lightproof containers with 4% PFA until sectioning. The retinal segments were rinsed repeatedly with 0.1M PBS and used with tangential section by a standard cryosectioning procedure and H&E staining. Lengths of the rhabdom, dynamic movements of screening pigment granules were imaged using a Zeiss microscope (Axioscop- HBO 50) and measured using the software Fiji (NIH, USA).

Estimates of Photoreceptor Density at the Visual Axis Region

Young (1963) described the "simple cephalopod retina" where the major function of the photoreceptor layer was to receive photons, while all visual processing is conducted to the optic lobe. With this in mind, estimates of receptor density were therefore made using receptor nucleus counts within the selected retinal region of the inner segment layer. Estimates of nucleus density were modified from the protocol developed for octopus (Young, 1962) as follows: With the MRI retinal topographical map (**Figure 4**), estimates of nuclei density at the visual axis region were based on tangential sections (12 μm thickness). Each sample position represented a rectangular area [100 (W) × 50 (H) μm^2 – 100 (W) × 200 (H) μm^2] and within this area all nuclei in the inner segment area between the basal membrane and the retinal plexus layer were counted using the software Fiji. In addition, estimates of cell density were corrected with the equation suggested by Abercrombie (1946), eliminating counting bias particularly of those nuclei partially outside the section plane. Mean of nucleus densities were obtained from 6 to 8 consecutive slices and analyzed using the one-way ANOVA and the general linear model (GLM) for multiple comparisons.

Immuno-Histology of the Inner Segment Layer of Squid Retinae

The biomarker DiO was used to label lipid membranes of neurons, following a protocol developed for vertebrates (Köbbert et al., 2000). The staining protocol was modified from the basic protocol for the brain slice of mouse (Gan et al., 2000). The fixed squid eyes were isolated and DiO crystals were loaded into both the inner segment and rhabdomeric layers with the glass pipette tip (150 μm diameter) and kept in the light-proof box for 10 days. The two types of the inner segment layers were consecutively cut at a thickness of 25 μm using cryosection. The slices were then incubated with the primary antibody against synapsin (1:50; 3C11 anti SYNORF1, DSHB) in 1% NGS in 0.1M phosphate buffer saline (PBS) overnight at 4°C. The antibody was raised against a GST-synapsin fusion protein of fruit fly in the mouse (obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by Department of Biology, The University of Iowa, Iowa City, IA 52242, USA). After repeatedly rinsing with 0.1M PBS, the Alexa 568-conjugated secondary antibody (1:250; goat anti mouse, a11001, Invitrogen, USA) was applied for 1 h at 4°C. The slices were then embedded in the mounting medium with DAPI (Clear Mount ™ Mounting Solution, Invitrogen, USA) to visualize nuclei. The images were acquired using a confocal microscope (LSM710 META Violet, Zeiss, Germany) and analyzed using the software Fiji (NIH, USA).

Estimation of Resolution and Sensitivity of Squid Eyes

The optical resolution of squid eyes and the absolute sensitivity of an individual photoreceptor were estimated, respectively. The spatial cut-off frequency was determined by the size of the Airy disc (Land, 1981). The size of the Airy disc was suggested to be its half width (w). Estimations of the width (w) (μm) and the angular image size (θ) (radians) are described by the Equations (1 and 2).

$$w = f \times \frac{\lambda}{A} \tag{1}$$

$$\theta = 1.22 \times \frac{\lambda}{A} \tag{2}$$

where f is the focal distance; A is the diameter of the aperture; λ is the wavelength of light (485 nm for the mid-water species; 500 nm for the coastal species) (Chung and Marshall, 2016).

Visual capabilities were determined by the eye's resolving power R and the optical sensitivity S developed by Land (1981). Estimates of resolution and sensitivity of squid eyes were adapted from the equations in Land's work. The resolving power (rad^{-1}) is defined by Equation (3).

$$R = \frac{f}{2p} \tag{3}$$

where p is center-to-center receptor separation (μm).

The optical sensitivity S (μm^2 sr) of a simple eye to an extended scene of monochromatic light is defined by Equation

(4) as number of photons absorbed per receptor per unit of luminance in the visual scene:

$$S = \left(\frac{\pi}{4}\right)^2 A^2 \left(\frac{d}{f}\right)^2 \left(1 - e^{-kl}\right) \quad (4)$$

where d is the diameter of photoreceptor; l is the length of photoreceptor; k is the receptive coefficient of photoreceptor.

Chung and Marshall (2014) found that both laser lens tracing and MRI measurement obtain similar results of the focal distance of *Sepioteuthis lessoniana*, therefore, the focal distance, *f*, of this study was determined from the MRI imagery to measure the focal distance from the center of the lens to the hemispherical eyecup using the software Osirix.

RESULTS

A combination of MRI and histology uncovered several new adaptations of squid eyes: (1) Three types of eyeball deformation. (2) Differentials of screening pigment intensity and movement associating with the light environments. (3) A new form of the dual-layered inner segment retina and the underlying complex neural circuitry. These findings suggest that squid possess complex adaptations in response to vertically diverse aquatic visual environments and modes of life.

Comparisons between Magnetic Resonance Histology and Conventional Histology

By using MRI in combination with classical histology, a detailed brain atlas of *I. notoides* was initiated in three anatomical planes (**Figures 2**, **3**). Neuronal connections inside the central nervous system and the peripheral motor neuron can be traced along individual tracts. Within the optic lobe (OPL), darker regions and boundaries represent the areas containing dense nuclei (i.e., outer granular layer and inner granular layer of the OPL) (**Figures 2**, **3**). Within the retina, four different gray layers in MR images can be discriminated as rhabdom, basal membrane, inner segment, and cartilage layer, respectively (**Figure 3**). Aside from the detail of retinal structure, neuronal tracks, muscle fibers, brain lobes, other organs can also be discriminated (**Figures 2**, **3**) (Supplementary Videos S1–S4). For this paper, we concentrate only on those features relevant to the visual system comparison.

Comparisons of eyes, optic lobe, and motor center (supraesophageal + subesophageal mass) showed a strong relationship between sensory adaptations and the light conditions where they inhabit (**Tables 1, 2**). Eye size varied greatly amongst species. In coastal species, the combined eye volume was usually less than half of the head volume. In contrast, pelagic species showed significant eye enlargement relating to increased habitat depth (**Tables 1, 2**).

Non-hemispherical Cephalopod Eyes

MRI images indicate that the three mid-water species, *Abraliopsis falco, Pyroteuthis margaritifera*, and *S. spirula*, possessed a hemispherical eye (**Figure 4**). In contrast, non-hemispherical eyes were found in the other 4 species (**Figure 4**) which exhibit three types of regionally modified or deformed retinal structure. There are retinal bumps in two coastal squid species and a fovea-like structure of two deep-sea squid (**Figures 4–6**). The retinal bumps of the two coastal squid result from their enlarged optic lobe (OPL) pressing on the back of retina, forcing much of temporal retina close to the lens (**Figures 4A,B**). Previous work demonstrated that such eye deformation is not just the result of fixation shrinkage or other sagging artifacts during preparation (Chung and Marshall, 2014). The second type of retinal modification is found in *B. abyssicola*. This species possesses a tubular eye with a foveal pit, in which a patch of very long photoreceptors (>500 μm) is aligned with the central axis of the eye (**Figures 4G**, **5**). The third retinal modification is another invagination or deformation of the retinal hemisphere by a retinal ridge or pecten-like structure, akin to those found in bird eyes (Pettigrew et al., 1990) located in the naso-ventral retina of *S. spirula,* in both juvenile ($n = 2$) and adult stages ($n = 2$) (**Figure 6**). Anatomy, histology and MRI confirmed that the deformation of *S. spirula* eye is associated with a unique hard "connective" tissue in the orbit invading the back of the retina to form a sharp ridge (**Figure 6**).

Dynamic Movements of Screening Pigment Granules

Dynamic movements of screening pigment granules showed different patterns in light- and dark-adapted conditions (**Figures 7A,B**). This is in agreement with previous work (Young, 1963). In the dark-adapted state, screening pigment granules were concentrated at the basal membrane, leaving most of the photoreceptor unshielded (**Figure 7**). The thickness of the dark-adapted screening pigment layer in two coastal squid species covered 10–20% length of the rhabdomic layer. On the contrary, the dark-adapted mesopelagic squid possessed a reduced screening pigment layer where pigments cover only 1–7% of the length of the rhabdomic layer across the retina (**Figure 7C**).

In the light-adapted state, screening pigment granules of two coastal squid spread out toward the distal end of the outer segment (**Figure 7**). Screening pigment granules in the dorsal retina dispersed to approximately half the length of the outer segment (∼100 μm), leaving the distal region of photoreceptors unshielded by black granules. The ventral retina showed pigments evenly distributed along an entire outer segment (**Figure 7**). Additionally, a distinctive pigmented band was formed at the tip of the outer segment, known as the outer lamina (∼5–10 μm thickness) (**Figure 7A**). Unlike distinct granule movements seen in coastal squid, screening pigment granules showed no obvious movements in all mid-water species studied here, remaining in the basal region of photoreceptors (**Figure 7B**).

Variations of Retinal Features

Longitudinal sections of *I. notoides* retina (the red dash line in **Figure 4A**) showed a variance of rhabdom width between 4 and 7 μm. A thin (4 μm) and long rhabdom (200 μm) patch in this species in the dorso-posterior retina results in corresponding high visual resolution, assuming no photoreceptor coupling.

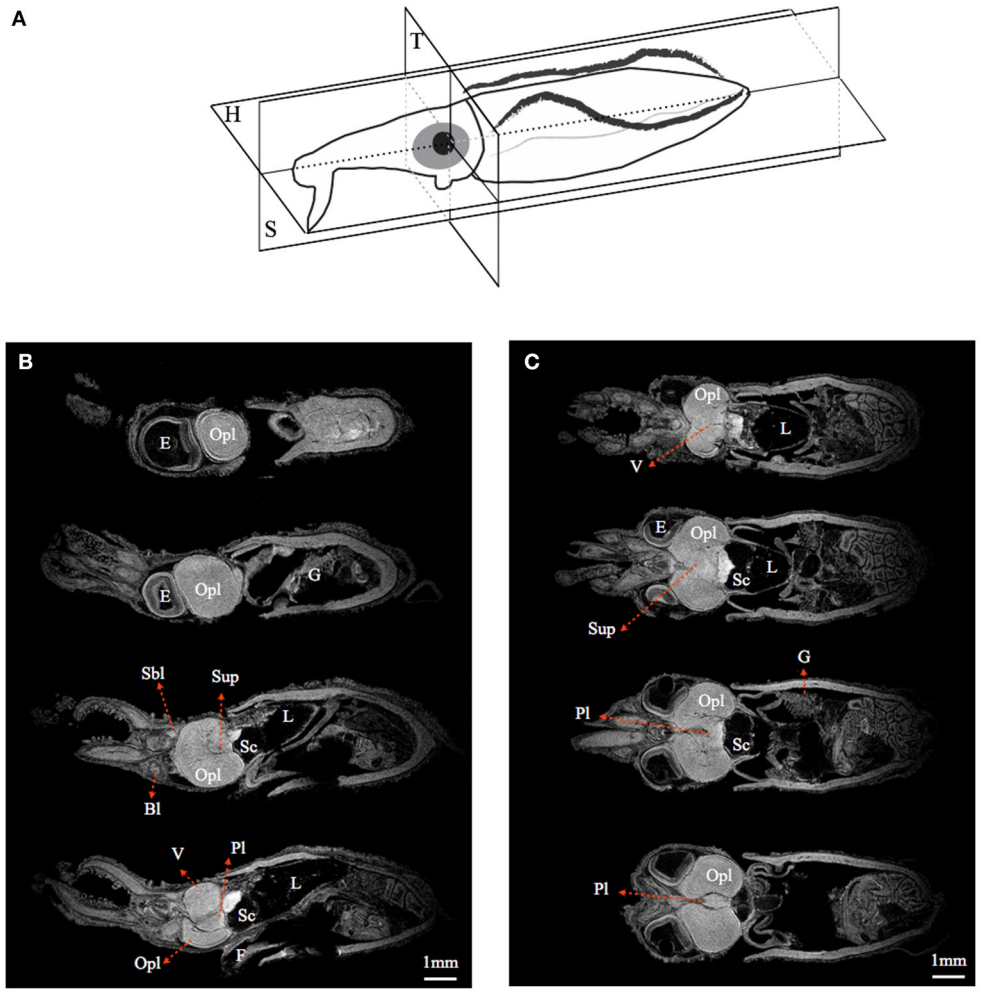

FIGURE 2 | Magnetic resonance histology of a squid, *Idiosepius notoides*. (A) Anatomical planes of squid. Horizontal plane (H). Sagittal plane (S). Transverse plane (T). **(B)** A series of sagittal sections at 200 μm intervals. **(C)** A series of horizontal sections at 200 μm intervals. (E) indicates eye; gill (G), liver (L), ventral lobe (V), brachial lobe (Bl), statocyst (Sc), optic lobe (Opl), pedal lobe (Pl), superior buccal lobe (Sbl), subesophageal mass (Sub), supraesophageal mass (Sup). Voxel resolution: 15μm. Scale bar: 1 mm.

This forward-looking area, like the foveal regions in fish, is likely used in predation (**Figure 4**). Using similar measures of photoreceptor packing density and size, squid retinae from this study can be categorized into three groups: (1) The densely-packed receptors located at dorso-posterior retina in two coastal squid (**Figures 4A,B**). (2) The densely-packed region located at ventro-posterior retina in 4 mid-water squid (**Figures 4C–F**). (3) The fovea of the tubular eye in *Bathyteuthis* (**Figures 4G**, **5**).

The width of rhabdoms in the densely-packed region among 7 species is 3–4 μm. We further examined the retinal cell density of these regions, and found that 2 mid-water squid species, *A. falco* and *Liocranchia reinhardti*, possess a thickened inner segment layer also exhibiting a 3–5-fold increase in retinal cell nuclei compared to the number of nuclei in the densely-packed retinal region of the other five species (One way ANOVA, $F = 119.28$, $p < 0.0001$) (**Figure 8**). Most unusually, the nuclei in the thickened inner segment layer of these two species can be grouped into two distinct morphological layers where large numbers of "round" nuclei are placed below a thinner layer of, more commonly observed, "oval" photoreceptor nuclei (**Figure 8**). As far as we know, this dual-layered inner segment retina has not been observed before in cephalopods.

Immunohistological staining showed an abundance of tubulin in this dual-layered inner segment presumably due to fiber-like supporting or connecting structures. Synapsin is also shown through immunohistochemistry located at junctions between dendrites, and between dendrite and soma (**Figure 8**). This indicates lateral synaptic connections exist within this dual-layered inner segment retina.

Optical Properties of Squid Eyes

The optical properties of squid eyes examined here are listed in **Table 3**. In all species, optical properties were generally similar, resulting the half width of the airy disc (w) between 0.5813 and

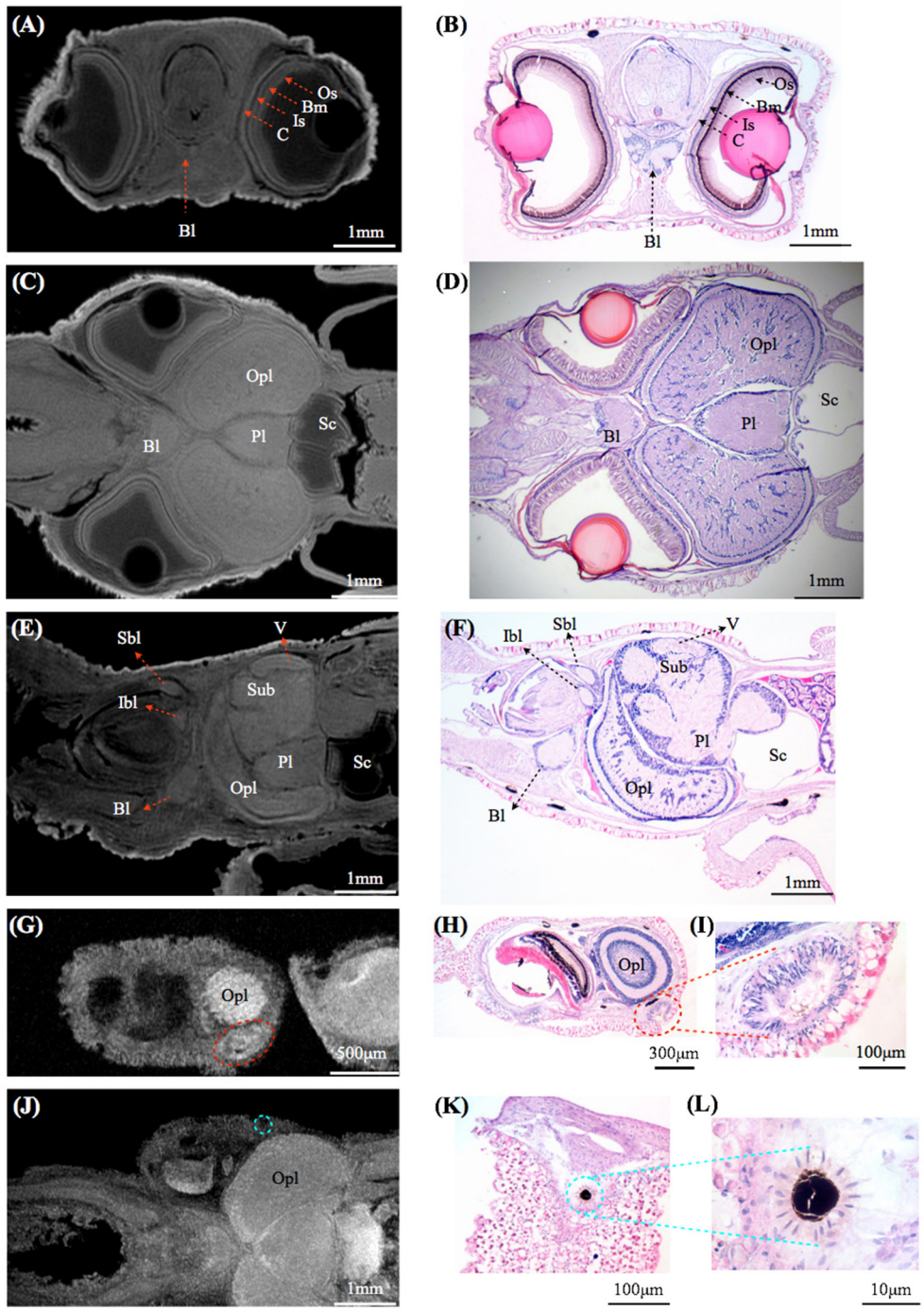

FIGURE 3 | Comparisons between magnetic resonance histology and conventional histology of a squid, *Idiosepius notoides*. (A,B) Transverse sections of squid head. Four different retinal layers can be identified, including the outer segment layer (Os), basal membrane (Bm), inner segment layer (Is), and cartilaginous eye cap (C). **(C,D)** Horizontal sections of squid head. **(E,F)** Sagittal sections of squid head. **(G–I)** Rhinophore. **(J–L)** Chromatophore. (E) indicates eye, gill (G), liver (L), ventral lobe (V), brachial lobe (Bl), statocyst (Sc), inferior buccal lobe (Ibl), optic lobe (Opl), pedal lobe (Pl), superior buccal lobe (Sbl), subesophageal mass (Sub), supraesophageal mass (Sup). The resolution of MRI slice at 16.4T (see Methods) is close to that of standard histology, however in contrast to histological sections that reveal cellular details, no individual cell can be identified in MRI images. Voxel resolution: 9 μm.

0.6379 μm. Angular image size and the resolving power per receptor were significantly influenced by the size of aperture, therefore, the tiny *I. notoides* has the lowest angular image size (0.00086 radians) and spatial resolution (92.5 cycles radian^{-1}). At the other end of the scale, the largest aperture, found in *S. spirula* rendered the finest angular size (0.00011 radians) and resolution

TABLE 2 | Volumetric comparisons of squid eyes and lobes.

Species	Mantle length (mm)	Supra-esophageal mass (mm^3)	Sub-esophageal mass (mm^3)	Optic lobes (mm^3)	Central nervous system (mm^3)	Eyes (mm^3)	Head (mm^3)	OPLs/CNS (%)	OPLs/Head (%)	Eyes/Head (%)
I. notoides	9	0.2634	0.1564	1.79	2.2104	0.8812	3.0916	81.01	57.92	28.50
S. lessoniana	19	1.9784	1.6941	13.2	16.8935	11.5146	28.4081	78.26	46.54	40.83
A. falco	20	1.2277	0.9854	6.28	8.4973	36.41	44.9073	73.96	13.99	81.08
P. margaritifera	19	0.6053	0.4213	2.68	3.709	19.6116	23.3206	72.32	11.50	84.10
L. reinhardti	20	0.1691	0.1305	0.71	1.0098	0.2156	1.2254	70.33	57.96	17.59
S. spirula	42	8.11	10.61	47.8	67.52	660.2	726.72	71.86	6.58	90.85
B. abyssicola	16	0.6298	0.641	2.45	3.7244	10.6218	14.3462	65.88	17.10	74.04

(721 cycles radian^{-1}). The optical sensitivity of an individual photoreceptor is between 3.91 and 6.73 μm^2 sr. Not surprisingly, the highest sensitivity is found in the eye of. *B. abyssicola* (6.73 μm^2 sr), the deepest living species and one showing a reduced-field tube-eye design associated with an attempt to boost sensitivity (Land, 1981).

DISCUSSION

The digitized neural atlas of squid central nervous system started here provides a rapid way to identify the gross anatomy of lobes, complex neural tracks and accurate volumetric estimates of different brain components. Systematic comparisons of volumetric estimates of eyes and visual system reveal that squid eye enlargement is reflected in habitat light conditions. Another important advantage of MRI is to guide or indeed prevent further sectioning of rare deep-sea specimens. In this study, our approach allows a comparative approach that has revealed several new aspects in cephalopod brain and eye structure. Expanded upon in the sections below our two main findings are:

(a) Adding to previous work on retinal deformation in squid eyes, we add a further two types of modification to cephalopod eye-cup shape. As with the defocused blur of the image for range-finding suggested by Chung and Marshall (2014), these changes in retinal structure appear associated with specific directional visual tasks.
(b) A combination of MRI and histology demonstrated previously unknown retinal layers. These modifications, are associated with deeper living species and may enhance sensitivity and visual flexibility for rhythmic vertical migration.

Advantages and Challenges of Cephalopod Brain Anatomy Using MRI

Comparisons of volumetric estimates of brain lobes with previous work (Maddock and Young, 1987) and results presented here reveal differences in some species (i.e., *B. abyssicola* and *S. spirula*). The animal size of two species used in Maddock and Young's and the current study was different (*B. abyssicola*, 30 vs. 16 mm ML and *S. spirula,* 21 vs. 42 mm ML), therefore, the variations of estimates of lobe volume (i.e., OPLs volume 15.6 mm^3 vs. 47.8 mm^3 in *Spirula*) could be due to the differential growth of lobes in different life stages (juvenile vs. adult in these two species). The different segmentation methods between two studies (subsampling sections vs. counting an entire series of sections) might also cause inconsistencies of volumetric estimates. For instance, two similar body size *P. margaritifera* were examined in two studies, however, estimates the OPLs volume showed significant differences as 15 mm^3 (ML 24 mm) in Maddock and Young (1987) vs. 2.68 mm^3 (ML 19 mm) in the current study. With classical histological technique in Maddock and Young (1987), the area of a segmentation was measured every 150 μm (every 10th slice for volumetric estimates) in small specimens and one per 600 μm (every 40th slice) for large specimens. In contrast, MRI segmentation in this study included all sequential sections and thus eliminated the problematic alignment of sections. Furthermore, a series of MRI images post-reconstruction allows us to generate 3D images along any stereotaxis plane for analysis, overcoming the methodological constraint of the classical histology of a single angle per specimen.

Although the current squid brain MRI shed new light to anatomical study, a live anatomy squid MRI or functional MRI using this classical neuroscience model animal have got limited progress mainly because of difficulties to keep this creature alive. Unlike successful MRI results using live *Aplysia* and crayfish which have relatively strong tolerance to hypoxia, a small holding chamber (i.e., 35 mm diameter of in our 16.4T scanner) and the associating challenges in oxygen supply and restriction of squid breathing movements during imaging need to be overcome.

Visual Adaptations in Different Light Conditions

A significant problem associated with many visual tasks underwater is maintaining enough sensitivity for various visual tasks in highly variable light environment. Our current study clearly showed that squid have developed visual adaptations rendering some of which are similar those found in fish and some of which appear to be unique to squid. Eye enlargement is a common feature in deep-sea visual predators (Marshall, 1979; de Busserolles et al., 2013). Although enlarged eyes ensure more photons reach to the retina, the eye of the largest pelagic fishes rarely exceeding 90 mm (i.e., swordfish) (Fritsches et al., 2005). On the contrary, the size of large mid-water squid eyes (i.e.,

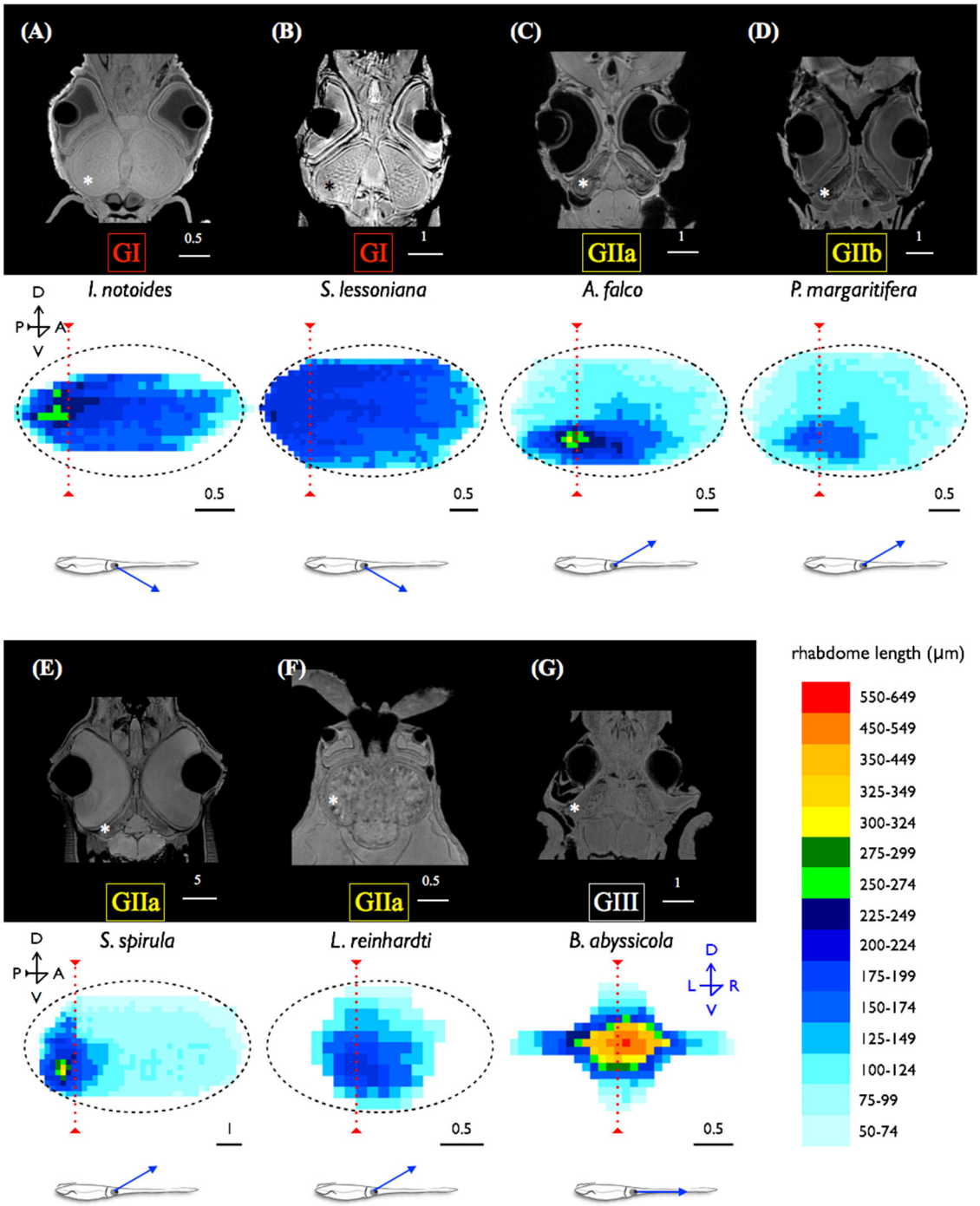

FIGURE 4 | Morphological variations of squid visual systems and the profile of retinal topography. Horizontal MRI sections of squid revealed several types of natural eye deformation in squid: non-hemispherical eyes **(A,B,F,G)**; hemispherical eyes **(C–E)**. Histological examinations combined with MRI results demonstrated that the thin (ca. 4 μm) and long rhabdom (>200 μm) patch results in corresponding high visual resolution along the longitudinal section of squid retina (the red dash line). The topography of squid retina revealed that the long and densely-packed receptors and the corresponding visual axis (blue arrow) reflect the visual adaptation to various light environments. *indicates the optic lobe, dorsal (D), ventral (V), anterior (A), posterior (P), left (L), right (R). Scale bar: mm.

Architeuthis, Dosidicus, Mesonychoteuthis, and *Octopoteuthis*) certainly exceeds the known largest fish eyes, with eye sizes recorded up to 27 cm (Nilsson et al., 2012).

Recently the giant squid eye, the largest eye on earth, has been suggested to be adapted for the detection of distant point light sources as well as detecting large predators (i.e., sperm

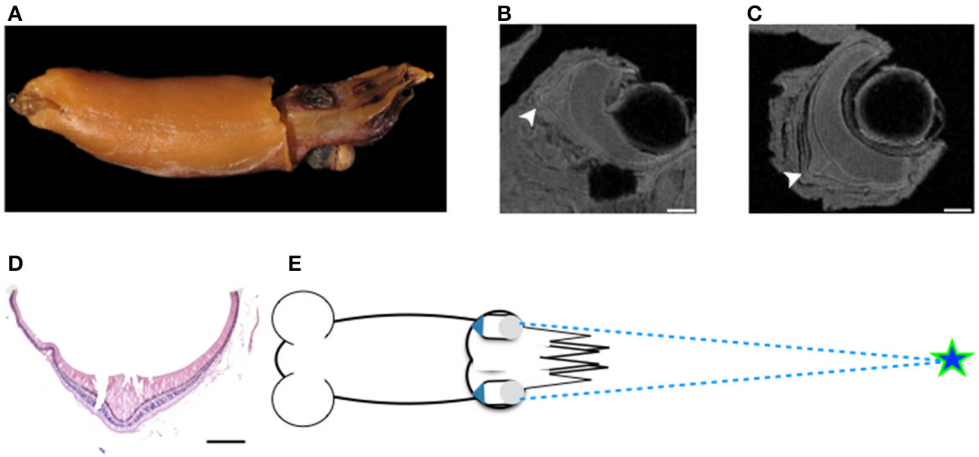

FIGURE 5 | Tubular eyes and fovea of *B. abyssicola* and potential function. The fovea and tubular eyes could maintain binocular stereopsis and preserve both resolution and sensitivity over a restricted frontal area where is critical for searching prey or mates. **(A–D)** The fovea of *B. abyssicola.* Arrows indicate the fovea. **(D)** A histological section of the fovea which the longest photoreceptor is c.a. 500 μm. **(E)** Illustration of potential function using the tubular eyes in detecting bioluminescent point sources. Scale bar: 500 μm.

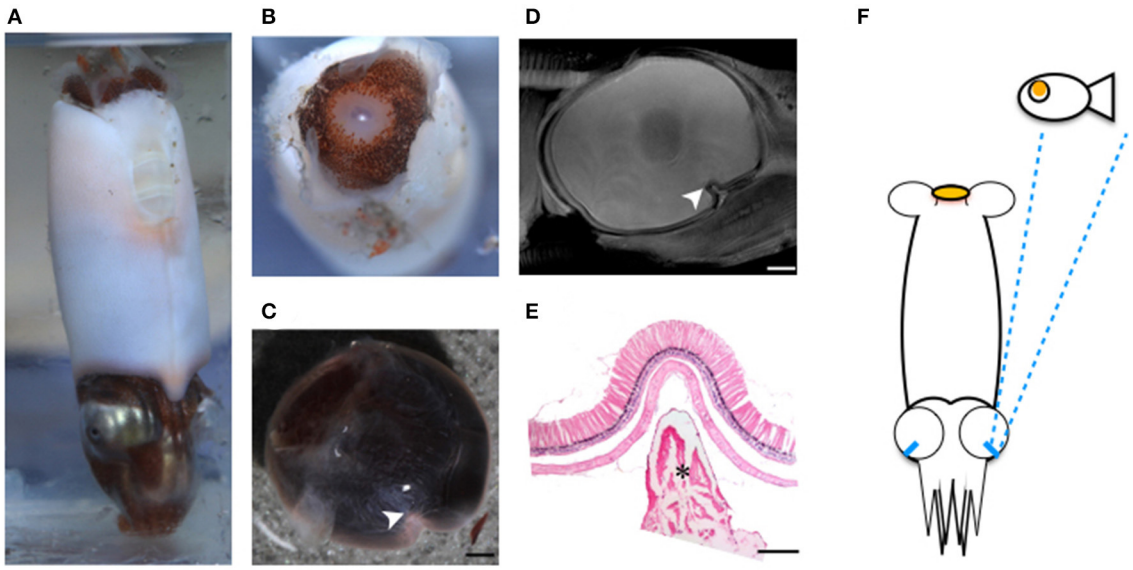

FIGURE 6 | Regional retinal specialization of *S. spirula* and potential function. With a large photophore emitting light upwards, the pecten-like structure located in the naso-ventral retina combined with its unique head-down posture could be used to detect the object moving overhead. **(A)** The natural head-down posture of live specimen. **(B)** A large photophore at the rear side of mantle. **(C–E)** The retinal ridge. **(C)** The back of the retina is invaded by connective tissue. **(D)** The ridge is located at the naso-ventral side of the eye. **(E)** Histological section of the retinal ridge. (arrow head: the retinal ridge; star: connective tissue). Scale bar: 200 μm. **(F)** Illustration of a potential foraging strategy to search for prey.

whales) illuminated by ambient bioluminescent flashes (Nilsson et al., 2012). Many small mesopelagic squid also possess large eyes relative to their body. For instance, relative eye size given by the eye diameter relative to mantle length was much larger in the firefly squid species studied here (a ratio of ∼0.22, 2 species) than those found in lantern fish of similar body size range (eye diameter vs. standard length, between 0.05 and 0.12, 61 species) (de Busserolles et al., 2013). Although enlarged eyes are certainly useful to increase light capture, there are constraints on eye design (especially maximal eye size) such that an extended receptive field rarely increases more than 3 log units of sensitivity (Land, 1981). Using an enlarged eye alone is therefore unlikely to maintain optimal vision during long distance vertical diving or over the day-night cycle. It is for this reason, among others, that many mesopelagic fish in fact migrate up and down in the water column (de Busserolles et al., 2013).

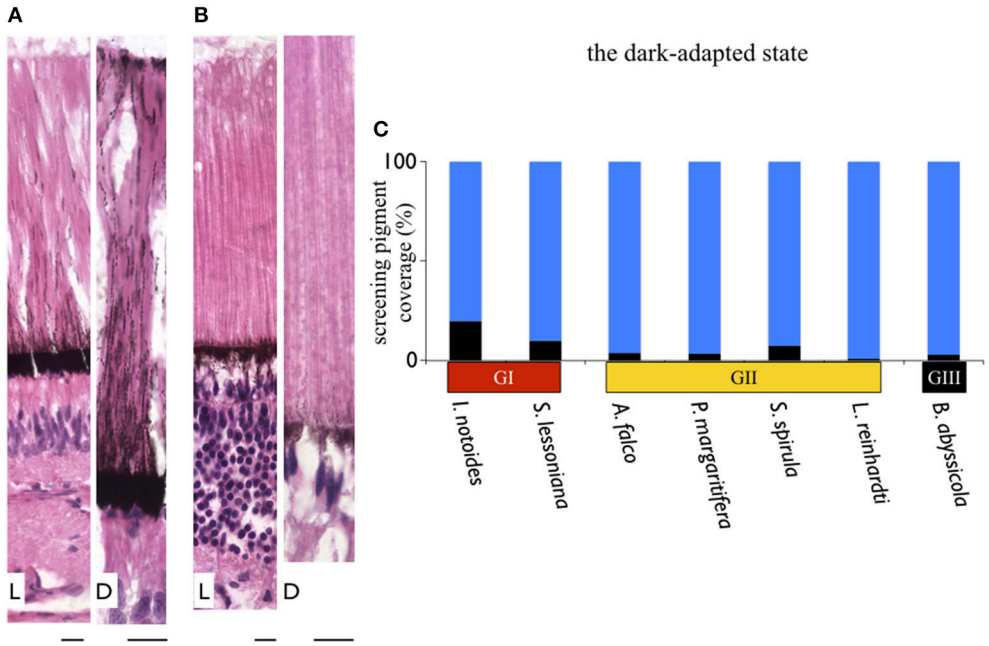

FIGURE 7 | Variation in thickness of the screening pigment layer in squid. Differentials of screening pigment density and movement are relating to their living light conditions. In contrast to coastal squid which possess dense and dynamic moving screening pigments to avoid bright sunlight, absence of screening pigment in mid-water squid maximize the amount of light reaching the retina. **(A)** A sample of dynamic movements of screening pigment granules in a coastal squid (*S. lessoniana*). L indicates the light-adapted retina; D as the dark-adapted retina. **(B)** A sample of absence of screening pigment movement in deep-sea squid (*A. falco*). Scale bars: 10 μm. **(C)** Varieties of thickness of the screening pigment layer at the ventral retina of the dark-adapted specimens. Black bars represent the thickness of the screening pigment layer in percentage relative to the length of the rhabdomeric layer.

The use of moveable screening pigments at the level of the photoreceptors as well as light-evoked pupillary activities in coastal squid allows a two-stage light attenuation capability and can theoretically tune different retinal regions to different light flux (Young, 1963; Douglas et al., 2005). The two coastal squid studied here possessing the crescent- or w-shaped pupil and its dynamic activities are likely able to offset the vertically uneven luminance from their natural habitat, filtering out large amounts of direct sunlight and improving image contrast in the visual scene as the similar mechanism suggested in cuttlefish by Mäthger et al. (2013).

Deep-sea squid eye design aims to maximize the amount of light reaching the retina at all times. In oegopsid squid, the absence of a corneal membrane and a reduced amount of screening pigment granules discovered in this study minimize these light-attenuated factors (**Figure 7**). Interestingly, sympatric competitors, fish, have developed a further optical adaptation, the tapetum. This reflective layer located below the retina allows the light that has not been absorbed by photoreceptors to pass through the retina a second time (Warrant and Locket, 2004). Although the reflection of deep-sea squid eye appears light red color, no such reflective layer analogous to the tapetum has been found in any squid studied so far. As the rhabdomeric photoreceptors of squid directly face to the light source and at 200–500 μm are much longer than known fish photoreceptors which are around 10–30 μm (even the extended or multi-bank rods of deep sea fish, which are around 100 μm), having a tapetum is probably not needed in squid.

The fovea of *Bathyteuthis* was previously described only briefly in Chun (1910) and Young (1972). The anatomical measurements provided here indicate this fovea or area is morphologically equivalent to those described in deep-sea fish. Over 50 deep-sea fish species are known to possess an area of higher resolution and this retinal region is presumed to be locked on objects of interest by eye or body movements, in a way similar to the use of the human fovea (Wagner et al., 1998; Warrant and Locket, 2004). The foveal structure (defined as a pit or mound as well as a local increase in photoreceptor density) found in some fish and in *Bathyteuthis* may increase the detection threshold for small bioluminescent object and/or the maintenance of binocular fixation to improve stereopsis (Pumphrey, 1948). Both the tubular eye and fovea in *B. abyssicola* seem to parallel these strategies in deep sea fish. As well as increasing resolution with a fovea-like structure, tubular eyes deliver higher sensitivity over a restricted angular area. So this eye (and those similar in deep-sea fish) provides both increased sensitivity and resolution (Land, 1981).

Aside from the optical adaptations of squid eyes found in the current study and previous investigations (Young, 1963; Sweeney et al., 2007), the molecular basis of spectral tuning in visual pigment has also evolved in response to the dominant spectra of their environment (Chung and Marshall, 2016). Providing both functional and opsin phylogenetic evidence, Chung and Marshall

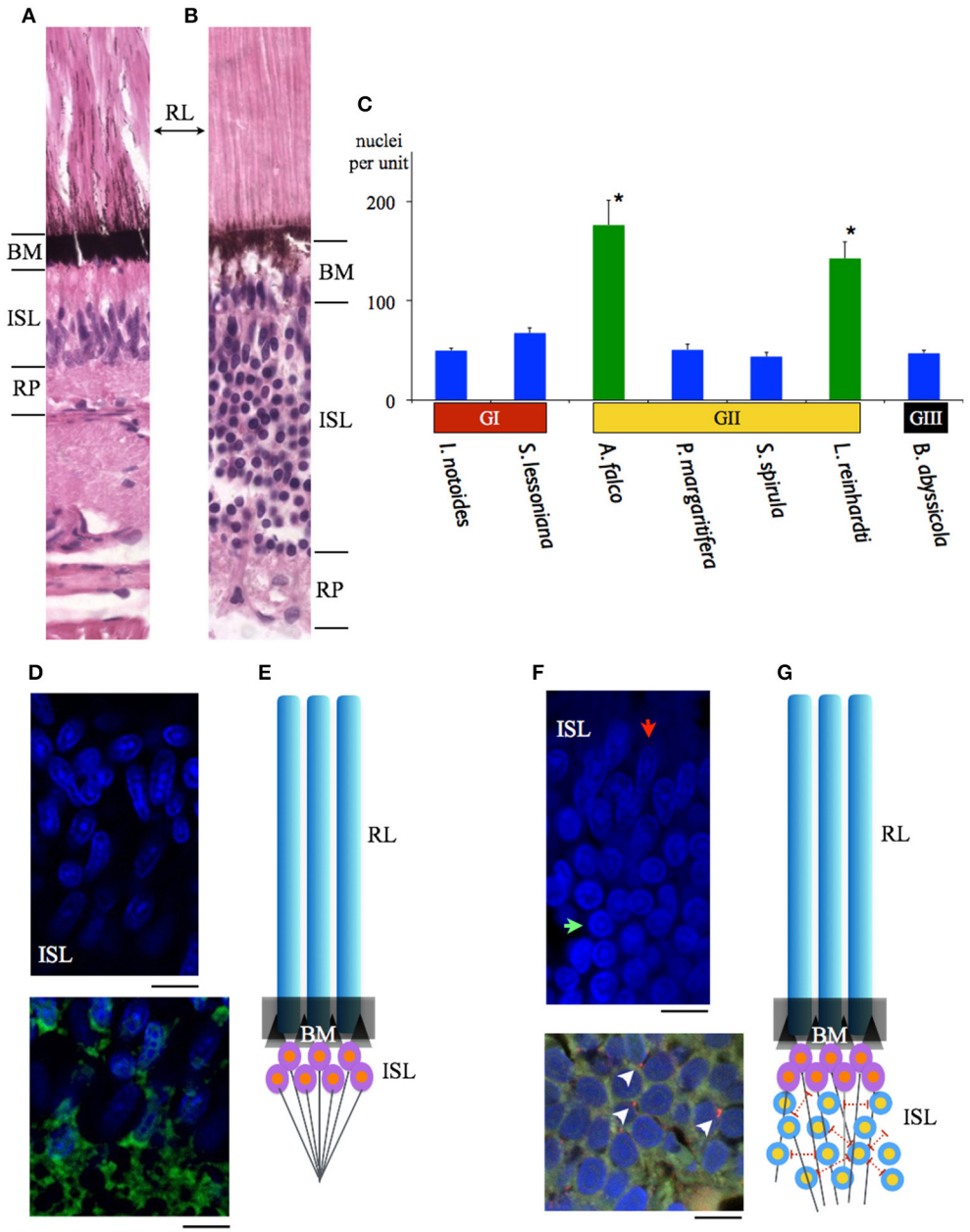

FIGURE 8 | Comparisons of the maximal density of nuclei amongst 7 squid species inhabiting different depths. (A) A sample of the previously recognized single inner segment layer of squid retina (*S. lessoniana*). **(B)** A sample of the new form of dual-layered inner segment squid retina (*A. falco*). **(C)** Blue bars indicate the squid which possess the regular single inner segment layer (nuclei density between 44 ± 3.98 – 68 ± 5.12 per unit). The retinal cell density showed no difference. Green bars represent the mid-water vertical migrants (distribution depth between 50 and 1000 m) (nuclei density between 143 ± 16.76 – 176 ± 21.61 per unit). Error bars are ± S.D. *Indicates that these two species possess significant increased cells in the inner segment layer rather than the other five squid. BM indicates basal membrane, inner segment layer (ISL), retinal plexus (RP), rhabdomeric layer (RL). **(D)** Immuno-staining of the regular single inner segment layer of *S. lessoniana.* DAPI (blue florescence) enhanced morpholgical feature of nuclei. DiO (Green fluorescence) showed nerve fibers in the retina plexus layer. **(E)** A schematic drawing of the single-layered inner segment retina. **(F)** Immuno-staining of the dual-layered inner segment retina of *A. falco.* The oval-shape nuclei are below the basal membrane and the round nuclei are located at the lower level of the inner segment layer. (red arrow: oval nucleus; green arrow: round nucleus). Positive synapsin labeling (arrow heads) (red florescence) represents synapse connections. **(G)** A schematic drawing of the dual-layered inner segment retina. Scale bar: 10 μm.

(2016) recently found that squid have evolved depth-dependent spectral tuning, including 4 species in this study, with maximal sensitivity to λmax at 500 nm in coastal squid and blue-shifted λmax of 485 nm in most known mid-water squid.

The optical sensitivity of squid studied here range between 3.91 and 6.73 μm^2 sr were relatively uniform given the great diversity of depths and habitat, and were similar to the previous estimate of octopus (4.23 μm^2 sr) in Land (1981). This optically

TABLE 3 | Optical properties of squid visual system.

Species	Focal length (μm)	Aperture (μm)	Receptor diameter (μm)	Receptor length (μm)	Absorption coefficient	Absolute sensitivity
I. notoides	740	580	4	200	0.0067	4.78
S. lessoniana	3,800	3,100	4	250	0.0067	5.34
A. falco	2,090	1,480	4	240	0.0067	3.96
P. margaritifera	3,510	2,930	4	210	0.0067	5.19
S. spirula	5,190	4,330	4	170	0.0067	4.67
L. reinhardti	5,770	4,560	4	150	0.0067	3.91
B. abyssicola	1,836	1,530	4	600	0.0067	6.73
Human[a] (cone)	16,700	2,000	2	30	0.035	0.02
(Rod)		8,000	20	30	0.035	36.81

[a] *The optical properties of human from Land (1981).*

calculated sensitivity is close to the cone cell of pelagic fish such as the blue marlin (1.5–5.6 μm^2 sr) (Fritsches et al., 2003). Surprisingly, the value of sensitivity of deep-sea squid is far less than the value of deep-sea shrimp (i.e., with *Oplophorus*, 3,300 μm^2 sr) (Land, 1981). Once optical improvement reaches its physics threshold (i.e., with the multibank retina alone, *Scopelarchus guntheri* is unlikely to increase the sensitivity up to 3 log units), the development of neural summation mechanisms (i.e., spatial or temporal summation) is a more efficient way to increase sensitivity (Warrant and Locket, 2004). For instance, visual sensitivity is improved by a high convergence ratio of photoreceptors to ganglion cells (i.e., the blue marlin, 40:1 at the foveal region) (Collin, 2008). Two mid-water squid species studied here possess a new form of complex retina that contains more and morphologically different nuclei in their dual-layered inner segment layer compared to those which have a regular single retinal layer. As the number of nuclei far exceeds the number of rhabdoms (Φ = 3–4 μm) (**Figure 8**), we suggest this new form of squid retina could be correlated with the development of a convergent neural circuits potentially providing the dynamic sensitivity adjustment mechanism needed for such a lifestyle.

A series of coleoid cephalopod vertical distribution studies in 1970s revealed that at least four families of mesopelagic squid (Chiroteuthidae, Cranchiidae, Enoploteuthidae, and Histioteuthidae) exhibit extensive diurnal vertical migration between the surface and the bottom level of the mesopelagic realm (c.a. 1,000 m depth) (Clarke and Lu, 1974, 1975; Lu and Clarke, 1975a,b). It is intriguing to note that the new type of the retinal circuit discovered here is associated with squid distribution depths, rather than any phylogenetic relationship. Although two enoploteuthid squid species have a close phylogenetic relationship (Young and Harman, 1998), their retinal design appears to be adapted to their photic ecology or at least depth range. *P. margaritifera* which is predominantly found between 50 and 400 m shows a regular, single-layered retina, while the other species, *A. falco*, possessing the dual-layered inner segment retina inhabit a greater range of depths (**Table 1** and **Figures 1**, **8**). Developing a retinal region over which photon catch is pooled (at the expense of spatial resolution) or with increased integration time (at the expense of temporal resolution) has been reported in a great range of taxa (Warrant and Locket, 2004). Thus, the species possessing the dual-layered inner segment retina indicates that they could have signal integration steps. Theoretically, the species with this new form of retinal networks may be more sensitive than those with a single-layered regular squid retina which could only have one convergent process from receptors to the optic lobe (Young, 1974). The neural connections within this type of squid retina are similar to the signal convergence mechanism of photoreceptors to ganglion cells known in deep-sea fish (Wagner et al., 1998).

The proposed signal summation of the dual-layered squid retina theoretically increases sensitivity, a likely adaptation to life in dim environments, but one which comes at the cost of losing spatial resolution. Neural superposition, or at least neural summation is known in the compound eyes of insects to enhance light sensitivity and resolution in nocturnal or crespuscular species, or to drive specific behaviors (Land, 1981; Warrant and Locket, 2004; Agi et al., 2014). The actual function of the dual-layered squid retina needs further evidence to determine and other functions such as polarization e-vector segregation or other signal processing remain possible.

Unique Visual Adaptations in Squid

Here we documented a second coastal squid, *I. notoides*, has the retinal bump resulting in intentional hyperopic defocus over a half of the frontal scene. Following the squid range-finding mechanism described by Chung and Marshall (2014), the retinal bump and the resulting image blur combined with head bobbing behavior is likely to provide reliable object size and distance information. In contrast to the retinal bump of *S. lessoniana* which disappears at the mature stage (Chung and Marshall, 2014), the retinal bump of the pygmy squid, *I. notoides*, appears in both juvenile and mature stages. This small predator has an adhesive organ to glue itself to the underside of seagrass and waits for prey. Along with this unique sit-and-wait behavior, their head bobbing driven by rhythmic breathing has been clearly recorded (Supplementary Video S5). Visually tracking prey combined with their accurate tentacular strikes despite this defocus suggests that retinal deformation and the resulting new range-finding mechanism might be more common in coastal squid than we expected.

The cranchid squid, *L. reinhardti*, possesses non-hemispherical eyes (**Figure 4**). Given the eye orientation

and the associating receptive visual scene, their vision might be largely restricted in lateral range. It is worth noting that cranchid squid often show significant ontogenetic changes of their morphology in both body shape and eyes (i.e., tubular eyes in larval stage but regular hemispherical eyes in adult) (Young, 1975b; Voss, 1980). Therefore, this non-hemispherical eye may only exist in juvenile stage but return to a regular hemispherical eye shape in adults. The relative requirements for vision at each life stage remain unknown.

The location of the pecten-like structure of *S. spirula* in the naso-ventral retina is unique among coleoids (**Figure 6**). The gas-chambered shell inside the posterior mantle cavity of *S. spirula*, enables this small cephalopod to float tentacles down in the oceans, resulting in a unique swimming posture of vertical jerky movements (Schmidt, 1922; Brunn, 1943) (Supplementary Video S6). A large photophore at the posterior end of the body may glow continuously, directed upwards (**Figure 6**). This photophore might work as a light lure to attract prey or as its own torch to emit light for foraging. Theoretically, the glow of its large photophore could result in a strong reflection from animals with tapetum-containing eyes (Warrant and Locket, 2004). *Spirula* might therefore notice the bright reflection from fish eye, enabling a unique foraging strategy. For example, as a point silhouette passes above the animal, the sharp retinal ridge would exaggerate the motion of the moving object (Pumphrey, 1948). In addition, *Spirula* possesses a densely-packed photoreceptor region on ventro-posterior retina as the retinal topography of many mid-water squid (Makino and Miyazaki, 2010), suggesting that this retinal region with its fine optical resolution are important for tentacular strikes (**Figure 4E**). Without behavioral observation, however, this remains a speculation for future investigation.

CONCLUSION

A combination of MRI and histology has discovered several new adaptations of squid eyes, including deformation of modification of eye shape, new photoreceptor arrangements, screening pigment movements, regionally differentiated retina, and a form of complex retina including interneuronal layers previously unknown. Also, a number of previously noted but only briefly described retinal modifications have been examined in more detail, including both pecten-like and fovea-like retinal structures in two deep-sea squid species. These adaptations indicate that squid have developed more complex visual adaptions than previously known in order to survive in various habitats.

DATA ACCESSIBILITY

Data supporting this article is available as an electronic Supplementary Material.

ETHICS STATEMENT

The maintenance and experimental protocol used here were covered by animal ethics permit (QBI/223/10/ARC/US AIRFORCE (NF)).

AUTHOR CONTRIBUTIONS

WC designed the study and prepared the dataset. WC and NM analyzed data and wrote the manuscript.

FUNDING

Authors were supported by funding from the Australian Research Council and the Airforce Office of Scientific Research.

ACKNOWLEDGMENTS

Thanks to Drs. Kerstin Fritsches and Marianne Keller for valuable comments in the early version of manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00105/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 27 July 2017
doi: 10.3389/fphys.2017.00538

Neural Organization of the Optic Lobe Changes Steadily from Late Embryonic Stage to Adulthood in Cuttlefish *Sepia pharaonis*

Yung-Chieh Liu[1,2], *Tsung-Han Liu*[3], *Chia-Hao Su*[4] *and Chuan-Chin Chiao*[1,2,3]*

[1] *Institute of Systems Neuroscience, National Tsing Hua University, Hsinchu, Taiwan,* [2] *Department of Life Science, National Tsing Hua University, Hsinchu, Taiwan,* [3] *Institute of Molecular Medicine, National Tsing Hua University, Hsinchu, Taiwan,* [4] *Institute for Translational Research in Biomedicine, Kaohsiung Chang Gung Memorial Hospital, Kaohsiung, Taiwan*

The optic lobe is the largest structure in the cuttlefish brain. While the general morphology of the optic lobe in adult cuttlefish has been well described, the 3D structure and ontogenetic development of its neural organization have not been characterized. To correlate observed behavioral changes within the brain structure along the development of this animal, optic lobes from the late embryonic stage to adulthood were examined systematically in the present study. The MRI scan revealed that the so called "cell islands" in the medulla of the cephalopod's optic lobe (Young, 1962, 1974) are in fact a contiguous tree-like structure. Quantification of the neural organizational development of optic lobes showed that structural features of the cortex and radial column zone were established earlier than those of the tangential zone during embryonic and post-hatching stages. Within the cell islands, the density of nuclei was decreased while the size of nuclei was increased during the development. Furthermore, the visual processing area in the optic lobe showed a significant variation in lateralization during embryonic and juvenile stages. Our observation of a continuous increase in neural fibers and nucleus size in the tangential zone of the optic lobe from late embryonic stage to adulthood indicates that the neural organization of the optic lobe is modified along the development of cuttlefish. These findings thus support that the ontogenetic change of the optic lobe is responsible for their continuously increased complexity in body patterning and visuomotor behaviors.

Keywords: ontogenetic development, neural connections, cell islands, visuomotor control, visual lateralization, cephalopods

OPEN ACCESS

Edited by:
Daniel Osorio,
University of Sussex, United Kingdom

Reviewed by:
Ana Turchetti-Maia,
Hebrew University of Jerusalem, Israel
Martin John How,
University of Bristol, United Kingdom

***Correspondence:**
Chuan-Chin Chiao
ccchiao@life.nthu.edu.tw

Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *18 February 2017*
Accepted: *11 July 2017*
Published: *27 July 2017*

Citation:
Liu Y-C, Liu T-H, Su C-H and Chiao C-C (2017) Neural Organization of the Optic Lobe Changes Steadily from Late Embryonic Stage to Adulthood in Cuttlefish Sepia pharaonis. Front. Physiol. 8:538. doi: 10.3389/fphys.2017.00538

INTRODUCTION

Cephalopods have the most sophisticated central nervous system (CNS) among all invertebrates (Nixon and Young, 2003). These complex brain structures reflect their intricate behaviors (Hanlon and Messenger, 1996). Within their CNS, a pair of optic lobes takes up about two-thirds of the total brain mass and these are known to have functions in visual processing and visuomotor control (Boycott, 1961; Young, 1962, 1974). Characterizing the neural structure of the optic lobes is thus essential for our understanding of the neural basis of cephalopod behavior.

The optic lobe in cephalopods is a kidney-shaped brain structure located behind the eye ball (**Figure 1A**). It can be divided into two parts, the outer cortex and the central medulla

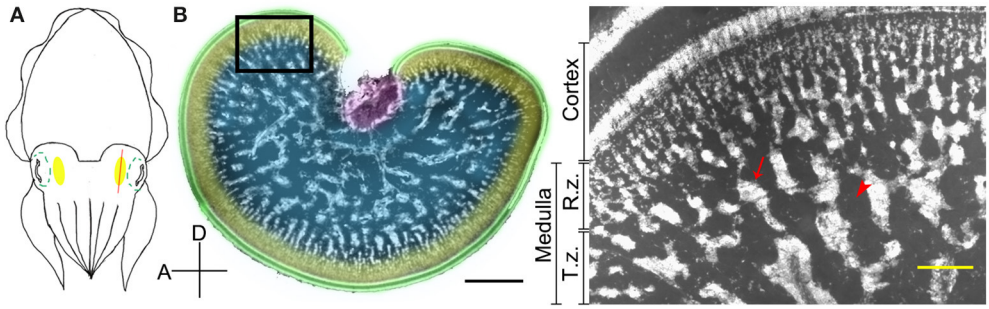

FIGURE 1 | The optic lobe of the cuttlefish, *Sepia pharaonis*. **(A)** A schematic diagram showing the position of the optic lobe (yellow area) relative to the eye (green dashed outlines) in a top view of the cuttlefish. The red line depicts the plane of the cross section. **(B)** A cross section of the optic lobe showing the general morphology. This cryosection of the left optic lobe of a cuttlefish (ML = 22.5 cm) was counterstained with a nuclear dye to visualize the cell organization. Green, cortex; Yellow, radial column zone (R.z.); Blue, tangential zone (T.z.); Purple, optic tract region. Inset, a high magnification image of the optic lobe from the same location of a different cuttlefish (ML = 8 cm) showing the various distinct zones. Arrow, cell islands; Arrow head, an area occupied mostly by neural fibers. D, dorsal; A, anterior. Scale bar, 2 mm (Inset, 200 μm).

(Boycott, 1961; **Figure 1B**). The cortex, also called the deep retina (Cajal, 1917), receives visual signals directly from the retina. It consists of two cell-rich granular layers with a single fiber-rich plexiform zone in-between (Young, 1962, 1974). It covers most of the optic lobe surface except for the optic tract region. In contrast, the medulla can be separated into two regions, the outer radial column zone and the central tangential zone (Young, 1962, 1974). The radial column zone, which contains numerous columnar structures of stacked cells and radially arranged neural fibers, lies beneath the outer cortex. When neural fibers from the radial column zone extend deeper into the center of the medulla, most of these fibers become tangentially arranged, thus this area is called the tangential zone (Young, 1974). Cell bodies within the tangential zone are clumped together into characteristic "cell islands" that are surrounded by neuropil, and there is no obvious histological differentiation in this region (Boycott, 1961; Young, 1962, 1974). Furthermore, it has been confirmed using phalloidin and alpha-tubulin staining that the space other than cell islands in the tangential zone are fully occupied by neural fibers in pygmy squids (Shigeno and Yamamoto, 2002; Wollesen et al., 2009). In cuttlefish, direct electrical stimulation of the cortex results in no obvious behavioral change, but stimulating the medulla evokes a range of body patterns unilaterally or bilaterally (Boycott, 1961). In addition, electrical stimulation of the medulla also produced various types of locomotive behavior (Chichery and Chanelet, 1976, 1978). These early experiments suggest that the cortex is responsible for visual information processing and the medulla is the motor command center for dynamic body patterning (Messenger, 2001). Despite our overall understanding of optic lobe structure and function, the detailed neural organization and the mechanisms underlying its control of body pattern generation have not been fully characterized.

"Live fast and die young" is an aphorism that well describes most modern cephalopods (O'Dor and Webber, 1986). Various lines of evidence indicate that most cephalopods complete their life cycles in one to two years (Boyle, 1983). This life style suggests that their brains must develop rapidly to meet their behavioral needs. Thus, characterizing the ontogenetic changes within the optic lobe in cephalopods and comparing with other fast growing animals may provide insights into the evolution of neural adaptation. Furthermore, it is known that early visual experience in embryos or hatchlings is important for the development of various visual behaviors (Dickel et al., 2000; Poirier et al., 2005; Darmaillacq et al., 2006; Lee et al., 2010, 2012; Guibe and Dickel, 2011; Romagny et al., 2012). Thus, examining the optic lobe structure from late embryonic to juvenile stages may also shed light on the neural plasticity of these observed behavioral adaptations. Earlier ontogenetic studies of brain structure in other cephalopod species are informative as they have helped to elucidate the growth pattern of the optic lobe (Meister, 1972; Marquis, 1989). In sepiolid squids, it has been found that both the volume of the optic lobe and its proportion in the brain keep increasing throughout the animal's embryonic stages (Kerbl et al., 2013). In oegopsid, lolignid, and pygmy squids, structural studies also revealed that, in the optic lobe, neuropil appear earlier in the cortex and tangential zone than in the radial column zone (Shigeno et al., 2001b,c; Yamamoto et al., 2003). Furthermore, it has been shown that the tangential zone of oegopsid and lolignid squids undergo a significant morphological change as they move from the embryonic to the juvenile stage (Shigeno et al., 2001a; Kobayashi et al., 2013). Despite the success of these early descriptive studies in squids, a systematic and quantitative study of the optic lobe from embryonic stage to adulthood in cuttlefish is crucial for correlating the observed behavioral plasticity with the optic lobe structure at various developmental stages of the animal.

In addition, behavioral lateralization was recently reported in the cuttlefish wherein the animals show side-turning preferences in a T-shape apparatus (Jozet-Alves et al., 2012b). This observed visual lateralization is task and age dependent in juvenile cuttlefish. It has been suggested that the turning bias in cuttlefish results from an eye use preference. Further analysis has revealed that there is an individual variation in the magnitude of the optic lobe asymmetry (Jozet-Alves et al., 2012a). Although the cerebral

correlates of visual lateralization are apparent, it is important to further compare the internal structures of the left and right sides of the optic lobe in developing cuttlefish and to determine the structural basis of this observed visual behavioral asymmetry.

In the present study, the optic lobes of the pharaoh cuttlefish *Sepia pharaonis* were collected at different time points from late embryonic stage to adulthood. An MRI scan was used to reconstruct the internal structure of the optic lobe in an adult cuttlefish and the so called "cell islands" in the medulla of cephalopod's optic lobe were found to be contiguous. Histological examination of the optic lobes confirmed that the morphological features of the cortex and radial column zone were established earlier than those of the tangential zone during the embryonic and post-hatching stages, and neural fibers and cellular organization in the tangential zone increased and modified along the development of the cuttlefish. Furthermore, comparing the internal structures of the left and right optic lobes revealed that lateralization is evident in the cortex and radial column zone during the embryonic and juvenile stages. These morphological observations are discussed with respect to the behavioral development of the cuttlefish and adaptation by the cuttlefish.

MATERIALS AND METHODS

Animals

Embryonic and early post-hatching cuttlefish, *S. pharaonis*, were reared from eggs collected at Keelung, Taiwan. All eggs were transported to the National Tsing Hua University and maintained in the laboratory using two closed-circulation aquarium systems (700 L each; water temperature approximately 24°C). The room was kept on a 12 h light and 12 h dark cycle. Sub-adult and adult cuttlefish (sex undetermined) were obtained from local fishermen at Keelung, Taiwan. For embryonic and juvenile cuttlefish, the optic lobes were collected when they attained an appropriate stage or the required mantle length (**Table S1**). The embryonic stages were determined based on developmental characterizations of *S. pharaonis* (Lee et al., 2016) and *S. officinalis* (Lemaire, 1970). The post-hatching stages (juvenile, sub-adult, and adult) were determined based on developmental processes recorded in a previous *S. pharaonis* culture study (Minton et al., 2001). In a separate experiment, additional optic lobes from cuttlefish of embryonic stage 24 ($N = 6$), mantle length 2.8 cm ($N = 2$), 4 cm ($N = 2$), 17.7 cm ($N = 2$), and 19 cm ($N = 2$) were used for immunostaining studies (see below). These samples were also included in the analysis of cell size in the optic lobe.

Histology

All animals were anesthetized using 3% $MgCl_2$ added to sea water (Mooney et al., 2010). Each pair of optic lobes located behind the eyes (**Figure 1A**) was carefully dissected out and fixed immediately using 10% formalin in sea water for at least 3 days. The samples were then placed in 70% ethanol for storage. A day before cryosectioning, the optic lobes were incubated with a mixture of OCT (tissue freezing medium) and 30% sucrose solution. Immediately before sectioning, the samples were embedded in OCT and placed on the stage of a cryostat (CM3050S, Leica). A series of 30 μm slices was cut along the sagittal plane from the lateral side to the medial side of the optic lobe (**Figure 1**). To visualize the internal structure of the optic lobe consistently, only the middle section (50% of sections from the lateral side) was collected, unless stated otherwise. In a separate experiment, 10 μm slices were collected for the immunostaining study. The optic lobe slices were rinsed using PBS (phosphate-buffered saline), and then stained with a nuclear dye, either DAPI (4′,6-diamidino-2-phenylindole) or PI (propidium iodide), to visualize the cell organization (**Table S1**). In the immunostaining experiments, the optic lobe slices were incubated with 10% normal donkey serum, 0.5% Triton X-100, and 0.1% sodium azide in PBS for 1 h at room temperature. After blocking, the slices were incubated with the primary antibody against acetyl α-tubulin (dilution 1:200; T7451, Sigma) for 1 day at 4°C to label neural fibers (Klagges et al., 1996; Shigeno and Yamamoto, 2002). After extensive rinsing with PBS, the secondary antibody, donkey anti-mouse IgG conjugated with DyLight fluorophore 488 (dilution 1:250; Jackson), was applied overnight at 4°C to visualize the immunoreactivity. To ensure the specificity of the primary antibodies, a control experiment of only the secondary antibody without the primary antibody was conducted. The results confirmed that neural fibers in the optic lobe can be labeled only when the primary antibodies were applied (data not shown). Finally, after rinsing with PBS, the samples were mounted with glycerol mounting medium for fluorescent imaging.

Image Acquisition

Histological and immunostaining images of the optic lobe slices were acquired on an upright fluorescent microscope (Axioskop 2 mot plus, Zeiss) using either a 5X (A-Plan, 0.12 NA, Zeiss) or a 10X (Plan-Neofluor, 0.3 NA; Zeiss) objective lens depending on the sample size, or on a fluorescent dissecting microscope (Stemi SV11, Zeiss). The high resolution fluorescent images of showing nuclei and neuropil were acquired on a confocal microscope (LSM 510, Zeiss) using a 40X objective lens (Plan-NEOFLUAR, NA 0.75, Zeiss). In addition, the left optic lobe of a sub-adult cuttlefish *S. pharaonis* (ML = 16 cm) was subjected to the MRI scanning at the Kaohsiung Chang Gung Memorial Hospital (9.4T, Bruker BioSpec 94/20 USR) to obtain its 3D structure. Before scanning, the sample was embedded in agar containing ferric ions to reduce background noise. The MRI scanning system is made up of a self-shielded magnet with a 20 cm clear bore and a BGA-12S gradient insert (12 cm inner diameter) that offered a maximal gradient strength of 675 mT m^{-1} and a minimum slew rate of 4,673 $Tm^{-1}s^{-1}$. The optic lobe was imaged at high resolution with TurboRARE-3D-torun sequence (TR/TE = 3,000/48 ms, NEX = 2). The stack of MRI data was then processed to make a movie of the stereo image of the optic lobe.

Image Analysis

Quantifications of neural organization from the histological images of the optic lobe slices were done using ImageJ (National Institutes of Health, USA). The thicknesses of the cortex and of the radial column zone were measured separately (**Figure 1B**).

The width of the radial column was determined by measuring the lateral spread of the columnar-like stacked nuclei, and the density of the radial columns was obtained by counting the number of radial columns along the circumference of the medulla in the optic lobe. The cross-sectional areas of the optic lobe, medulla, tangential zone, and cell islands were also determined accordingly (**Figure 1B**). Since all measurements from the left and right optic lobes were monotonically related (assessed by the Spearman's rank correlation coefficients; **Table S2**), the data from both left and right optic lobes were combined during analysis. However, to assess the optic lobe lateralization of individual cuttlefish, the measurements from the left and right sides were compared at each developmental stage.

To quantify the complexity of the cell islands in the MRI scan, the tree-like structure (see below) was analyzed by measuring the shortest distance of each branch point from the optic tract region. To examine the proportions of neural fibers in the optic lobes of cuttlefish at different ontogenetic stages, fluorescent signals of the immunostaining images were first thresholded and the areas were then measured. To quantify the cell size and density in the cell islands, the tangential zone was divided into two groups, peripheral and central areas, except for the optic lobe of embryonic stages where the cell islands are relatively homogeneous. The average nucleus size in the cell islands was determined for cuttlefish at each different ontogenetic stage by measuring the areas of randomly selected individual nuclei ($N = 30$) in the peripheral and central regions separately. Similarly, the average nucleus density in the cell islands was determined by counting the number of nuclei within three randomly assigned ROIs in the peripheral and central islands separately. The average nucleus size in the cortex and the radial column zone was also determined using the similar approach. For statistical analysis, the one-way ANOVA and *post hoc* Tukey's test were used to determine the significant difference after assessing the data normality with the Shapiro-Wilk test (SigmaPlot, NA).

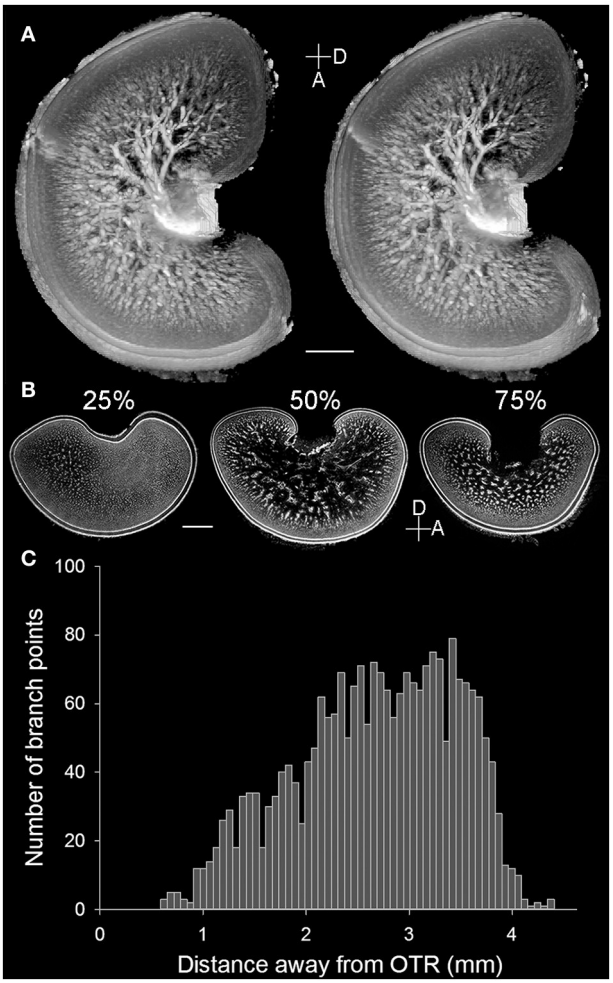

FIGURE 2 | Cell islands in the medulla of the optic lobe are a continuous structure. **(A)** A pair of stereo images enhanced and reconstructed from the MRI scan showing the 3D structure of cell islands (light areas) in the medulla of the left optic lobe of a cuttlefish (ML = 16 cm). Note that the small opening on the left side of the optic lobe was an artifact caused by the electrode penetration from a separate study. **(B)** Three cross cryosections at different percentages from the lateral to the medial side of the right optic lobe of a cuttlefish (ML = 19.5 cm) showing the distinct morphologies of the cell islands. D, dorsal; A, anterior. Scale bar, 2 mm. **(C)** The branch point numbers of the 3D structure of cell islands shown in **(A)** increase steadily as the distance from the optic tract region (OTR) increases.

RESULTS

Cell Islands Are Contiguous throughout the Medulla of the Optic Lobe

The present study showed the detailed neural organization in the optic lobe of the pharaoh cuttlefish *S. pharaonis*, which is consistent with the early Cajal staining study of functional brain organization of the common European cuttlefish *Sepia officinalis* (Boycott, 1961). However, the MRI scan of the optic lobe from a sub-adult *S. pharaonis* revealed for the first time that the so called "cell islands" present in the medulla (Young, 1962, 1974) are contiguous and have a tree-like structure (**Figure 2A**; see the **Movie S1** in the Supplementary Information). A careful examination of these cell islands showed that this tree-like structure spread out from the optic tract region (OTR) with a continuously increasing number of branch points until reaching the radial column zone (**Figure 2C**).

Detailed examination of the histological images of the optic lobe slices showed that the cellular organization and fine structures are conserved throughout the optic lobe, except for the optic tract region (the main output region of the optic lobe) which is located closer to the medial and dorsal sides. Furthermore, the radial column zone (the main input region which receives visual signals from the eyes) is slightly thicker on the lateral and ventral sides of the optic lobe. As a result, the tangential zone could hardly be observed in the sagittal section of the optic lobe at 25% from the lateral side (**Figure 2B**, left), and the boundary between the radial column zone and the tangential zone were difficult to discern in the section at 75% from the lateral side (**Figure 2B**, right). Since the middle section of the optic lobe slices (50% from the lateral side) exhibits the general characteristics of the optic lobe morphology most clearly, such as

the cortex, radial column zone, and tangential zone (**Figure 1B**), which represents a canonical view or a vantage point of the optic lobe, only the middle sections were used to examine the ontogenetic changes that occur during the development of the neural organization of the optic lobe.

Features of the Cortex and Radial Column Zone Are Established Earlier than Those of the Tangential Zone during Embryonic Development

Since the retina of cuttlefish embryos becomes reddish and the optic lobes become enlarged at stage 22 (Lee et al., 2016), the morphological development of the optic lobe was examined from this stage onward (**Figure 3**). At stage 22, the entire optic lobe was filled with cells and there was hardly any space for neuropil. The boundary between the cortex and the medulla was less distinct. Similarly, the boundary between the radial column zone and tangential zone was unrecognizable at this stage. At stage 23, although the characteristics of the radial column zone were still missing, space for neuropil appeared in the center of the medulla. The boundary between the cortex and the medulla was discernible and the two granular layers of the cortex were distinct. At stage 24, the feature of the radial column zone first appeared and the tangential zone had even more space for neuropil. The size of the optic lobe was also found to have enlarged significantly. The basic structures of the optic lobe were established at this embryonic stage. From stage 25 to stage 29, while the size of the optic lobe only increased moderately, the space for neuropil in the tangential zone continuously expanded. Note that the optic lobe in stage 27 appeared to be slightly larger than those in stages 28 and 29 due to individual size differences (**Figure 3**). Nevertheless, this observation supports that the size of the optic lobe does not increase significantly at late embryonic stages. At hatching (or stage 30), the size of the optic lobe was much larger than during previous embryonic stages, and the boundaries between the cortex and medulla, as well as between the radial column zone and tangential zone were much more distinct when compared with the embryos.

To quantify morphological changes of neural organization during the development of the optic lobe, the thickness of the cortex and radial column zone was measured from stage 22 to hatching (**Figure 4A**). It is apparent that the cortex increased steadily during embryonic development except from stage 29 to hatching, but the radial column zone was not recognized until stage 24 and showed a steeper increase in size throughout the latter embryonic stages. A careful examination of the organization of the radial column zone showed that the width of radial columns decreased significantly throughout the late embryonic development (**Figure 4C**). As a consequence, the density of radial columns increased steadily from stage 24 to hatching (**Figure 4D**). When the areas of the optic lobe, medulla, tangential zone, and cell islands were compared, the results confirmed previous observations that the size of the optic lobe showed two distinct fast growing periods, from stage 23 to stage 24 and from stage 29 to hatching (**Figure 4B**). While the areas of the medulla and tangential zone followed a similar growing pattern to that of the optic lobe, the area made up of the cell islands increased relatively slowly. As a consequence, the proportion of the cortex and medulla in the optic lobe remained moderately stable throughout embryonic development, but the radial column zone took up a significant space from stage 24 onward and the tangential zone became relatively smaller as the embryos grew (**Figure 4E** and **Table S3**). Since the increase in the cell islands was slower than that of the tangential zone, this resulted in the density of cell islands in the tangential zone of the optic lobe decreasing continuously throughout embryonic development (**Figure 4F** and **Table S3**).

Neural Fibers in the Tangential Zone Increase Continuously from Post-hatching to Adulthood

Although the general morphology of the optic lobe from different sizes of cuttlefish was similar to the one observed in late embryos,

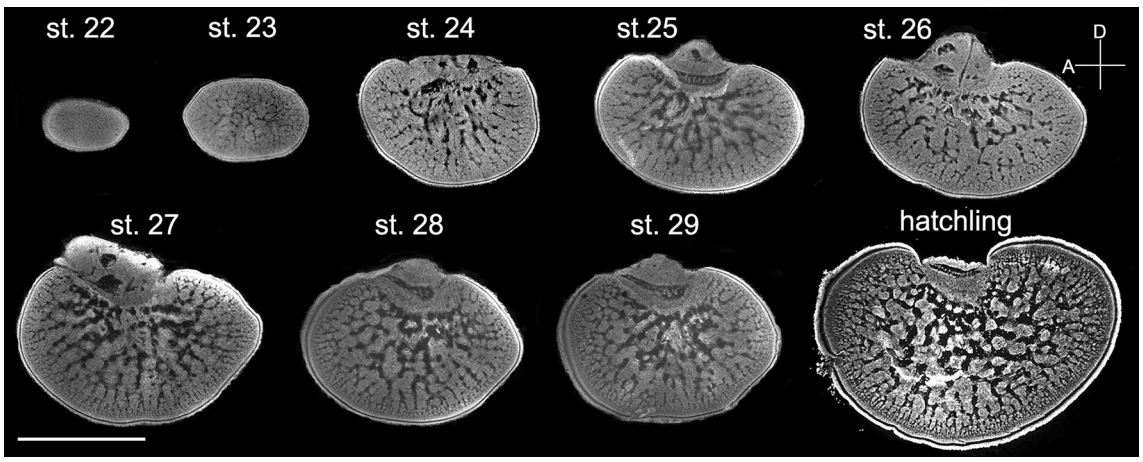

FIGURE 3 | The distinct structures of the optic lobes gradually appear during the late embryonic stages. Middle sections of the left optic lobes from different embryonic stages of cuttlefish. Nuclear staining was used to visualize the cell organization in the optic lobe. D, dorsal; A, anterior. Scale bar, 1 mm.

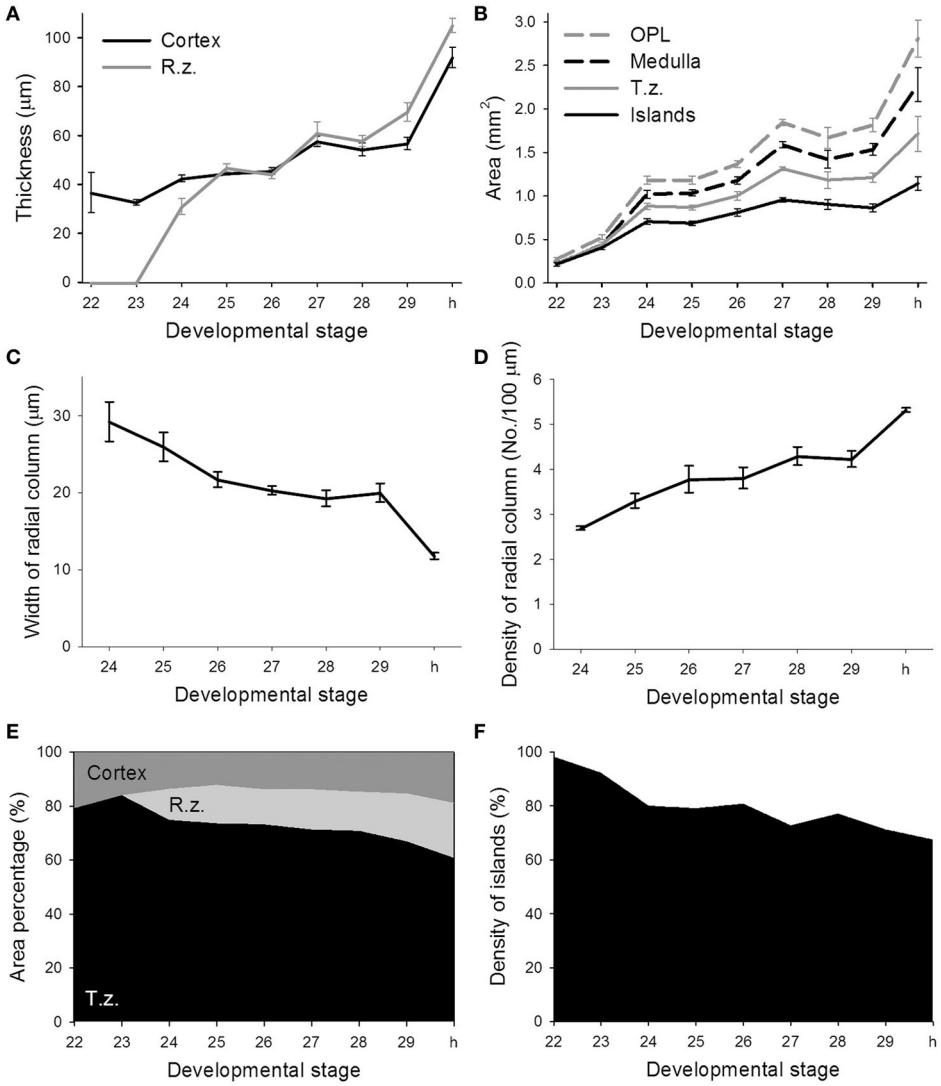

FIGURE 4 | The features of the radial column zone first appear at stage 24, while the proportion of cell islands decreases throughout the embryonic development of the optic lobe. **(A)** The thickness of the cortex and radial column zone (R.z.) of the optic lobe steadily increases during embryonic development. **(B)** Various areas of the optic lobe (OPL), medulla, tangential zone (T.z.), and cell islands gradually increase throughout embryonic development. **(C)** The width of the radial column steadily decreases during embryonic development. **(D)** Density of the radial columns gradually increases throughout embryonic development. **(E)** The proportions of the cortex, radial column zone, and tangential zone within the optic lobe change during embryonic development. **(F)** Density of cell islands in the tangential zone of the optic lobe decreases as the embryo develops. h, hatchling.

the proportion of neuropil in the tangential zone was found to transform continuously throughout the entire post-hatching life (**Figure 5**). Specifically, much of the size increase of the optic lobe was a result of an expansion of the medulla rather than the cortex, and much of the area expansion of the medulla at adulthood was due to the growth of the tangential zone rather than the radial column zone. Furthermore, the space for neuropil in the tangential zone enlarged steadily, indicating that there was a continuous increase in neural fibers among the cell islands as cuttlefish grew.

To quantify these morphological changes in the neural organization during the development of the optic lobe, the thickness of the cortex and radial column zone was measured from animals of mantle length 1.0–30.2 cm (**Figure 6A**). The results showed that both the cortex and radial column zone have two distinct growth phases, the first one being when the cuttlefish's mantle length is below 5 cm, and the second one being when the mantle length exceeds 5 cm. It should be noted that the relation between size and age of the animals is not strictly linear (Minton et al., 2001), thus the observed two growth phases may not correlate with the cuttlefish's age. When the cuttlefish were in the post-hatching stages, the thickness of the cortex and radial column zone increased significantly, but when the cuttlefish reached the sub-adult and adult stages,

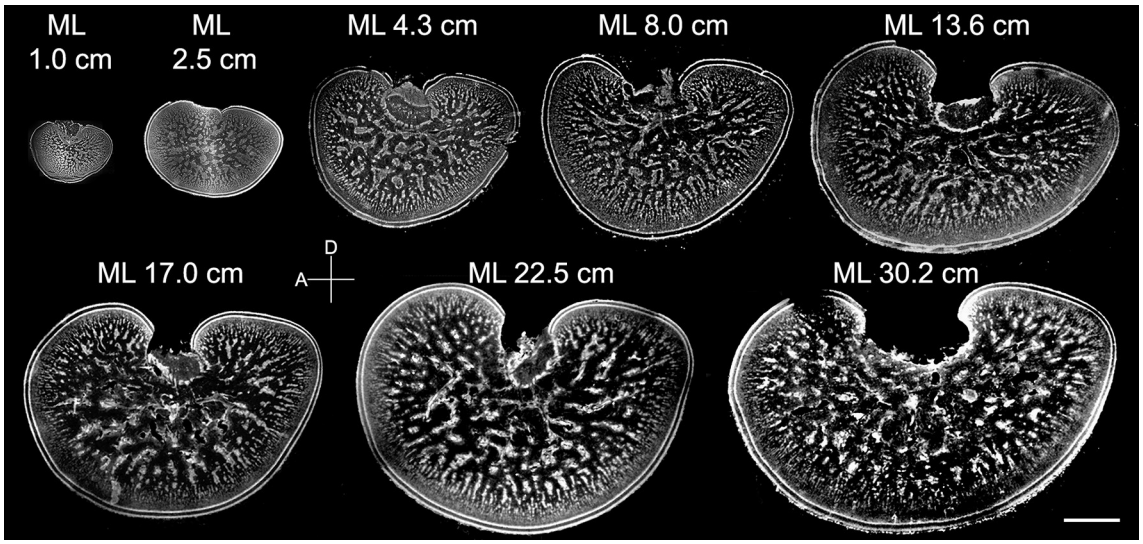

FIGURE 5 | The neural organization within the medulla of the optic lobe changes continuously from hatching to adulthood. Middle sections of the left optic lobes from different mantle lengths (ML) of cuttlefish. Nuclear staining was used to visualize the cell organization in the optic lobe. D, dorsal; A, anterior. Scale bar, 2 mm.

the thickness of the cortex and radial column zone remained relatively stable. A careful examination of the organization of the radial column zone revealed that both the width and the density of radial columns did not change drastically from hatching to adulthood (**Figures 6C,D**). When the areas of the optic lobe, medulla, tangential zone, and cell islands were compared, the results showed that the expansion of the optic lobe, medulla, and tangential zone was fast when the mantle length was below 5 cm, and continued even when cuttlefish reached sub-adult and adult stages, though the size increase in the tangential zone was relatively slower at these later stages (**Figure 6B**). In contrast, the expansion of the cell islands was steady during the juvenile stage, but stopped after the mantle length of cuttlefish was above 5 cm. As a result of these relative area changes, the proportion of the cortex in the optic lobe showed a continuous decrease as cuttlefish grew (**Figure 6E** and **Table S4**). However, the proportion of the radial column zone increased significantly when the mantle length was less than 5 cm, but decreased gradually when cuttlefish reached adult stage. Furthermore, the proportion of the tangential zone was increased moderately after cuttlefish reached the sub-adult and adult stages. Since the increase in cell islands was slower than that of the tangential zone, the density of cell islands in the tangential zone decreased continuously throughout the entire post-hatching development period (**Figure 6F** and **Table S4**). It should be noted that the density of cell islands decreased from 60% to about 40% when the mantle length of cuttlefish was below 5 cm, indicating that the tangential zone is transformed from cell soma dominant to neuropil dominant. In addition, the density of the cell islands continued to decrease from about 40–20% when the mantle length was above 5 cm, indicating that neural fibers among cell islands are increasing without there being significant cell proliferation during the sub-adult and adult stages.

The development of the Optic Lobe Is Accompanied by Increases of Cell Soma Size and Neural Fibers

To verify the observation that the expansion of the neuropil area is indeed a result of the increase of neural processes in the tangential zone, acetyl-α-tubulin which labels neural fibers was used to visualize the development of the optic lobe in embryonic, juvenile, and adult cuttlefish (**Figure 7**). Complementary to the images in **Figures 3**, **5** which showed the distribution of the cell somata, immunostaining images of acetyl-α-tubulin revealed neural processes in the optic lobe. It is apparent that the neural fibers increased continuously in the tangential zone throughout different developmental stages. To distinguish the origin of the increased neural fibers in the tangential zone during development, the neural fibers in the input region (the cortex and radial column zone), the tangential zone, and the output region (the optic tract region) of the optic lobe were characterized separately at three different developmental stages. It was found that the proportion of neural fibers of the tangential zone remained stable throughout developmental stages (**Figure S1** and **Table S5**). This suggests that the increase of neural fibers in the tangential zone as cuttlefish growing is equally contributed by the fibers from the input region, tangential zone, and output region of the optic lobe.

In addition to the increase of neural fibers during development, the cell soma size in different areas of the optic lobe was also expanded. It is apparent that the cell soma size and cell density in the cell islands appeared smaller and packed, respectively, when cuttlefish were in earlier developmental stages (**Figure 8**). This observation indicates that the organization and cell size within the cell islands of the optic lobe change continuously from the embryonic stage to the adulthood. To quantify the morphological and organizational changes down

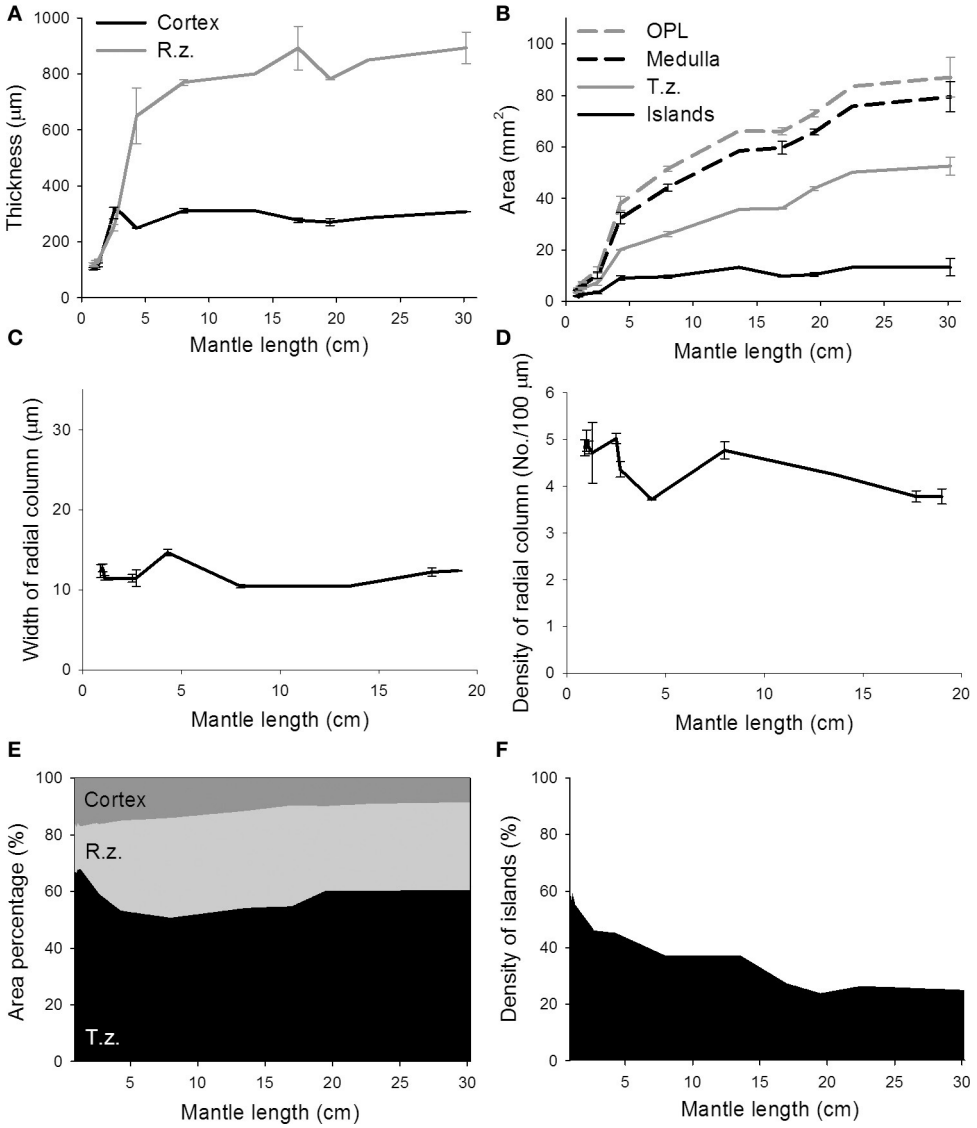

FIGURE 6 | The proportion of cell islands in the medulla decreases continuously from hatching to adulthood, while the growth of the cortex and radial column zone remains stable. **(A)** The thickness of the cortex and radial column zone (R.z.) of the optic lobe increases when the mantle length of cuttlefish is less than 5 cm, but stops increasing during the sub-adult and adult stages. **(B)** The areas of the optic lobe (OPL), medulla, and tangential zone (T.z.) increase continuously throughout post-hatching development, but the area of cell islands stops increasing after the mantle length of cuttlefish exceeds 5 cm. **(C)** The width of the radial column stays relatively constant during most of post-hatching stages. **(D)** Density of the radial columns also maintains in a moderate range throughout most of post-hatching stages. **(E)** The proportion of the radial column zone increases steadily when the mantle length of cuttlefish is less than 5 cm, but decreases gradually when cuttlefish reach the adult stage. **(F)** The density of cell islands in the tangential zone of the optic lobe decreases continuously as cuttlefish develop into adulthood.

to the cellular level in the optic lobe during development, the nucleus size (a proxy to estimate the cell soma size) and the nucleus density in the cell islands were estimated. The nucleus size in the cortex increased only from juvenile to adult cuttlefish (**Figure 9A**), while that of the radial column zone increased significantly from embryonic stage to adult cuttlefish (**Figure 9B**). In addition, it is evident that the average nucleus size in the cell islands of the peripheral region increased significantly from juvenile to adult cuttlefish (**Figure 9C**). In contrast, the average nucleus size in the cell islands of the central region increased significantly from embryo to juvenile cuttlefish. Similarly, the average nucleus density in the cell islands of the peripheral region decreased significantly from juvenile to adult cuttlefish (**Figure 9D**). In contrast, the average nucleus density in the cell islands of the central region decreased significantly throughout all three developmental stages. These findings suggest that the entire optic lobe, especially the cell islands, have gone through a significant change during development and this reorganization in the optic lobe may be important for behavioral changes throughout the life.

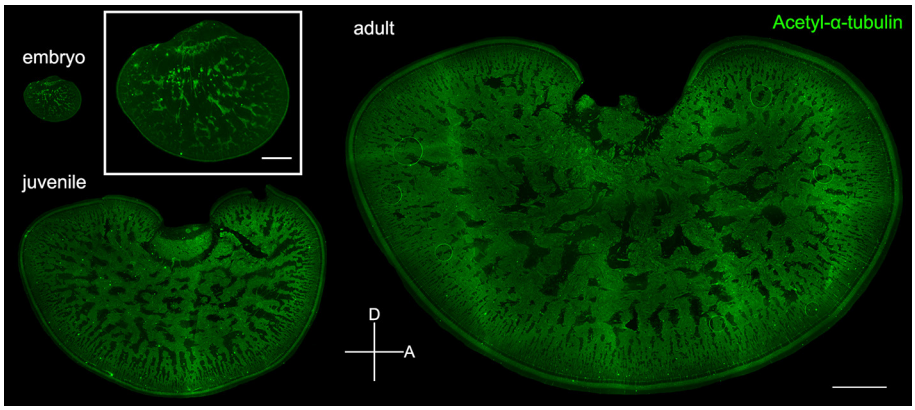

FIGURE 7 | Neural fibers in the tangential zone are complementary to the cell islands in the optic lobe. Fluorescence images of acetyl-α-tubulin which labels the neural fibers in the tangential zone of embryo (stage 24), juvenile (ML = 4 cm), and adult (ML = 17.7 cm). Inset shows the enlarged image of the optic lobe in embryo. D, dorsal; A, anterior. Scale bar, 1 mm (Inset, 200 μm).

Lateralization of the Optic Lobes Is Evident in the Cortex and Radial Column Zone during the Embryonic and Juvenile Stages

Previous studies on visual lateralization in the common European cuttlefish *S. officinalis* found that the sizes of the left and right optic lobes were not identical and this cerebral asymmetry creates a bias with respect to their side-turning behavior (Jozet-Alves et al., 2012a,b). In the present study, although the developmental pace of the left and right optic lobes in pharaoh cuttlefish *S. pharaonis* was similar at various life stages (**Table S2**), we found that the internal structures of the optic lobe in *S. pharaonis* showed a prominent variability in left/right asymmetry when the two sides of the brain were compared throughout the embryonic and juvenile stages (**Figures 10**, **11**). Specifically, the relative areas of the cortex and radial column zone showed a large variation with respect to the lateralization index throughout all late embryonic stages (**Figures 10A,B**) and when the mantle length of cuttlefish was less than 5 cm (**Figures 11A,B**). Interestingly, the internal structures of the optic lobe on the two sides of the brain became more symmetrical during the sub-adult and adult stages. Note that the sample size for the ML > 5 cm was small, thus the data should be treated cautiously. In contrast, the relative area of the tangential zone and the density of the cell islands were largely symmetrical during the late embryonic stages (**Figures 10C,D**) and throughout all post-hatching life (**Figures 11C,D**). These findings regarding the optic lobe indicate that the visual processing areas (the cortex and radial column zone) are more prone to lateralization than the visuomotor control area (the tangential zone) during the development of the cuttlefish.

DISCUSSION

Ontogenetic Development of the Optic Lobe

The present study reveals that the maturation of the various different regions in the optic lobe of cuttlefish is a non-uniform process. Specifically, morphological changes are most significant around embryonic stage 24, hatching, and at the time when the mantle length reaches 5 cm (**Figures 4**, **6**). In other words, the basic neural organization of the cuttlefish optic lobe is established when the animals are still young, but neural fibers among cell islands increase continuously from juvenile to adulthood. This type of brain maturation pattern is common in other animals. For example, mice complete neuronal differentiation and migration within the cerebral cortex a few days before birth, while neural connections among the various different cortical areas continue to form well beyond birth (Johnson et al., 2002). It has also been reported that most neurons in the ventral nerve cord of adult fruit flies are created during the larval stage, but the neural connections are continuously increased during later developmental stages (Truman and Bate, 1988).

Cuttlefish are a semelparous species, which means that eggs and juveniles develop without parental care. As a consequence, eggs are very vulnerable, and hatchlings need to cope on their own to find food and avoid predators. At embryonic stage 24, the layered structure of the optic lobe becomes evident with the first appearance of the radial column zone (**Figure 3**). This time point corresponds roughly to the observation that the visual system is functional from stage 25 of the cuttlefish *S. officinalis* (Romagny et al., 2012). More importantly, it is known that embryonic visual experience has a significant impact on the development of post-hatching behavior (Darmaillacq et al., 2006, 2008; Guibe and Dickel, 2011). Thus, early development of the visual processing area within the optic lobe, including the cortex and radial column zone, is crucial to allowing cuttlefish to detect visual stimuli and adapt to different visual environments very early in life. In addition, the finding that the radial columns decrease the width and increase the density throughout embryonic development (**Figures 4C,D**) suggests that the spatial resolution of the visual information processing is also continuously increased before hatching. Future studies of examining the effect of visual deprivation on the development of optic lobes and visual behaviors of hatchlings will provide further evidence of neural plasticity at this critical stage.

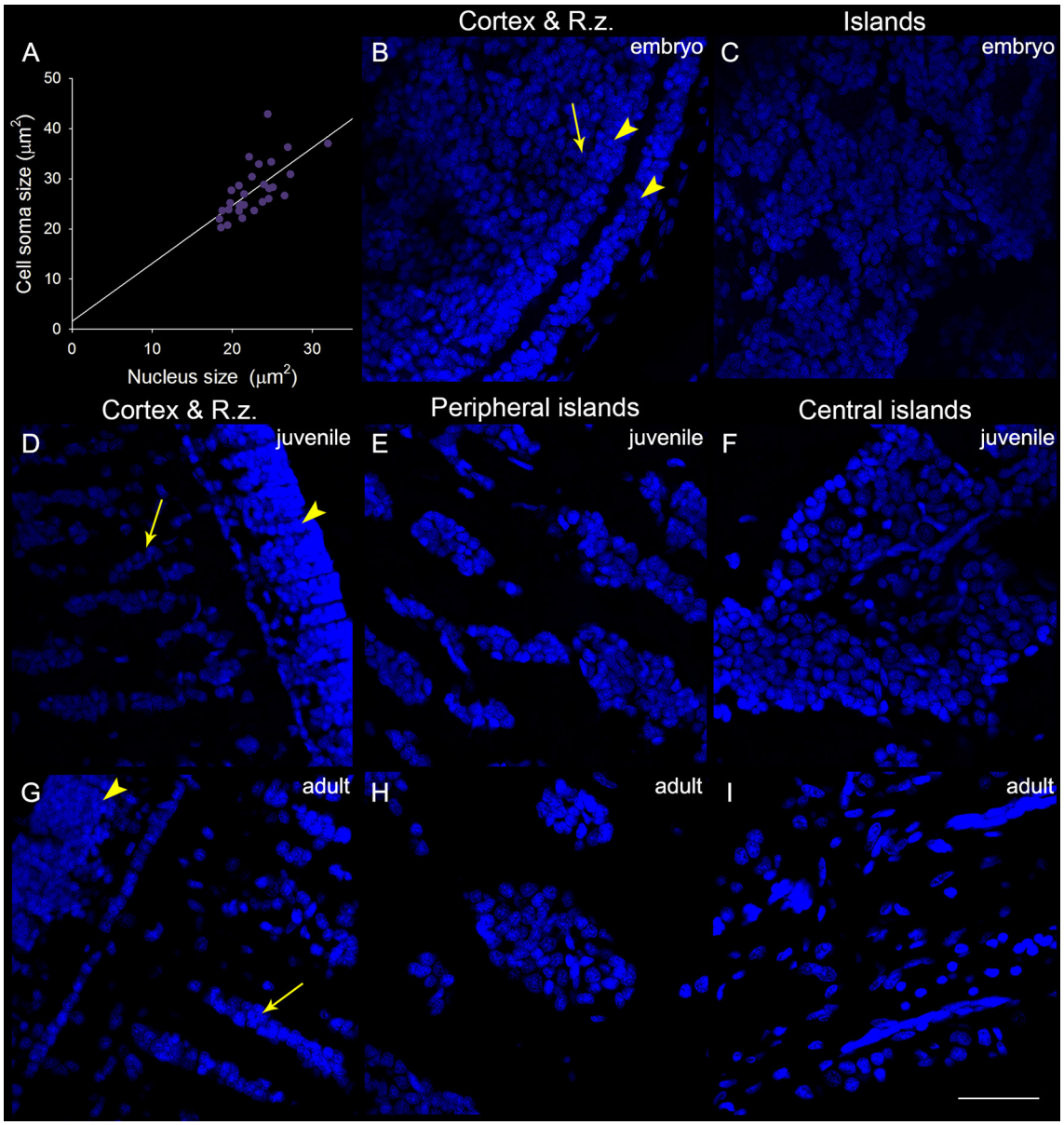

FIGURE 8 | The organization and cell size within the optic lobe change significantly from the embryonic stage to the adulthood. **(A)** A positive correlation ($R^2 = 0.454$, $p < 0.001$) between the nucleus size (estimated from the DAPI staining images) and the cell soma size (estimated from the acetyl-α-tubulin staining images and confirmed with the DIC images) obtained from the measurements (30 nuclei) of the cell islands in a juvenile cuttlefish (ML = 4 cm). This relationship allows us to use the nucleus size as a proxy to estimate the cell soma size in the cell islands. **(B)** The DAPI staining image of the cortex and radial column zone (R.z.) at the embryonic stage 24. **(C)** Nuclei (DAPI staining) in the tangential zone are not yet separable into the cell islands at the embryonic stage 24. **(D–F)** The DAPI staining images of the input region (the cortex and radial column zone) as well as cell islands in the peripheral and central regions of the optic lobe from a juvenile cuttlefish (ML = 4 cm), respectively. **(G–I)** The DAPI staining images of the input region as well as cell islands in the peripheral and central regions of the optic lobe from an adult cuttlefish (ML = 17.7 cm), respectively. Scale bar, 50 μm; Arrowhead, the granular layer of the cortex; Arrow, the stacked nuclei in the radial column zone.

From hatching to a mantle length of 5 cm is another fast growing period that involves a significant increase in the size of the optic lobe (**Figure 5**). However, much of this size increase in the optic lobe is attributable to an expansion of the medulla rather than of the cortex. Although previous developmental studies of the squid's brain have shown that the neuropil appear earlier in the tangential zone than in the radial column zone (Shigeno et al., 2001b,c; Yamamoto et al., 2003), the area of radial column zone actually grows faster than that of the tangential zone before the mantle length reaches 5 cm (**Figure 6**). Despite the slower expansion rate of the tangential zone, this area is gradually transformed from cell soma dominant to neuropil dominant. These results suggest that neural fibers among cell islands are increasing without significant cell proliferation. It

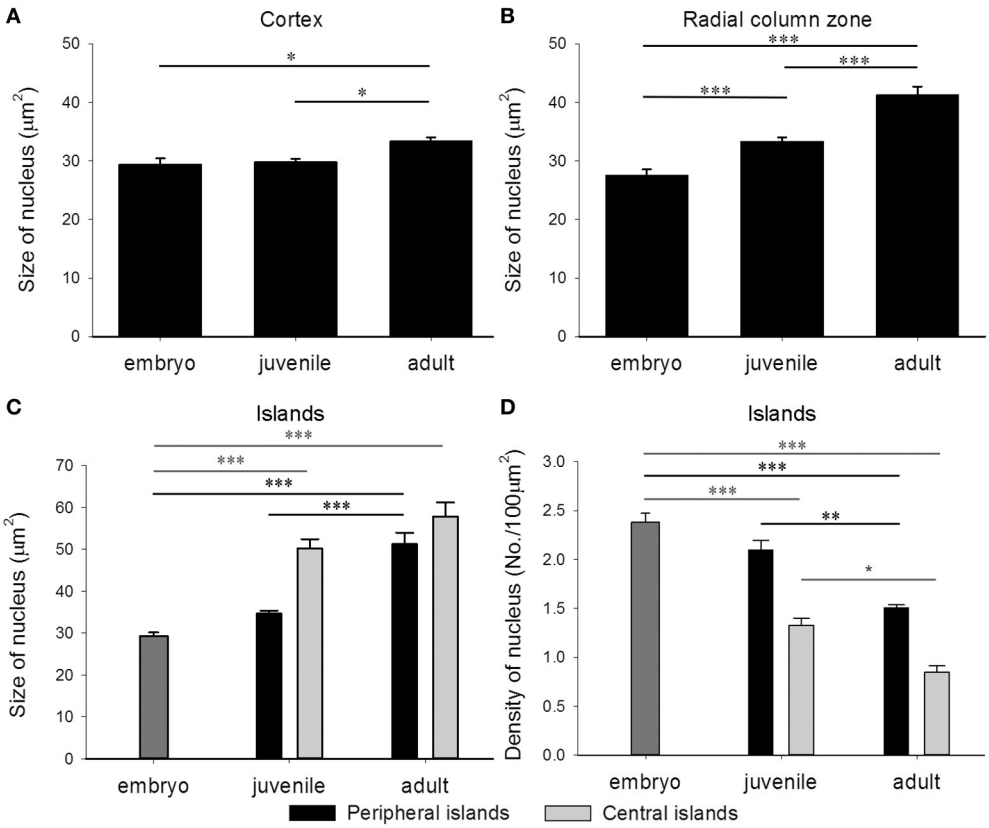

FIGURE 9 | The nucleus size in the optic lobe increases while the nucleus density in the cell islands decreases during development. **(A)** The average nucleus size in the cortex of the optic lobe increases significantly from juvenile to adult cuttlefish. **(B)** The average nucleus size in the radial column zone of the optic lobe increases significantly throughout all developmental stages of cuttlefish. **(C)** Due to the volume difference of the cell islands at various regions of the tangential zone in juvenile and adult cuttlefish, the peripheral and central regions were analyzed separately. The average nucleus size in the cell islands of the peripheral region increases significantly from juvenile to adult cuttlefish. In contrast, the average nucleus size in the cell islands of the central region increases significantly from embryo to juvenile cuttlefish. **(D)** The average nucleus density in the cell islands of the peripheral region decreases significantly from juvenile to adult cuttlefish. In contrast, the average nucleus density in the cell islands of the central region decreases significantly throughout all three developmental stages. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$.

has been well documented that post-hatching visual experience is important to shape the visual behavior of juvenile cuttlefish (Dickel et al., 2000; Poirier et al., 2005; Lee et al., 2010, 2012). The finding that the radial column zone and neural fibers among cell islands are disproportionally increased during this post-hatching period further supports the hypothesis that visual perception and visuomotor control of body patterning are crucial to juvenile cuttlefish as part of their camouflage and other defensive behaviors (Hanlon and Messenger, 1988).

After the mantle length reaches 5 cm and beyond, the optic lobe of cuttlefish continues to grow, but much of the size increase is a result of tangential zone growth rather than radial column zone growth (**Figure 6**). Furthermore, the density of the cell islands continuously decreases, suggesting that neural fibers among cell islands are increasing continuously without significant cell proliferation during the sub-adult and adult stages. It is known that the function of body patterning in cuttlefish continuously changes from hatching to adulthood; specifically there is a change from primarily being used for concealment as a defensive behavior to being mainly used for visual communication during reproductive behavior (Hanlon and Messenger, 1996). The observation that the tangential zone and the neural fibers among the cell islands are increased during the sub-adult and adult stages further supports the hypothesis that visuomotor control of dynamic body patterning depends on neural processing among the cell islands in the medulla of the optic lobe. These results are similar to studies on birds in which it has been found that the brain size is highly correlated with the development of novel foraging techniques (Overington et al., 2009). Furthermore, it has been shown that the volume of the song-related nuclei and the size of the associated neural tissues in songbirds are correlated with their song length and repertoire size (Garamszegi and Eens, 2004). These examples support a strong correlation between neural reorganization and behavioral modification throughout the life of animals.

In a close examination of cell morphology and fibers distribution in the optic lobe (**Figure 7**), we found that the proportion of neural fibers of the tangential zone remains stable throughout developmental stages (**Figure S1**). This suggests that the origin of the increased neural fibers observed in the tangential

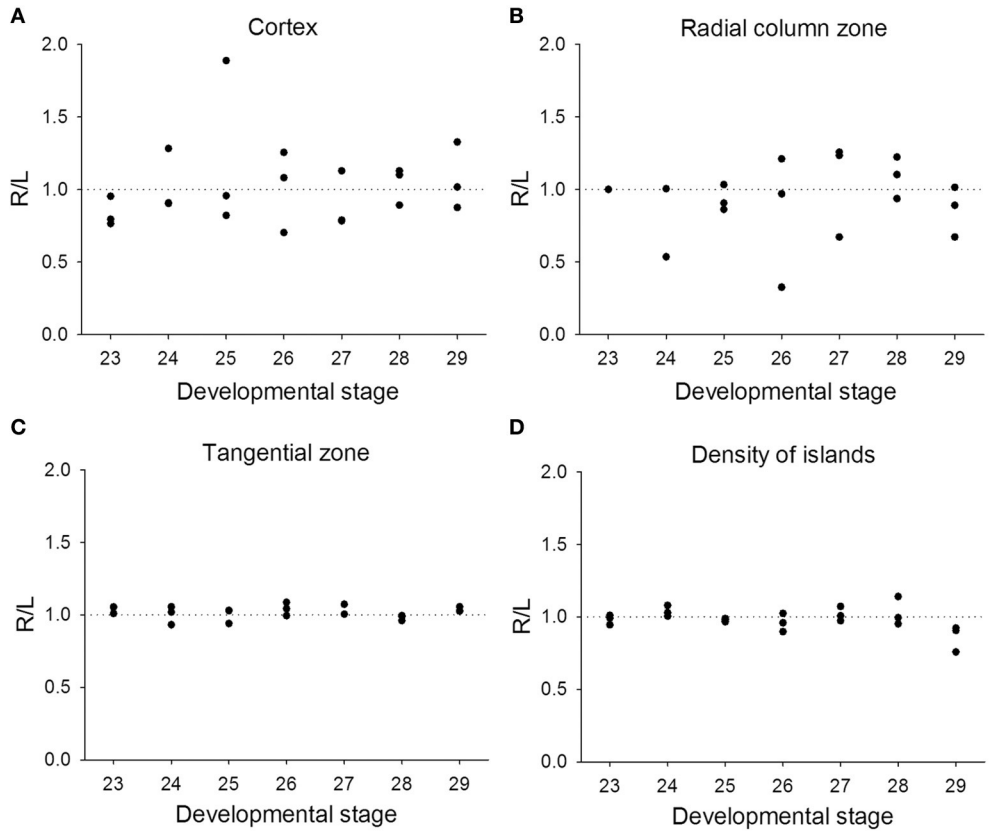

FIGURE 10 | Lateralization of the optic lobe is more pronounced in the cortex and radial column zone during the embryonic stages. The lateralization index is defined as Right/Left. **(A,B)** The relative areas of the cortex and radial column zone show a large variation in lateralization index throughout all late embryonic stages. **(C,D)** The relative area of the tangential zone and the density of cell islands are mostly symmetric across the various embryonic stages.

zone during development (**Figures 4**, **6**) is equally contributed by the growth of neural processes from the input and output regions as well as within the tangential zone itself. It also supports that information transfer in and out of the tangential zone is increased proportionally with the information processing within the tangential zone during cuttlefish development. Surprisingly, by examining the nucleus size in different areas of the optic lobe and the density of the cell islands at different developmental stages, we found that the organization and cell size within the optic lobe change significantly from the embryonic stage to adulthood (**Figures 8**, **9**). This finding suggests that specific areas in the optic lobe, especially the cell islands, are continuously reorganized to account for behavioral changes throughout life. It has been reported that the robust nucleus of the archistriatum (RA), an anatomically discrete brain region that is known to be involved with song production in birds, increase greatly in volume during a restricted period of song development in male zebra finches, and the growth of the RA is due to an increase in the cell soma size and a decrease in the cell density (Bottjer et al., 1986). This result suggests that the cells in the RA are undergoing fundamental maturational changes as the song behavior is beginning to acquire its adult form. This observation in the RA of zebra finches is parallel with our finding in the optic lobe of cuttlefish. The increase in the cell size may indicate that the metabolic activity of these neurons is increasing, whereas the decrease in the cell density may indicate that the dendritic arbor of these neurons is increasing, and/or that neural fibers from other regions are growing into this area. Further studies are needed to elucidate the underlying mechanism of morphological changes in the optic lobe during cuttlefish development.

Neural Basis of Visual Lateralization

In the present study, we confirmed that lateralization of the optic lobe may be a general feature of cuttlefish and that it is age dependent. Our results are consistent with the previous study showing that there is individual variation in the magnitude of the optic lobe asymmetry among the common European cuttlefish *S. officinalis* (Jozet-Alves et al., 2012a), and we have also shown that the lateralization indices vary greatly in both the cortex and radial column zone during the embryonic and juvenile stages of the pharaoh cuttlefish *S. pharaonis*. In contrast to the above, the variation was much less in the tangential zone and in the density of the cell islands (**Figures 10**, **11**). In the aforementioned study, these authors found that these anatomical brain asymmetries were correlated with behavioral asymmetries,

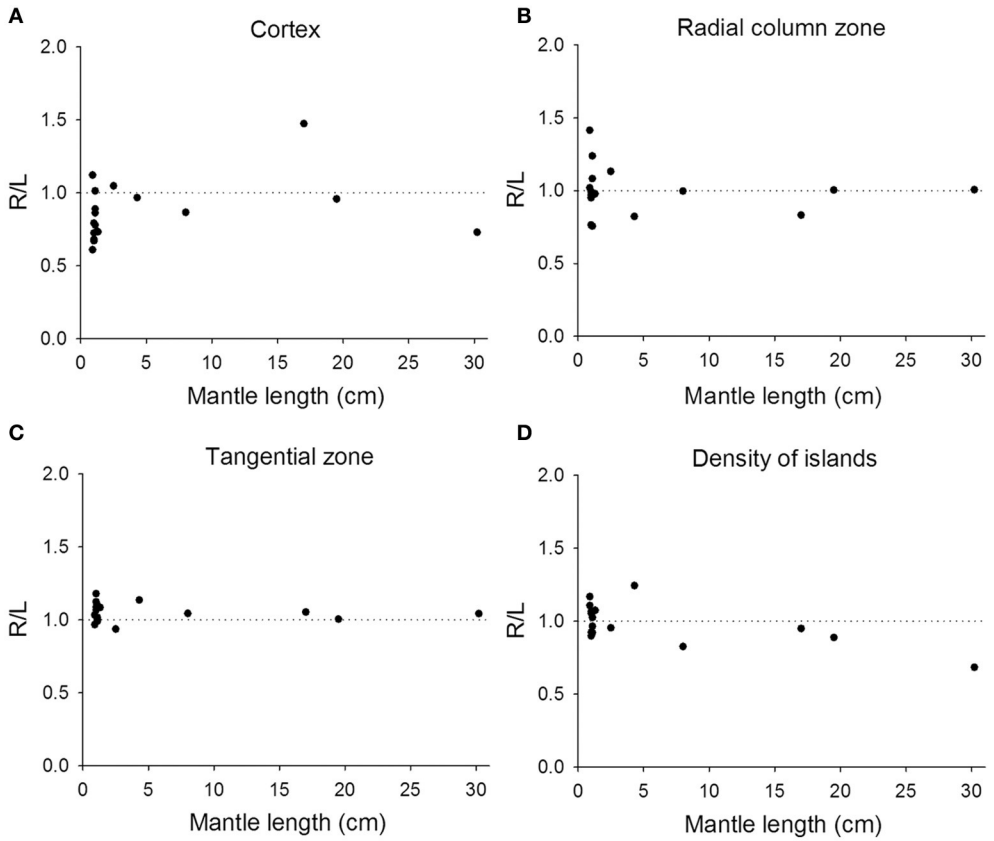

FIGURE 11 | Lateralization of the optic lobe is more apparent in juvenile cuttlefish. **(A,B)** The relative areas of the cortex and radial column zone show a large variation in the lateralization index when the mantle length of cuttlefish is less than 5 cm. **(C,D)** The relative area of the tangential zone and the density of cell islands have a reduced lateralization index variation compared to the cortex and radial column zone.

that is the larger the right optic lobe, the stronger the bias toward turning leftwards (Jozet-Alves et al., 2012a). In a separate study, it was also suggested that this left-turning bias observed in juvenile cuttlefish was the result of an eye use preference (Jozet-Alves et al., 2012b). Although, we did not carry out behavioral experiments to examine the correlation between anatomical asymmetry and turning bias, the fact that the cortex and radial column zone (the visual processing areas) are more prone to lateralization than the tangential zone (the visuomotor control area) in the optic lobe of developing cuttlefish supports the idea that eye use preference brings about the visual experience-dependent enhancement of side-turning preference. This is akin to neural plasticity observed in primate visual cortex and frog tectum (Hubel and Wiesel, 1977; Constantine-Paton and Law, 1978).

Neural Organization of the Optic Lobe

Earlier morphological studies of the optic lobes in octopus and squid (Young, 1962, 1974) suggested that the columnar organization of the radial column zone is likely to retain retinotopic information and thus it functions as a visual feature processing center. This neural organization pattern is similar to the visual cortex of the mammalian brain (Hubel and Wiesel, 1977), and the lamina and outer medulla of the insect's optic lobe (Strausfeld, 1970), which suggests a convergent evolution of these animal's brain organizations. Despite this similarity, there are some obvious differences in their neural systems (Breidbach and Kutsch, 1995). For example, the axons from the retina of cephalopods and insects do not form a bundle before projecting to the optic lobe, and the dorsoventral chiasma located behind the eye of cephalopods reverses the optically inverted image back in the optic lobe (Young, 1974). This latter feature ensures that the cortex and radial column zone in cephalopods retain upright retinotopic information, unlike the one in the visual cortex of mammalian brains. Furthermore, the optic nerves are mostly projected to the ipsilateral side of the optic lobe in cephalopods and insects, while they are largely projected to the contralateral side of the brain in vertebrates (Nixon and Young, 2003). These observations suggest that the visual system of cephalopods and insects are more alike when compared with vertebrates, despite the convergent evolution of the optics of the eyes in cephalopods and vertebrates (Packard, 1972).

In the tangential zone during the present study the so called "cell islands" (Young, 1962, 1974) were found to be contiguous in the MRI data, forming a tree-like structure

(**Figure 2A**). Since the internal organization of the optic lobe is quite conserved in most species of cephalopods (Nixon and Young, 2003), this 3D structure of the optic lobe from a sub-adult cuttlefish suggests that such an organization of neurons within the tangential zone could be a general feature in all optic lobes of cephalopods (Boycott, 1961; Young, 1962, 1974). By electrically stimulating the medulla of the optic lobe, previous studies have shown that various body patterns can be evoked unilaterally or bilaterally in cuttlefish (Boycott, 1961). Further studies have shown that the medulla is responsible for producing various types of locomotive behavior (Chichery and Chanelet, 1976, 1978). Our recent study also supports that various body pattern components can be selectively activated together when the medulla of the optic lobe from the oval squid *Sepioteuthis lessoniana* was stimulated electrically (Liu and Chiao, 2017). These findings suggest that the medulla, specifically the tangential zone, is the motor command center for locomotion and dynamic body patterning in cephalopods (Messenger, 2001). The motor signals generated by the medulla are the major input for the basal lobe, which is also an important motor controlling center in cephalopods and have been suggested to be functionally similar to the basal ganglia in vertebrates (Gleadall, 1990). Collectively, the functions of the tangential zone of the optic lobe and the basal lobe in cephalopods seem to be similar to that of the basal ganglia in vertebrates, where the primary function is to receive the sensory signals from various cortical layer, and generate motor signals to downstream brain structures for controlling and regulating the activities of the motor and premotor cortical areas during voluntary movements (Alexander et al., 1986; Reiner et al., 1998). However, the neural organizations of these two motor command centers are significantly different. In the optic lobe of cephalopods, neurons are clustered together and formed a contiguous tree-like structure, whereas neural organization in the striatum of basal ganglia have no apparent regional differences despite of the perfect topography and neurotransmitter-related neuronal distribution in different regions or nuclei (Squire et al., 2003). Nevertheless, both brain structures receive information from the sensory areas and select actions by sending control signals to the motor areas. Thus, the optic lobe with the downstream basal lobe in cephalopods and the basal ganglia in vertebrates are both crucial to the control of the motor movements required for specific behaviors. Alternatively, it has been suggested that the optic tectum of vertebrates, particularly fish and amphibians, has many output neurons to the midbrain territories that regulate motor programs (reaction, orientation, attack, and escape, etc.), thus the function and projection might be similar between the optic lobe in cephalopods and the optic tectum in fish and amphibians (Butler and Hodos, 2005). Finally, it has been suggested that the arthropod central complex and vertebrate basal ganglia circuitries that underlie the selection and maintenance of behavioral actions are evolutionarily conserved (Strausfeld and Hirth, 2013; Fiore et al., 2015). Thus it is possible that the optic lobe of cephalopods shares deep homology with the central complex of arthropods and the basal ganglia of vertebrates.

ETHICS STATEMENT

This study was exempt from one or more of the above requirements, because cephalopods are invertebrates and do not need to be approved by the institutional animal care and use committee in Taiwan.

AUTHOR CONTRIBUTIONS

YL conceived, designed, carried out the work, and drafted the manuscript. TL helped plan experiments and interpreted data. CS collected MRI data. CC helped plan experiments, interpreted data, and revised the manuscript.

ACKNOWLEDGMENTS

We are grateful to Prof. Samuel H. H. Chan at the Kaohsiung Chang Gung Memorial Hospital (Kaohsiung, Taiwan) for his generous support of the MRI scan. We thank Dr. Stephen L. Senft at the Marine Biological Laboratory (Woods Hole, MA, USA) for processing the MRI data and making a movie of the stereo image of the optic lobe. We also appreciate his invaluable comments on the manuscript. In addition, we thank Mr. Chun-Yen Lin and Yao-Chen Lee for collecting the cuttlefish eggs. This study was supported by the Ministry of Science and Technology of Taiwan NSC-102-2628-B-007-001-MY3 (to CC).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00538/full#supplementary-material

Figure S1 | Proportions of neural fibers of the input region (the cortex and radial column zone), the tangential zone (T.z.), and the output region (the optic tract region) of the optic lobe have no significant difference among different developmental stages.

Table S1 | The optic lobe samples and their nuclear staining methods.

Table S2 | All measurements from the left and right optic lobes are monotonically related.

Table S3 | Standard errors not shown in **Figures 4E,F**.

Table S4 | Standard errors not shown in **Figures 6E,F**.

Table S5 | Standard errors not shown in **Figure S1**.

Movie S1 | A pair of stereo images enhanced and reconstructed from the MRI scan showing the 3D structure of cell islands (light areas) in the medulla of the left optic lobe of a cuttlefish (ML = 16 cm). This MRI scan reveals for the first time that the so called "cell islands" present in the medulla (Young, 1962, 1974) are contiguous and have a tree-like structure.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

METHODS
published: 01 March 2017
doi: 10.3389/fphys.2017.00106

SpotMetrics: An Open-Source Image-Analysis Software Plugin for Automatic Chromatophore Detection and Measurement

Stavros P. Hadjisolomou[1, 2]* *and George El-Haddad*[3]

[1] Department of Social and Behavioral Sciences, American University of Kuwait, Salmiya, Kuwait, [2] First Year Experience Program, American University of Kuwait, Salmiya, Kuwait, [3] Scientific Software Engineer, Scientific Software Consultancy and Training, Jeita, Lebanon

Coleoid cephalopods (squid, octopus, and sepia) are renowned for their elaborate body patterning capabilities, which are employed for camouflage or communication. The specific chromatic appearance of a cephalopod, at any given moment, is a direct result of the combined action of their intradermal pigmented chromatophore organs and reflecting cells. Therefore, a lot can be learned about the cephalopod coloration system by video recording and analyzing the activation of individual chromatophores in time. The fact that adult cephalopods have small chromatophores, up to several hundred thousand in number, makes measurement and analysis over several seconds a difficult task. However, current advancements in videography enable high-resolution and high framerate recording, which can be used to record chromatophore activity in more detail and accuracy in both space and time domains. In turn, the additional pixel information and extra frames per video from such recordings result in large video files of several gigabytes, even when the recording spans only few minutes. We created a software plugin, "SpotMetrics," that can automatically analyze high resolution, high framerate video of chromatophore organ activation in time. This image analysis software can track hundreds of individual chromatophores over several hundred frames to provide measurements of size and color. This software may also be used to measure differences in chromatophore activation during different behaviors which will contribute to our understanding of the cephalopod sensorimotor integration system. In addition, this software can potentially be utilized to detect numbers of round objects and size changes in time, such as eye pupil size or number of bacteria in a sample. Thus, we are making this software plugin freely available as open-source because we believe it will be of benefit to other colleagues both in the cephalopod biology field and also within other disciplines.

Keywords: chromatophore, cephalopod, image-analysis, Fiji, software, spot, SpotMetrics

OPEN ACCESS

Edited by:
Daniel Osorio,
University of Sussex, UK

Reviewed by:
Najmeh Sahebzadeh,
Zabol University, Iran
Jay J. Culligan,
University of Sussex, UK

***Correspondence:**
Stavros P. Hadjisolomou
shadjisolomou@auk.edu.kw

Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *15 September 2016*
Accepted: *09 February 2017*
Published: *01 March 2017*

Citation:
Hadjisolomou SP and El-Haddad G (2017) SpotMetrics: An Open-Source Image-Analysis Software Plugin for Automatic Chromatophore Detection and Measurement. Front. Physiol. 8:106. doi: 10.3389/fphys.2017.00106

INTRODUCTION

Cephalopods are renowned for their rapid body pattern change capabilities utilized in camouflage or communication (Adamo et al., 2006; Shohet et al., 2007; Hanlon et al., 2011). Sub-second body pattern transformations are enabled by the combined activity of neurally controlled intra-dermal chromatophores and reflectors (Cloney and Brocco, 1983; Sutherland et al., 2008).

A lot can be learned about the sensorimotor control of body patterns by stimulating the visual system of coleoid cephalopods and measuring the resulting chromatophore activity. Since coleoid cephalopods have hundreds to thousands of tiny chromatophore organs, manual measurements and analyses of continuous chromatophore activity can become extremely complicated tasks. In this paper, we present a software plugin we developed for cephalopod researchers, "SpotMetrics," that can automatically detect, track, and measure chromatophore activity.

Sensorimotor Control in Cephalopod Body Patterning

Visual camouflage and signaling in coleoid cephalopods is driven by a sensorimotor system consisting of visual input of their surroundings, a sophisticated central nervous system (CNS) for information processing, and a muscular skin for generating patterns (Novicki et al., 1990). Coleoids have well-developed eyes and acute vision, which feed lobes in the CNS with input on spatial patterning, contrast, and luminance of the environment. The CNS directly controls dynamic skin changes on a massively parallel distributed system of effectors. Motor neurons selectively activate radial muscles, which in turn, produce sub-second retraction or expansion of thousands of chromatophore organs (Florey, 1969).

Effectors

Chromatophore organs are arranged in layers with a vertical hierarchy, each layer carrying a different pigment color. For example, in the squid *Doryteuthis pealeii* (Lesueur, 1821), there are three layers: (1) the top consists of yellow chromatophore which are the smallest, (2) the middle layer with red chromatophores which are intermediate in size while, (3) the lower layer is made up of brown chromatophores which are either the same size as red chromatophores or larger (Bell et al., 2013). The organization of chromatophore layers varies between cephalopod species.

A lot can be learned about the sensorimotor control of body patterning in cephalopods by examining the chromatic changes manifested at the individual chromatophore level. This can be achieved by video recording and analyzing the expansion and contraction patterns which generate or hide colors as responses to visual stimuli. Up until now, the main difficulties that emerged from having to record and analyze individual chromatophores on cephalopods had to do with the small sizes and the vast numbers of chromatophores. For example, *Sepia officinalis* (Linnaeus, 1758) carry smaller chromatophores compared to *Doryteuthis pealeii* (Lesueur, 1821) and require a microscope or an appropriately powerful camera lens to be viewed in adequate detail. Any small movement generated by the cuttlefish may shift chromatophores outside of the point of focus. For this reason, many observations of individual chromatophores are generally done by using either dead specimens or live chromatophores on excised skin (Goodwin and Tublitz, 2013). A solution to this issue is to use high definition video standards to capture data of higher quality and use customized software to detect, track, and measure chromatophores.

Video Acquisition

Data acquisition can be improved by recording chromatophores in High Definition (HD) resolutions and at a high frame rates. Current HD commercial cameras (and even some smartphone devices) can record videos at 1,280 × 720, 1,920 × 1,080, and 2,560 × 1,440 pixel resolutions. These cameras can also record footage at 60, 120, and 240 frames per second (FPS) in NTSC format. More expensive camcorders can record at even higher resolutions (Ultra HD) and higher frame rates.

Recording at higher resolutions helps improve data acquisition, as each chromatophore is represented by more pixels and has more detail compared to lower resolutions. A higher framerate of acquisition has a shorter interval between consecutive frames and collects more images of chromatophores per second. The amount of detail per image and the rate of continuation between frames become extremely important for the task of detecting, tracking, and measuring individual chromatophores. This enables researchers to study specifics of the chromatophore sensorimotor system as a whole (for example, the pathway from eyes to brain to chromatophores) in a living animal. Therefore, higher quality video acquisition improves data collection and makes data analysis more manageable.

However, because of the additional information being stored per frame, and with more frames being recorded per second, the size of HD videos at high frame rates climbs to several GB per minute of recording. This is an important point because a larger video file will require additional processing time and computing resources which may disrupt or end altogether the normal software analysis process.

Software to Measure Chromatophore Activity

The following steps are necessary to extract chromatophore activity data from a series of images: (1) detect individual chromatophores and remove unnecessary background information, (2) track each chromatophore in time and space (if the footage is from an animal that moves) and, (3) measure chromatophore surface area and color. One of the main issues with analyzing individual chromatophore data has to do with the large number of objects of interest to be measured. Depending on cephalopod species and magnification level used for video recording, the observer may be looking at a few individual chromatophores on a newly hatched animal or up to a few hundred to thousands in an adult animal. Manual processing of all the chromatophores becomes extremely cumbersome, if not impossible. Therefore, an automatic system of detecting, numbering, and tracking each chromatophore (both in space and time) becomes essential for such analysis.

Availability of Software for Analyzing Chromatophore Activity

There have been a few published studies in the cephalopod biology field which mention use of image analysis tools to measure individual chromatophore activity from video recordings (Suzuki et al., 2011; Goodwin and Tublitz, 2013; Brown, 2015; Ramirez and Oakley, 2015). These researchers

either developed their own customized scripts to be used within the Matlab® software package or made use of freely available software such as Fiji (Fiji Is Just ImageJ) (Schindelin et al., 2012) and Image J (Schindelin et al., 2015). However, with the exception of a few cases, these scripts and procedures are not being made readily available online to other researchers. Any researcher interested in analyzing videos of individual chromatophore activity would have to re-invent identical methods because of lack of access to such software. In other cases, customized software uploaded on university websites may become inaccessible during site server changes.

These issues can be prevented by following software development guidelines on sharing code and data and properly documenting the software on repository sites such as GitHub or SourceForge. Another factor which limits accessibility to software has to do with licenses and limited installations of paid-for applications. Commercial software solutions can be very powerful for data collection or analysis and are more likely to be continuously supported by dedicated developer teams compared to free open sourced software. However, the high cost per license may be prohibitive for students without sufficient funding. As a solution to this problem, students can choose to use free software, so the idea of developing and publishing programs on open-source platforms is well worth pursuing. Research can be accelerated by having methods and data publicly accessible to any scientist who may benefit from it. For this reason, we designed and created a software plug-in, SpotMetrics, that can analyze and process large HD video files in a freely available image analysis program.

SpotMetrics

The main objective of SpotMetrics is to process and analyze large HD video recordings of chromatophores. The plug-in automatically detects and numbers individual chromatophores, tracks them for the duration of the video, and provides information about their surface area and color properties. We would like to emphasize here that such a software solution has been published (Goodwin and Tublitz, 2013) as a customized script to be run within the Matlab® software package. The differences between this publication and Goodwin and Tublitz (2013) is that SpotMetrics is based on completely free and open-source software. Also, we are expanding on the ideas presented in that paper by adding a system that can track hundreds of chromatophores moving within the 2D space domain. We are working with a freely available software, Fiji, which does not require a license purchase for usage since it is under General Public License (GPL). In the same manner, we are making SpotMetrics software available to all under a GPL. Also, we are welcoming others to contribute to this software by contacting us with suggestions and requests to be worked into the existing plugin. Additionally, the plugin will be uploaded and maintained on a GitHub server which will contain periodic updates.

METHODS

SpotMetrics is a software plugin developed in JAVA specifically to be used with the image analysis suite Fiji.

Fiji is a suite of plugins and most of those plugins are a collection of algorithms themselves. The main reason that Fiji is so successful in the scientific community is that it allows the combination and reuse of other software and algorithms.

Libraries

SpotMetrics makes use of the following libraries which are used to detect, track, and measure the spot properties within a particular video:

AVI_Reader (https://imagej.nih.gov/ij/plugins/avi-reader.html) is used to import and read video files (.avi format) of chromatophores or other objects of interest to be analyzed.

TrackMate (http://imagej.net/TrackMate) detects and tracks every spot for the entire length of the video. Also, TrackMate keeps a record of every spot's X, Y coordinates from every frame for later reference. Lastly, it allows users to filter out spots that are of no interest.

Particle Analyzer (http://imagej.net/Particle_Analysis) analyzes and keeps track of the Regions of Interest (ROIs) in each frame and measures the area of each spot.

Measure Color (author: George El Haddad) is a simple algorithm that retrieves the central X, Y coordinate of each spot (with the help of TrackMate) and return the Red, Green, and Blue (RGB) values for the spot.

Apache POI (https://poi.apache.org/) is a fast and highly scalable library used to export data to a Microsoft Excel file (.xlsx). It is capable of generating large Excel files, depending on the amount of data to be exported.

Protocol

The following steps outline the procedure used by SpotMetrics to filter images and enhance chromatophore detection, tracking, and measurement.

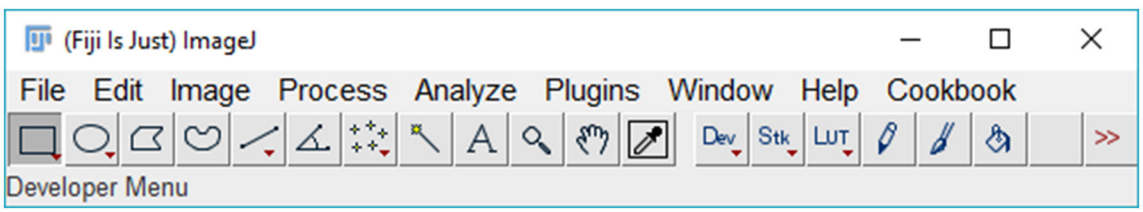

FIGURE 1 | Main menu of Fiji software.

SpotMetrics Procedure for Analyzing Spots/Chromatophores in Videos

1. Image processing and background subtraction:
   a. Program processes the imported video by converting all frames to 8-bit gray scale.
   b. Uses Fiji's Auto Threshold on every frame (method = "Default") to remove background and keep spots (chromatophores) for further analysis.
2. Spot detection and tracking:
   a. Analyze objects in image to identify/detect spots in each frame.
   b. Based on user settings (spot size), filters out spots that do not fit in selected category.
   c. Each spot is identified by a unique number.
   d. Track spots of interest over the length of video to collect data on size and color.
3. Data output.
   a. The program will generate an Excel file containing two spreadsheets with size and color measurements for further analysis:
      i. First spreadsheet lists the surface area (in pixels squared) of each spot per each frame.
      ii. Second spreadsheet lists the RGB values of each spot per each frame.

Step-by-Step Instructions to Run SpotMetrics Analysis

For first-time users, we recommend to run the program using the default options initially and to adjust settings for subsequent analyses based on results.

1. Run Fiji (**Figure 1**).
2. Click on "Plugins" menu and select "SpotMetrics."
3. Click "..." button and browse to folder containing the video you want to import. Choose video and click "Open" (**Figures 2**, **3**).

Note on video file format compatibility: The user must first convert all video files to be used with SpotMetrics into the.avi format. This is a requirement of the AVI_Reader library that is used here to import video files: "*PC users can use the free VirtualDub program to uncompress AVI files. Macintosh users can use QuickTime Pro to convert QuickTime movies into uncompressed AVI movies. Note that AVI files with audio tracks may fail to open.*" (https://imagej.nih.gov/ij/plugins/avi-reader.html).

4. The user has the option to crop part of the video at this point by using the Fiji available editing tools.
5. Click on the "Processing" tab to set video processing options (**Figure 4**).
   a. Keep the default settings for the initial run. The default settings will be "subtract background: 50" and "dark background" unchecked. Threshold method will be set to "default."
6. Click on the "Tracking" tab to set the spot tracking options (**Figure 5**).
   a. All measurements are in pixels, so set the "blob diameter" to a value close to each spot. For example, the default blob diameter is set to "10" pixels.
   b. The "blob threshold" value. Any chromatophore smaller than this diameter will not be tracked. Thus, if the user is only interested on larger spots, then a lot of the smaller ones can be filtered out using this setting.
   c. For the remaining options, we recommend leaving the default values as is:
      i. Linking max distance set to "15"
      ii. Gap closing max distance set to "15"
      iii. Gap closing max frame gap set to "2"

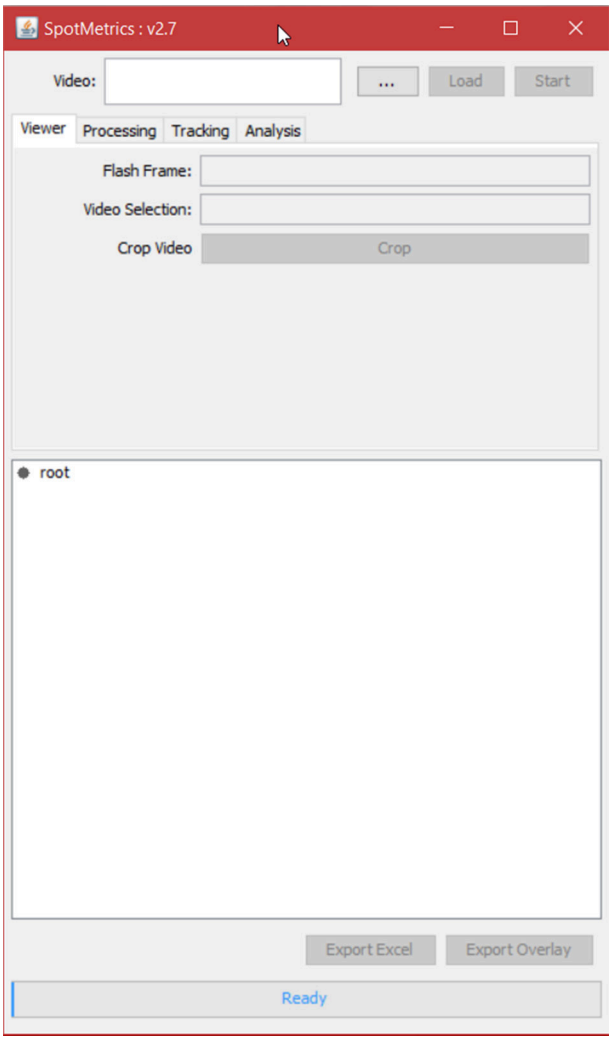

FIGURE 2 | Main menu of SpotMetrics.

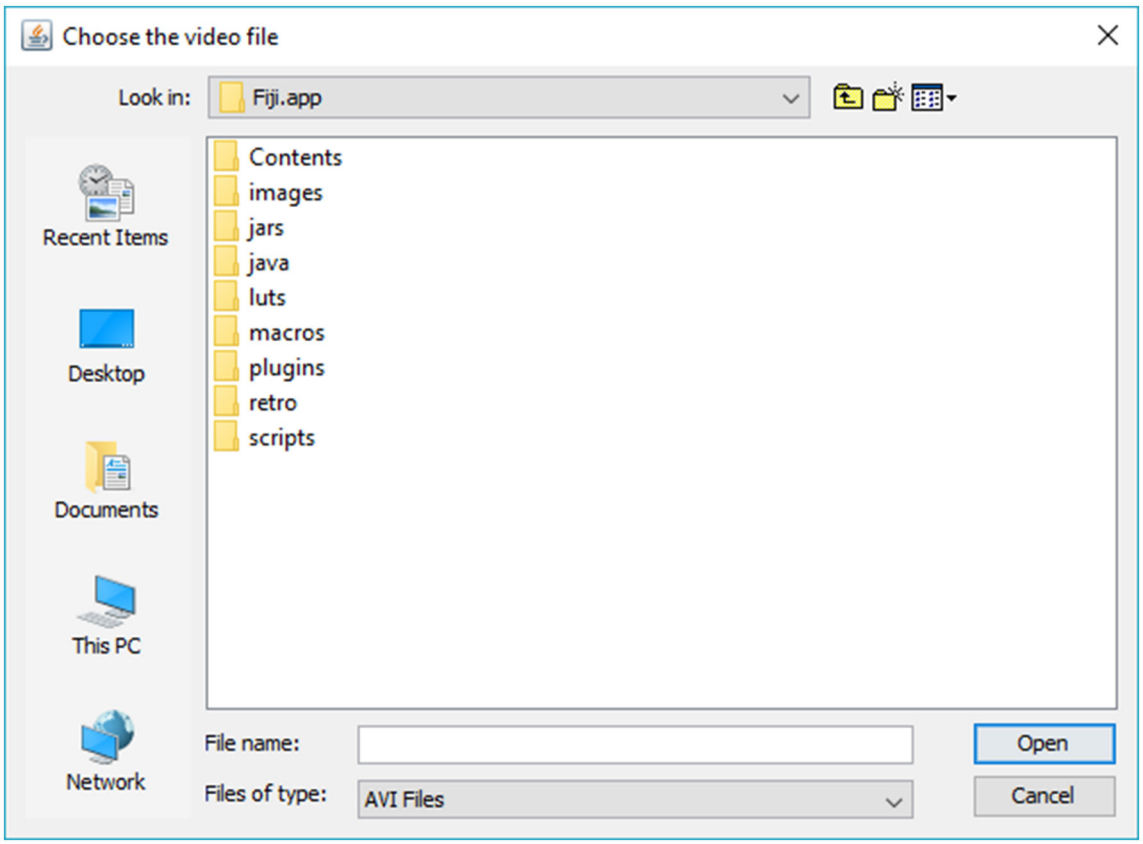

FIGURE 3 | Browse to desired directory to load video file.

iv. Initial spot filter value set to "0"

7. Click on the "Analysis" tab to set particle analysis options (**Figure 6**).
   a. Set the square area of the particle to detect (this is in pixels squared).
   b. Set the circularity of the particle being detected where 1.0 is a perfect circle.
8. Set the initial ROI offset for each spot that the particle analyzer will scan inside of. This is the initial box that will be drawn around each spot; the particle analyzer will scan inside this box so this has to be more or less accurate. If not, it can be tweaked later.
9. Click "Start" to initiate analysis.

Post-Analysis Steps to Improve Measurements Manually

Once the program has analyzed the video and measured spot properties, the user can run diagnostic tests to see how many spots have missing data and apply edits to fix the issues.

When analysis is done and the spots are listed in a tree menu: (**Figures 7**, **8**).

1. In the tree menu, right click on any track and select menu item "Diagnose All Tracks"
   a. This instructs the plugin to perform a particle analysis based on the "Particle Analyzer" options under the "Analysis" tab.
2. The "scan results" report indicates:
   a. Number of spots per track.
   b. Number of tracks with missing spots.

From this point in analysis, the user can manually edit each track that has missing spot measurements in specific frames.

1. Right click and select menu item "Edit Track"
   a. The ROI around the spot will turn from magenta to blue to indicate it is being edited.
   b. Click the "Next" button to scan frames forward to find the spot that is not being detected by the particle analyzer.
   c. Normally the issue is that the spot touches the ROI boundary, either because the is ROI is too small or slightly offset.
   d. Adjust the blue ROI with the mouse so that it properly encapsulates the spot and has at least a one-pixel gap between.

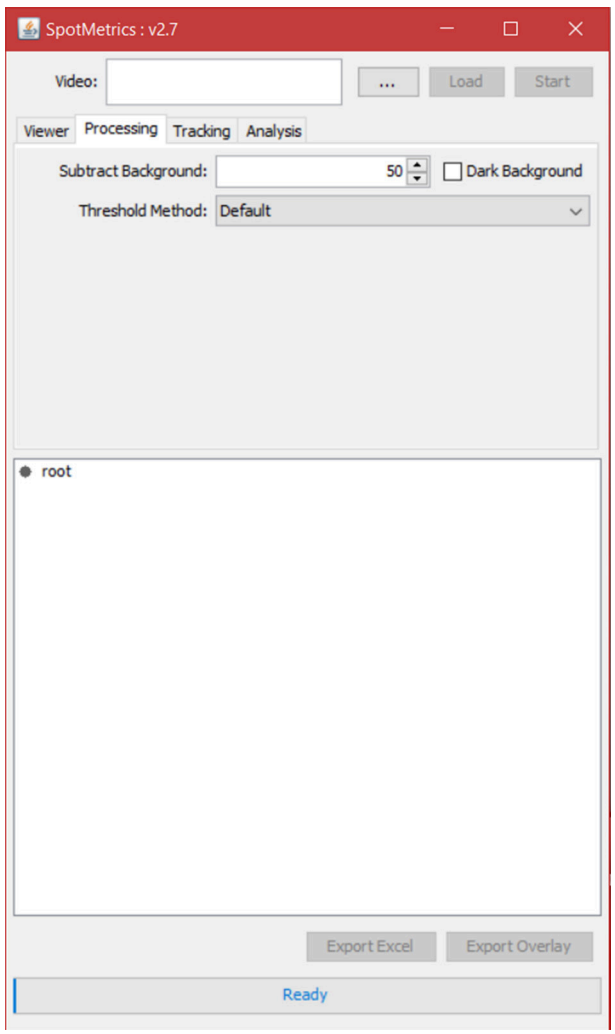

FIGURE 4 | Processing tab. Default settings will remove light background and keep dark objects.

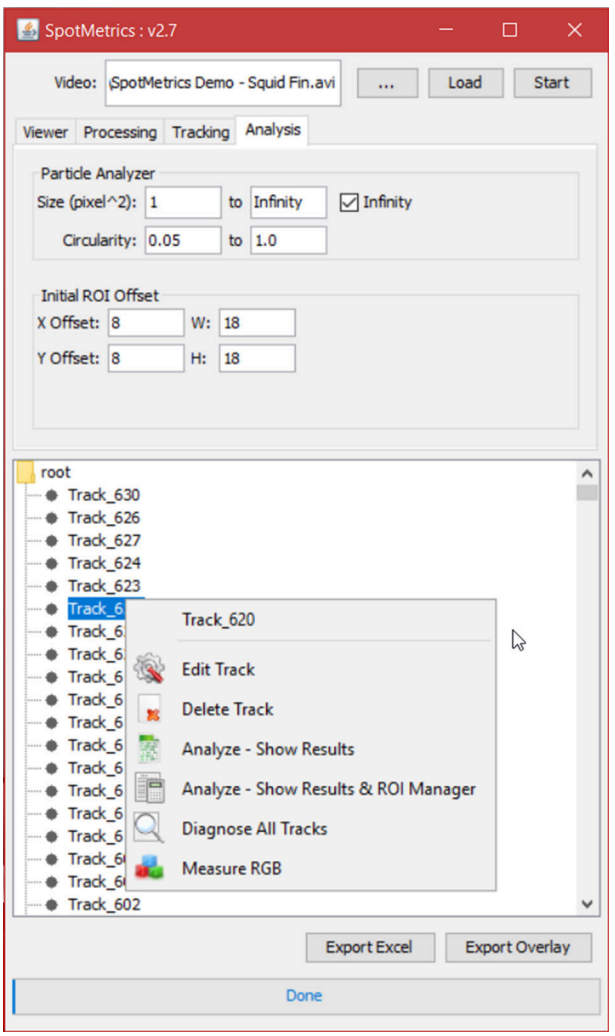

FIGURE 5 | Tracking tab. Choose blob diameter in pixels to track chromatophores of such diameter. Choose threshold value to exclude all chromatophores with diameters smaller than threshold value.

e. Click "Update ROI."
f. Continue until all frames are accounted for then close the "Edit Track" pop-up window.

2. Right click and select menu item "Analyze—Show Results."

a. Make sure that all the spots are properly detected and there is no missing information from any frames.

Also, the user can delete any track from the tree menu:

3. Right click and select menu item "Delete Track."
4. Click the "Export Excel" button and choose a directory and filename to save the chromatophore data in an Excel sheet. You can create graphs to visually inspect the chromatophore activity (**Figure 9**).
5. Click on the "Export Overlay" button to render a new video in which each tracked spot is shown with its outline as an overlay (**Figure 10**). Each spot is labeled based on two things: (1) the numbered ID of the track, and (2) the surface area measurement in pixels for each track. This is a valuable feature for reviewing each analysis, either to detect any errors, or to visually investigate each tracked chromatophore activity compared to others in time. This video can be saved for presentation purposes (Videos 1 and 2).

LIMITATIONS AND CONSIDERATIONS

System Requirements

SpotMetrics makes heavy use of CPU and RAM resources which are reallocated to speed up the analysis. The processing time of SpotMetrics is estimated by the number of chromatophores/spots that are detected within a given video file. If the plugin has to analyze 100,000 spots, the processing time will be significantly longer than analyzing 1,000 spots. We highly recommend using a PC with a modern processor to run this plugin. In addition, users

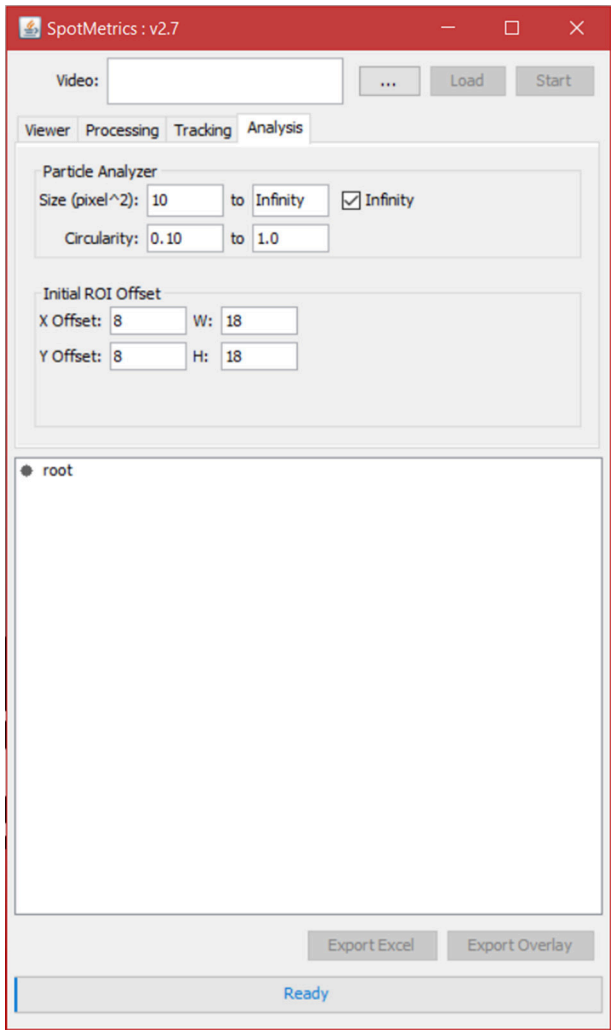

FIGURE 6 | Analysis tab. Set Search Region dimensions (ROI offset).

should avoid having other utilities run in the background while SpotMetrics is processing data.

Image Quality

The success of this plugin depends heavily on the image quality of the imported video files. For example, chromatophores/spots that appear out-of-focus/blurry will be more difficult to be detected and tracked by the plugin. In turn, the plugin will extract less information from such videos compared to recordings in which chromatophores/spots appear in more detail (**Figure 9**).

In addition, shaky footage, or lots of movement of chromatophores in the video can decrease the efficiency of detection and tracking over time. If the chromatophores/spots are displaced in the 2D domain rapidly, then the program will have difficulty tracking them for the entirety of the video. However, this can be corrected, up to a point, by applying a stabilization tool over the original video, using popular video editing software such as Sony Vegas Pro or Adobe After Effects. Nevertheless, we advise to proceed with caution whenever using video effects in this manner as to avoid modifying the recorded video in ways which may alter the image and add artifacts.

Possible Chromatophore Omission Due to Top-Down Perspective of Camera Angle

Cephalopod chromatophores are stacked in a vertical hierarchy (see section Effectors). Depending on the species, the top layer consists of either the largest chromatophore type (such as in some octopus species) or the smallest (such as in some squid species). The specific chromatophore arrangement becomes important when considering SpotMetrics detects and tracks objects within a 2D image; if two objects in a 3D environment are situated in such a way that the larger object is directly above a smaller object, then when converting the image to 2D, the larger object will appear to cover the one below when viewed from a top-down perspective. In this case, the smaller object below is not available for observation and thus SpotMetrics will not detect it.

When examining cephalopod body patterning, if a video sequence consists of fully expanded chromatophores within the top layer of Octopus skin, and given this type of chromatophores is the largest, then the smaller chromatophores below could be completely covered and hidden from observation from a top-down perspective. In this example, SpotMetrics would not be able to detect the hidden chromatophores, and would instead detect, track, and measure only the top layer of chromatophores. Therefore, to prevent this ommission when studying the chromatophore activity from a top-down perspective, it's vital to consider this fact to choose an appropriate choice of cephalopod species to study.

SIGNIFICANCE OF SPOTMETRICS AS A RESEARCH TOOL

SpotMetrics' value as a research tool comes in the form of automation and simplification for the researcher. This plugin would take several days' worth of manual processing down to an hour with just a few clicks in a user-friendly graphical environment. It does so by utilizing the power of existing algorithms, tools, and libraries provided by the scientific imaging open-source community and combining them for the use of tracking and analyzing spots.

Without a plugin such as SpotMetrics, a researcher would have to use a different plugin for each of the following steps: (1) to filter video data based on the contrast of the image and to remove unnecessary background information, (2) to identify and use the right method to detect spots in a video, (3) track spots over a duration of time and through space, (4) to analyze the spots that have been tracked for their color properties, (5) to analyze spots for surface area changes, and (6) to extract and collect the data from each individual tool and consolidate them into an organized spreadsheet ready for statistical analyses of results. Undertaking each of the above steps manually is technically possible. However, in this scenario, the researcher would have to spend a considerable amount of time on research, and trial-and-error process, in getting the output from each plugin to be formatted appropriately to be used as

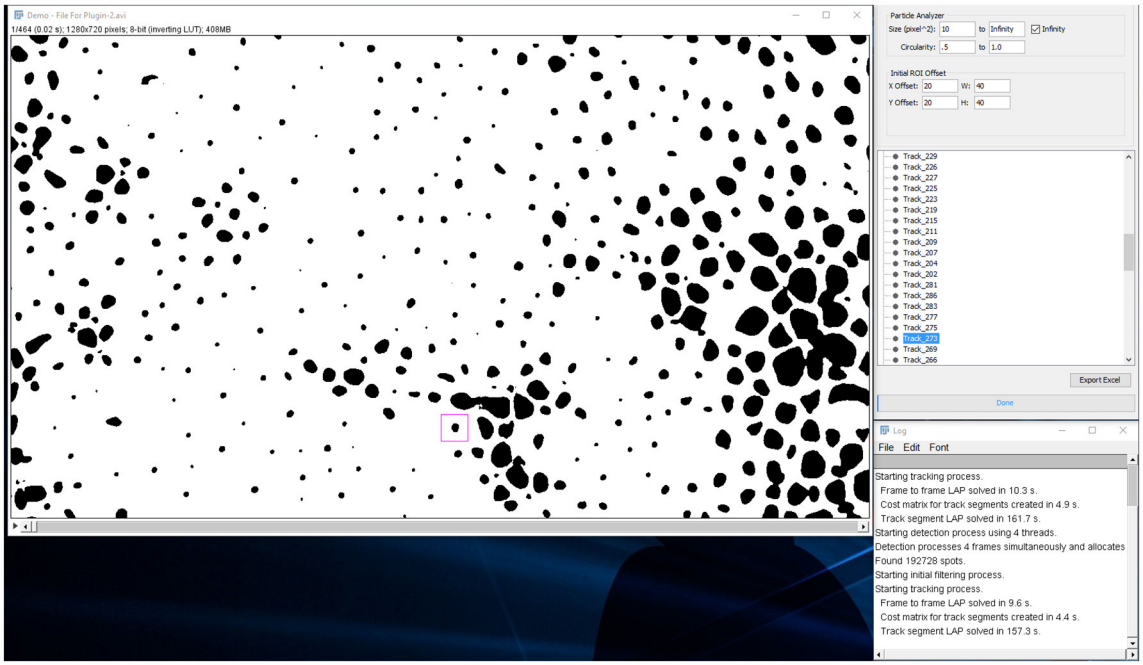

FIGURE 7 | Analysis tab. Results tree with individual chromatophores being represented as tracks.

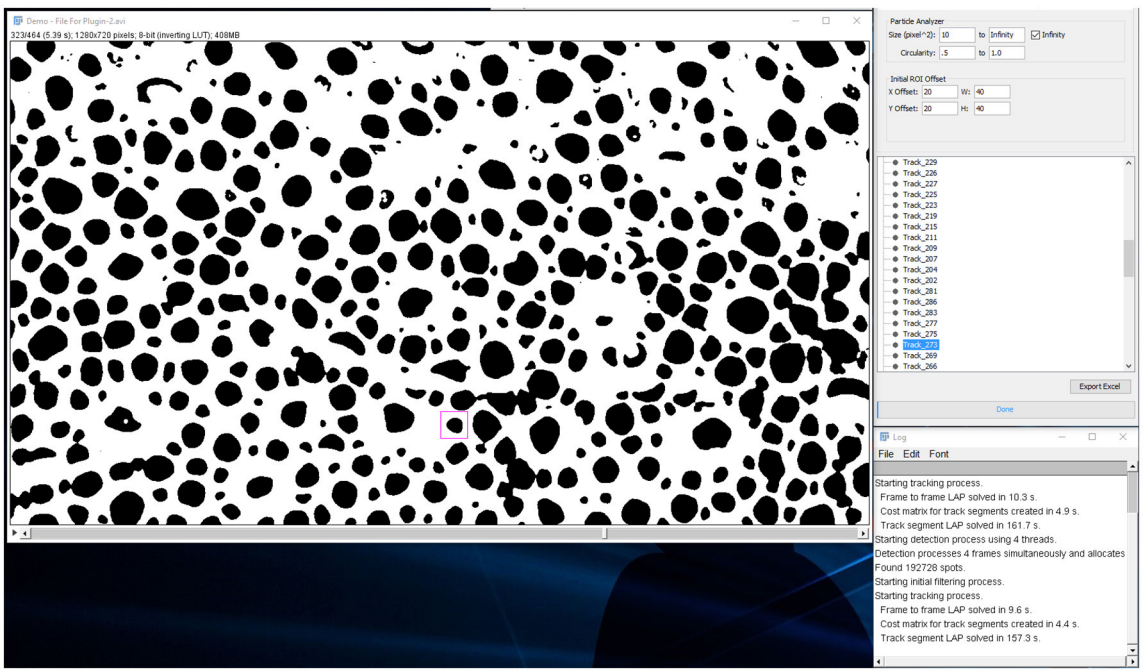

FIGURE 8 | Analysis tab. Users can playback the video and inspect individual tracks as chromatophores expand or retract.

input for the next step. SpotMetrics automates all the above processes, saving the researcher valuable time which would have otherwise been spent on researching and troubleshooting each method.

APPLICATIONS AND CONCLUSIONS

SpotMetrics can be used in a variety of image analysis procedures with the goal of detecting, tracking, and measuring circular

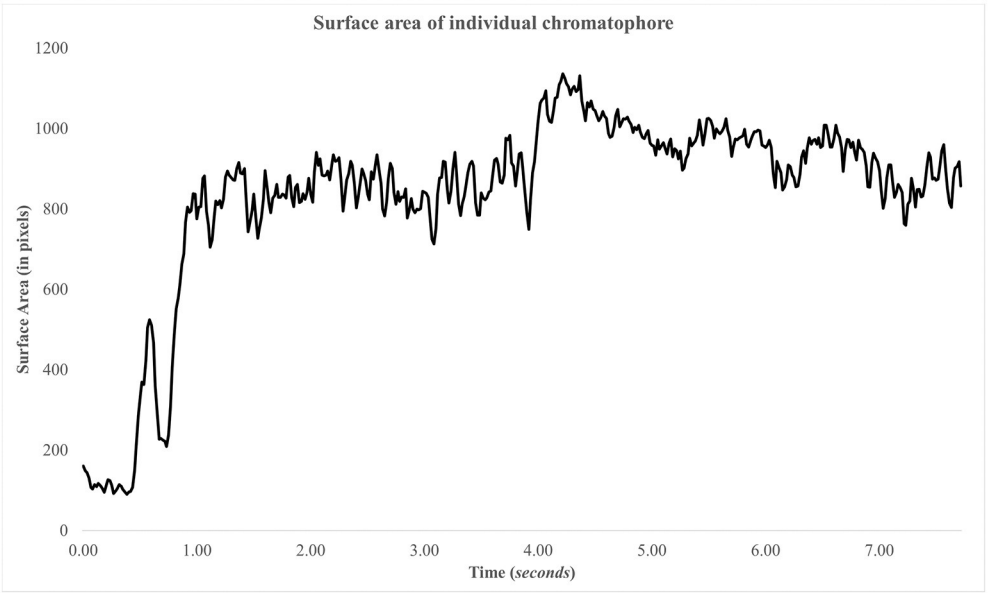

FIGURE 9 | Surface area of chromatophore over time. The chromatophore expands rapidly within the first second and remains expanded. There's another expansion, minor, at ~4 s.

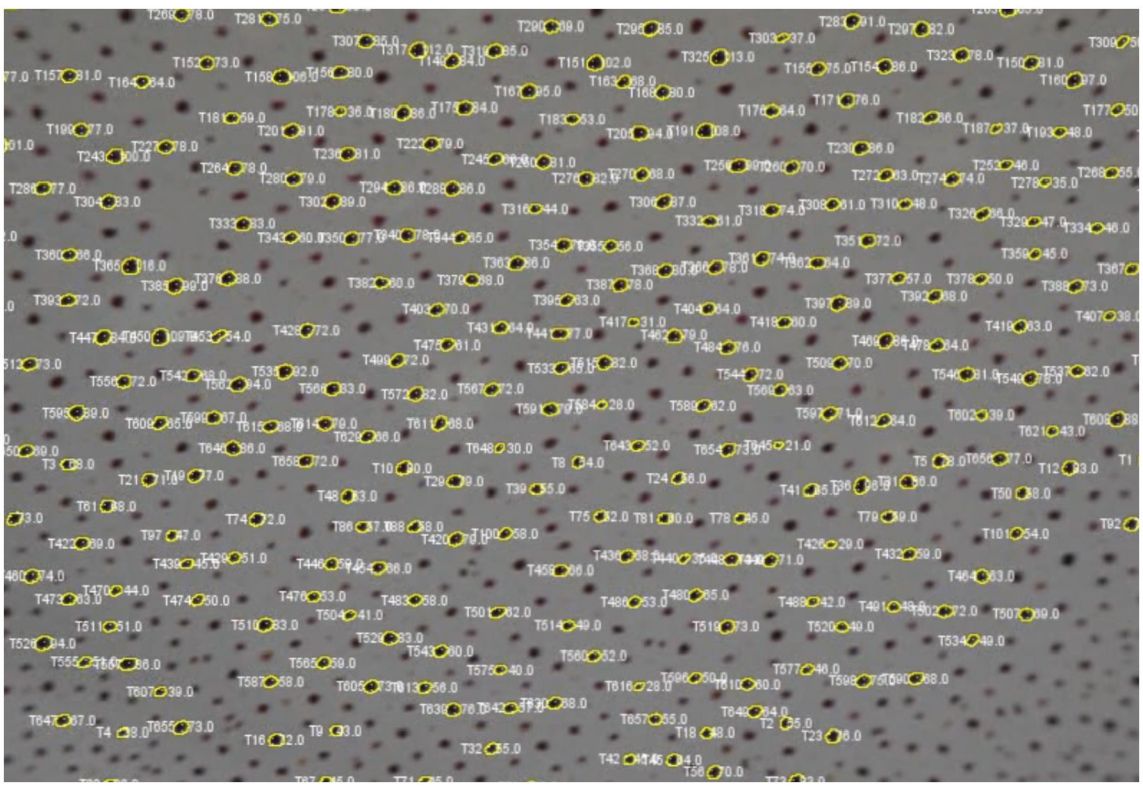

FIGURE 10 | Screenshot of video with each tracked chromatophore and corresponding outlines as overlays.

objects over a period of time. This plugin was developed with the primary goal to contribute to existing studies on sensorimotor integration control of the chromatophore system in cephalopods. We believe this tool will be helpful to researchers who wish to examine chromatophore activity as a response to sensory stimulation to better understand the underlying mechanisms of both sensors and effectors. For example, SpotMetrics can be used to measure chromatophore responses when animals are tested behaviorally under different chemical agents, presented with different background information, or presented with a potential predator, prey, mate, or competitor.

Similar to cephalopod biology studies, SpotMetrics can be of benefit to researchers who study chromatophore and melanophore systems in fish and reptiles. Lastly, this software can potentially be utilized to detect numbers of round objects and size changes in time, such as pupil dilation studies or number of bacteria in a sample.

We are making SpotMetrics freely available under the GPL license. The source code will be made available on the GitHub repository which will enable easier access to the latest update of the program. We'd like to extend an invitation to interested parties who may want to collaborate on improving this plugin by adding customized features and expanding the scope of the software in analyzing experimental data.

ETHICS STATEMENT

The animals used in the present study were squid. There was no requirement for this study to be approved by an ethical committee since at the time this study took place (July, 2014) the Institutional Animal Care and Use Committee (IACUC) protocols were not issued for invertebrate research in the USA. Nevertheless, we took every precaution to ensure the animals were under the least amount of stress possible and were not harmed in this study. Furthermore, the animals were allowed to acclimate when transported from housing to the experimental rig and vice versa. The study was observational and the animals were not subjected to any invasive methods.

AUTHOR CONTRIBUTIONS

SH contributed to the conception and design of the work. SH contributed to the drafting the manuscript. SH gave final approval for this version to be published. SH agrees to be accountable of all aspects of work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. GE contributed to the software development presented in this paper. GE contributed to revising the manuscript. GE gave final approval for this version to be published. GE agrees to be accountable of all aspects of work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

FUNDING

The study was supported by a doctoral student research grant from the Graduate Center of the City University of New York to SH.

ACKNOWLEDGMENTS

SH would like to thank Rita El-Haddad for providing valuable feedback on the manuscript draft. GE would like to thank Joelle El-Khoury and Emma El-Haddad for providing support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00106/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer JJC and handling Editor declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

frontiers in Physiology

ORIGINAL RESEARCH
published: 07 February 2017
doi: 10.3389/fphys.2017.00054

Reconsideration of Serial Visual Reversal Learning in Octopus *(Octopus vulgaris)* from a Methodological Perspective

*Alexander Bublitz, Severine R. Weinhold, Sophia Strobel, Guido Dehnhardt and Frederike D. Hanke **

Institute for Biosciences, Sensory and Cognitive Ecology, University of Rostock, Rostock, Germany

Octopuses *(Octopus vulgaris)* are generally considered to possess extraordinary cognitive abilities including the ability to successfully perform in a serial reversal learning task. During reversal learning, an animal is presented with a discrimination problem and after reaching a learning criterion, the signs of the stimuli are reversed: the former positive becomes the negative stimulus and vice versa. If an animal improves its performance over reversals, it is ascribed advanced cognitive abilities. Reversal learning has been tested in octopus in a number of studies. However, the experimental procedures adopted in these studies involved pre-training on the new positive stimulus after a reversal, strong negative reinforcement or might have enabled secondary cueing by the experimenter. These procedures could have all affected the outcome of reversal learning. Thus, in this study, serial visual reversal learning was revisited in octopus. We trained four common octopuses (*O. vulgaris*) to discriminate between 2-dimensional stimuli presented on a monitor in a simultaneous visual discrimination task and reversed the signs of the stimuli each time the animals reached the learning criterion of ≥80% in two consecutive sessions. The animals were trained using operant conditioning techniques including a secondary reinforcer, a rod that was pushed up and down the feeding tube, which signaled the correctness of a response and preceded the subsequent primary reinforcement of food. The experimental protocol did not involve negative reinforcement. One animal completed four reversals and showed progressive improvement, i.e., it decreased its errors to criterion the more reversals it experienced. This animal developed a generalized response strategy. In contrast, another animal completed only one reversal, whereas two animals did not learn to reverse during the first reversal. In conclusion, some octopus individuals can learn to reverse in a visual task demonstrating behavioral flexibility even with a refined methodology.

Keywords: reversal learning, simultaneous visual discrimination, operant conditioning, behavioral flexibility, secondary reinforcer

OPEN ACCESS

Edited by:
Graziano Fiorito, Stazione Zoologica Anton Dohrn, Italy

Reviewed by:
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Ildikó Kemenes, University of Sussex, UK
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Yuzuru Ikeda, University of the Ryukyus, Japan

***Correspondence:**
Frederike D. Hanke
frederike.hanke@uni-rostock.de

Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *18 August 2016*
Accepted: *23 January 2017*
Published: *07 February 2017*

Citation:
Bublitz A, Weinhold SR, Strobel S, Dehnhardt G and Hanke FD (2017) Reconsideration of Serial Visual Reversal Learning in Octopus (Octopus vulgaris) from a Methodological Perspective. Front. Physiol. 8:54. doi: 10.3389/fphys.2017.00054

INTRODUCTION

During reversal learning, an animal has to discriminate between two stimuli. However, after successfully responding to one stimulus with a high performance, the animal has to switch its response pattern because the stimuli will be redefined. The previous positive stimulus (S+), the animal was rewarded for upon choosing, becomes the negative stimulus (S−), and the previous

S− becomes the new S+. In a serial reversal learning experiment, the signatures of the stimuli are changed repeatedly every time the animal reaches a specific performance level. The way an animal solves a serial reversal learning experiment tells the experimenter if it has learnt stimulus specific responses or if it has learned to learn (Harlow, 1949; Shettleworth, 1998). The latter would be clear if the animal adopted a win-stay/loose-shift strategy, which could lead to the optimal performance of only one error after a reversal has taken place. By running a reversal learning experiment, behavioral flexibility of a species can be evaluated. Behavioral flexibility is the ability of a species or an individual to develop a new response pattern to unknown stimuli or to alter and adapt an existing response pattern to familiar stimuli. A high degree of flexibility in behavioral response patterns is often required to cope with challenges that animals are confronted with due to environmental changes or unpredictable resources. Behavioral flexibility and the ability to learn more than a mere associate of inhibitory and excitatory reactions to two stimuli as shown when an animal is successful during reversal learning experiments is commonly associated with advanced cognitive abilities (Shettleworth, 1998) beyond mere discrimination learning.

Reversal learning has been studied in numerous vertebrate species including monkeys (Warren, 1966; Milner and Ettlinger, 1970), mice and rats (Mackintosh, 1963; Bissonette and Powell, 2012), cats (Cronholm et al., 1960; Warren, 1966), horses (Fiske and Potter, 1979), kangaroos (Munn, 1964), birds (Bullock and Bitterman, 1962; Gonzalez et al., 1967; Boogert et al., 2010), reptiles (Day et al., 1999; Leal and Powell, 2011), fish (Gonzalez et al., 1967; Parker et al., 2012) and amphibians (Jenkin and Laberge, 2010) among others. In invertebrates, honey bees (Meineke, 1978), crayfish (Capretta and Rea, 1967), cockroaches (Longo, 1964), spiders (Liedtke and Schneider, 2014), and also octopus (Boycott and Young, 1957; Mackintosh, 1964; Young, 1962; Mackintosh and Mackintosh, 1963) have already been confronted with reversal tasks. Experiments on serial reversals in octopus (for overview see **Table 1**) revealed the ability of the animals to perform multiple reversals (Mackintosh, 1964; Mackintosh and Mackintosh, 1964). In Mackintosh and Mackintosh (1964), the octopods even showed an increase in performance, i.e., the number of errors decreased the more reversals were experienced. This performance compares favorably with a number of vertebrate and invertebrate species tested so far, in rats (Lawrence and Mason, 1955), lizards (Gaalema, 2011), corvids (Bond et al., 2007), pigeons (Gonzalez et al., 1967), isopods (Morrow and Smithson, 1969) as well as bumblebees (Strang and Sherry, 2014). However, in other studies with octopus, no improvement in a series of subsequent reversals could be documented, instead it was found that later reversals took the octopus longer to learn (Mackintosh, 1964; Young, 1962), which compares with the performance of other invertebrates including honey bees (Meineke, 1978) and crayfish (Capretta and Rea, 1967). During reversal learning experiments with octopus, training was often continued for a certain number of trials or sessions after reaching the predefined learning criterion, in order to test whether overtraining had an influence on the reversal learning performance. Mackintosh and Mackintosh (1963) demonstrated in a brightness discrimination task, including a black and white rectangle as stimuli, and by documenting the performance within a single reversal after the acquisition of the original task, that overtrained animals learnt the reversal significantly faster than non-overtrained subjects. However, this phenomenon could only be observed in the presence of irrelevant cues, for instance, the animal could have additionally used either the position or the orientation of the stimuli as an additional cue. Young (1962) investigated repeated reversals in octopuses in a brightness discrimination task, including a black and white circle as stimuli, with the sign of the stimuli being reversed every day for eight reversals without setting any learning criterion. When considering the proportion of errors to trials, performance became progressively worse with repeated reversals. Most likely this was due to a decreasing number of total attacks with subsequent reversals.

Previous studies on reversal learning in the octopus include some methodological aspects that need to be focused on. First, reversal learning in octopus has only been performed with 3-dimensional stimuli cut mostly from Perspex and fixed to a transparent rod for presentation purposes. They were submerged into the experimental tank probably manually, which might have resulted in the experimenter becoming visible to the experimental subjects. Thus, the experimenter could have provided secondary cues for solving the task. Second, the animals were rewarded with food for a correct response and a response to the S− was often followed by an electric shock. As a consequence, after a reversal, the animals usually had to be pre-trained on the new S+, the former S−, by solely presenting the new S+ for a fixed number of trials or until a certain learning criterion was met (Mackintosh, 1964; Mackintosh and Mackintosh, 1963). This procedure was adopted in order to prevent the animals from stopping to attack directly after a reversal. A cessation of cooperation immediately after a reversal of the experimental animal might happen if, after a reversal, it responded incorrectly because it continued to respond according to the previous definitions of the stimuli, which would ultimately lead to a punishment on the first trial. However, pre-training is considered detrimental in an investigation of learning abilities as the animal learns from every feedback given.

In order to overcome the aforementioned methodological implications, we conducted a visual serial reversal learning experiment with four octopuses as proof of concept for the new methodology and accomplished the following: We presented computer-generated stimuli on monitors and could thus shade the whole aquarium with curtains or carpets in order to avoid secondary cueing by the experimenter. We did not pre-train the animals after a reversal, which was facilitated by using positive reinforcement alone. For reinforcement, we introduced a visual secondary reinforcer, which has never been applied in octopus training before. In conclusion, we could obtain first insight into the serial reversal learning abilities of four octopus individuals with a refined approach.

TABLE 1 | Overview of the previous visual reversal learning studies including *Octopus vulgaris*.

Reference	Focus of the study	Stimuli	Number of animals	Pre-training	Electric shock	Learning criterion	Number of completed reversals
Boycott and Young, 1957	Reversal of learned responses and effect of vertical lobe removal	Circles, rectangles, L-shaped (Plastic)	9	No	Yes	–	1[+]
Young, 1962	Repeated reversals with a reversal every day comparing performance of animals trained with different stimuli to performance of animals without vertical lobe	Circles, rectangles, squares (Plastic)	26 (in 3 groups) 9 without vertical lobe	No	Yes	–	4–8[#]
Mackintosh, 1964	Effect of overtraining on reversal performance	Rectangles	18 (in 4 groups)	Yes	Yes	80% (in 20 trials)	2–9
Mackintosh and Mackintosh, 1963	Effect of overtraining on reversal performance with and without irrelevant cues	Rectangles (Perspex)	24 (in 3 groups)	Yes	Yes/No	90% (in 20 trials)	1*
Mackintosh and Mackintosh, 1964	Reversal learning with and without irrelevant cues (simultaneous stimulus presentation)	Rectangles (Perspex)	10	No	No	80% (in 10 trials)	7–14

[+]*No classic reversal learning procedure, for details see reference.*

#The signs of the stimuli were reversed every day for nine days without that the performance of the octopuses had reached a specific learning criterion.

**Experimenters stopped the reversal training after the first reversal.*

MATERIALS AND METHODS

Ethical Statement

This study was carried out in accordance with the directive 2010/63/EU. This study involved a procedure with the severity classification "mild" (Annex VIII). The experiments conducted in this study were approved (6712GH00113) by local authorities (Staatliches Amt für Umwelt und Natur Rostock) according to § 42 of the German law on nature protection. The ARRIVE guidelines checklist (Kilkenny et al., 2010) was the basis for the preparation of this manuscript.

Experimental Subjects

Experimental subjects were four common octopus individuals (*Octopus vulgaris*), four females with a mantle length of 4–8 cm (**Table 2**), which were subadult at the beginning of the experiment. Three animals were experimentally naïve animals but one, experimental subject Ov3, was already familiar with the experimental procedure and had already received some training in a former visual discrimination task examining concept formation (unpublished data). They were captured in the Mediterranean Sea in the waters of the Tuscan Archipelago, Italy, in spring, and training started with the first phase, feeding by the experimenter (**Table 3**), as soon as the animal showed interest in food. The animals were kept following the information on maintenance, care, and welfare given for invertebrates in general and cephalopods in particular (Oestmann et al., 1997; Dunlop and King, 2009; Smith et al., 2011; Andrews et al., 2013; Fiorito et al., 2014, 2015). Two subjects, Ov1 and Ov3, were kept in individual 250 l glass tanks (100 × 50 × 50 cm). Subjects Ov2 and Ov4, were housed in a 3000 l sea water aquarium system with individual compartments for the animals (130 × 73 × 86 cm; **Table 2**). The experiments were conducted in the respective home tanks of the individuals. The tanks were filled with continuously circulating sea water (salinity 33 g/kg, temperature 19–23°C). Artificial illumination was provided mimicking a natural day-night cycle (10/14 h or 12/12 h). To ensure a balanced diet, subjects were given freshly thawed pieces of great northern prawns (*Pandalus borealis*), thawed smelts (*Osmerus eperlanus*), common mussels (*Mytilus edulis*) as well as mussels of the genus *Veneridae* and common shrimps (*Crangon crangon)*. Food was provided to the subjects at least twice a day mainly during the experiments. Individuals were either rewarded with approximately 1 g of northern prawn or mussel per correct response. The type of reward was chosen according to the availability of mussels and to individual preference but was kept constant for one individual over the whole experimental period. Thus, the animals received food according to their performance, which was usually less than 5% of their body weight per day. With a daily food intake of 5% body weight, octopus seems to be fed near satiation (Chapela et al., 2006).

Experiments lasted from 30 min up to 2 h, depending on the individual and its motivation. They were carried out 5–7 days a week over a total period of approximately 6 months per individual. The experimental phases (**Table 3**) followed each other without any large break.

Experimental Setup

The general experimental setup is shown in **Figure 1**. It was installed in the home tank before the arrival of the animal and remained there throughout the experimental period. For stimulus presentation purposes, an LCD monitor was used (21.5 inch, E2251 Full HD, LG electronics, Inc., Seoul, Korea). It was attached to one side wall of the tank from outside. In the middle of the screen, a vertical divider was installed within the tank, which ensured that the animal was giving a precise response either to the left or to the right side of the monitor.

TABLE 2 | Details on the experimental subjects including sex (F female, M male), size as mantle length (in cm), size of the home tank (in l), experimental past, if applicable.

Ov	Sex	Size (cm)	Tank size (l)	Experimental past
1	F	5	250	No
2	F	6	800	No
3	F	6	250	Yes
4	F	8	800	No

Unlike former studies, in which the use of a transparent door kept experimental subjects at a certain distance to the location of stimulus presentation (see e.g., Mackintosh and Mackintosh, 1963; Sutherland and Carr, 1963), a terracotta flower pot was positioned at approximately 50 cm distance to the monitor and was aligned with the center of the monitor. It served as a starting point for each single trial during experiments and ensured that the subjects always had the same viewing angle on the display and the same distance to the stimuli at the beginning of each trial. Close to the flower pot, a transparent acrylic tube (length 55 cm, diameter 3 cm) was inserted through the lid of the aquarium. This tube served to provide the food reward to the subjects. This procedure helped to avoid problems with practicability of food delivery as reported in Boal (1996) and Crancher and King (1972). During experiments, an opaque curtain around the aquarium as well as an opaque cover on the lid of the tank served to keep the experimenter out of sight of the octopus in order to avoid unintentional secondary cueing. The experimenter observed the experimental procedure via a camera (Genius WideCam 1050, KYE System Corporation 2011, Taipei, Taiwan) equipped with a wide angle lens. The whole experimental area was illuminated with a lamp from above.

Stimuli

The stimuli (see inset in **Figure 2**) used in the experiments were designed with Corel DRAW X5 (Corel Corporation 2012, Ottawa, Canada) and presented to the animals within a Power Point presentation (Microsoft Office 2012, Microsoft Corporation, Redmond USA). All stimuli were presented as black shapes of identical surface area on a gray background on the LCD monitor as this stimulus/background combination elicited attacks by the animals. As an LCD monitor was used for stimulus presentation, octopus, being polarization sensitive (Shashar and Cronin, 1996), might use the polarization and/or luminance contrast for discriminating the stimuli. For all four animals, two different pairs of stimuli were used (**Table 4**; **Figure 2**). Three of the animals, Ov1, Ov2, and Ov3 had to discriminate between a vertical and horizontal rectangle (40 × 10 mm) of which two, Ov1 and Ov2, had the horizontal rectangle as S+ in the basic discrimination task (R0) while for subject Ov3 the vertical rectangle was defined as S+ in R0. The rectangles were chosen as octopuses are known to readily discriminate between these stimuli (Sutherland, 1957; Wells, 1978) and they are similar to stimuli used in reversal learning studies in octopus (Boycott and Young, 1957; Mackintosh, 1964). Stimuli were presented to the octopus in a two alternative forced choice experiment. Stimuli were chosen according to the outcome of a preference test with a maximum of 10 unrewarded trials that proceeded reversal training (**Tables 3**, **4**). A preference test was conducted (see Experimental procedure) as octopus has been reported to show pre-existing preferences for some stimuli over others (see e.g., Wells, 1978), which could interfere with learning or reversing in a reversal task. If an animal had a clear preference for one particular stimulus, that stimulus was defined as S−. Subject Ov4 had shown a high preference for the vertical rectangle. To compare the experimental outcome of this animal with the other animal that had also shown a high preference, we switched to a pair of stimuli that revealed no preference to one stimulus over the other, i.e., a bird-like and a house-like shape (both 60 × 60 mm).

The position of the S+ and the S− was pseudo-randomly changed from left to right after Gellermann (1933).

Experimental Procedure

Experiments with each subject were conducted by one experimenter throughout the complete period of training. As soon as the subjects approached the start location, the terracotta flower pot, the trial started by presenting both stimuli on the monitor. After 2 s, they were moved up and down within a range of approximately 3 cm to make the subjects readily attack the stimuli. Subjects were then supposed to respond to the S+ by swimming toward the screen and touching the stimulus within 10 s. The animals were rewarded for each correct response by moving a transparent rod with a black tip, the secondary reinforcer, up and down the feeding tube followed by a piece of food, the primary reinforcer, delivered through the tube. Incorrect choices were followed by directly switching off the stimulus presentation. In case of an inappropriate response i.e., withdrawal from the stimuli or approaching the feeding tube directly without responding, stimuli were switched off after approximately 10 s, and the trial was repeated. Inter-trial interval was limited to 10 min. If the animal did not return to the experiment within these 10 min, the session was ended.

Before initial training could start, all animals had to get used to the general experimental procedure i.e., to approach the start location, to await stimulus presentation, to respond to a stimulus on the monitor and return to the feeding tube and/or start location (**Table 3**). In order to establish the experimental procedure, only one stimulus was displayed on the monitor, which was a black circle with 4 cm in diameter. Animals were trained until following the experimental procedure for at least 10 times during one session.

Since octopuses have been reported to show pre-existing preferences for some stimuli over others (Boal, 1996), a preference test of maximally 10 unrewarded trials with the respective stimulus pair was performed prior to the training on the discrimination task (**Table 3**). Sometimes fewer preference trials were conducted (**Table 4**) as the animals stopped working most likely due to the absence of a reward.

After the preference test, reversal training was started (**Table 3**). In R0, the experimental subject was asked to respond to the stimulus it had not preferred during preference testing as S+. Subjects performed 16–20 trials a day. These trials were mostly

TABLE 3 | Illustration of the phases of the experiment with procedure and/or the predefined goal of the phase as well as the criterion to end a phase, if applicable.

Phases of the experiment	Procedure/Goal	Criterion
Training	Taking food from experimenter	
	Establishment of secondary reinforcer by pairing food and secondary reinforcer	
Establishment of experimental procedure	Stationing on the starting position (flower pot)	
	Attacking a moving stimulus (circle) on the right or left side of the monitor	10 attacks on circle/session
	Returning to the feeding tube after a response to the monitor for a reward	
Preference test	Presentation of stimuli planned to be used during reversal training in maximally 10 unrewarded trials; the animal's choices were documented to reveal a possible preference for one or the other stimulus	
Reversal 0 (R0)	Discrimination between the stimuli, stimulus not preferably chosen during the preference test was defined as the S+	Performance ≥ 80 % in 2 sessions of 16-20 trials
Reversal 1 (R1) - and every reversal with uneven number -	Discrimination between the stimuli reversed in sign: new S+ (=S− during R0) and new S− (=S+ during R0)	Performance ≥ 80 % in 2 sessions of 16–20 trials
Reversal 2 (R2) - and every reversal with even number -	Discrimination between the stimuli again reversed in sign: stimuli defined as during R0	Performance ≥ 80 % in 2 sessions of 16–20 trials

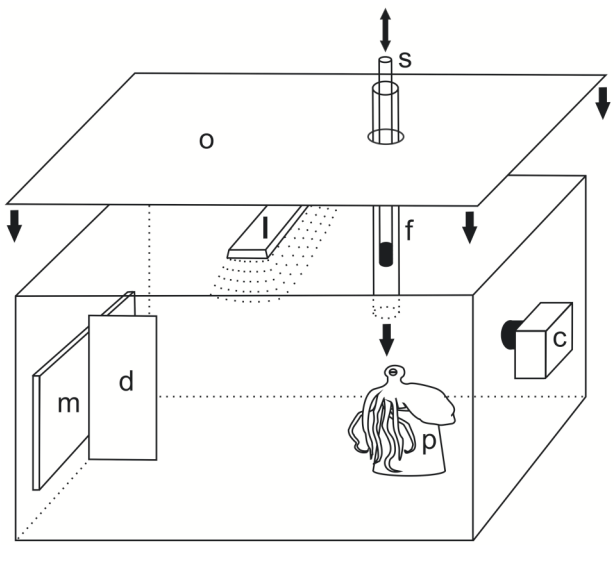

FIGURE 1 | Experimental setup. Stimuli were presented on a liquid crystal display (LCD) monitor m attached to the tank from outside. The left and right side of the monitor were separated by a divider **d.** For each single trial, the animal positioned itself on a flower pot **p** at approximately 50 cm distance to the monitor. Reward was provided through a transparent feeding tube **f** which was inserted through the lid of the aquarium. A transparent Perspex rod with a black tip **s** was used as secondary reinforcer. It was inserted into and moved up and down the feeding tube upon a response to the positive stimulus thus indicating a correct choice which was followed by food supply. The whole area was illuminated by a lamp **l**. To avoid secondary cues during experiments, the top of the aquarium as well as the side walls were shielded with an opaque cover **o** (side cover not shown for clarity). Experiments were observed and recorded with the help of a camera **c**. Not drawn to scale.

split off into two blocks of 8–10 trials, one block conducted in the morning and one in the afternoon, depending on the individual and its daily motivation. After the animals had reached the learning criterion, predefined as a performance of ≥ 80% correct choices (for a session of 16 trials: $p < 0.05$, for a session of 20 trials: $p < 0.01$, χ^2-test) in 2 sessions of 16 or 20 trials, the signs of the stimuli were reversed i.e., the former S+ was redefined as S− and the former S− was redefined as the new S+. This experimental stage is referred to as reversal 1 (R1). Apart from this, experimental conditions and procedures remained the same. If subjects again reached the learning criterion in R1, the second reversal (R2) was conducted by redefining the stimuli as in R0. Reversal training continued until experiments had to be stopped because of the (1) animals not responding anymore due to senescence, (2) animals not able to reach the learning criterion in one stage of reversal learning after extensive training or (3) animals' poor motivation during experiments.

Data Analysis

The performance of the individuals was analyzed as the total number of correct choices (in %) summarized for a 16 or 20-trials session. This performance was documented over time for every reversal resulting in classic learning curves (**Figure 2**). A reversal was considered to be completed if the animal achieved a performance at the preset learning criterion. The learning criterion was predefined with the help of a χ^2-test to assure that the animal's performance was statistically different from chance performance. For experimental subject Ov1, the number of errors to reach the criterion was additionally analyzed for each reversal separately (**Figure 2B**). The number of errors to criterion indicated in **Figure 2B** includes the number of errors made during the 2 sessions required to fulfill the learning criterion.

RESULTS

All experimental animals were able to discriminate between the given pair of stimuli and successfully completed R0 (**Figure 2**; **Table 4**). Ov1 finished the acquisition phase after 4 sessions, Ov2 after 30 sessions, Ov3 after 9 sessions, and Ov4 after 3 sessions. In the reversal training, the performance of the four animals differed in the numbers of completed reversals. Ov1 was

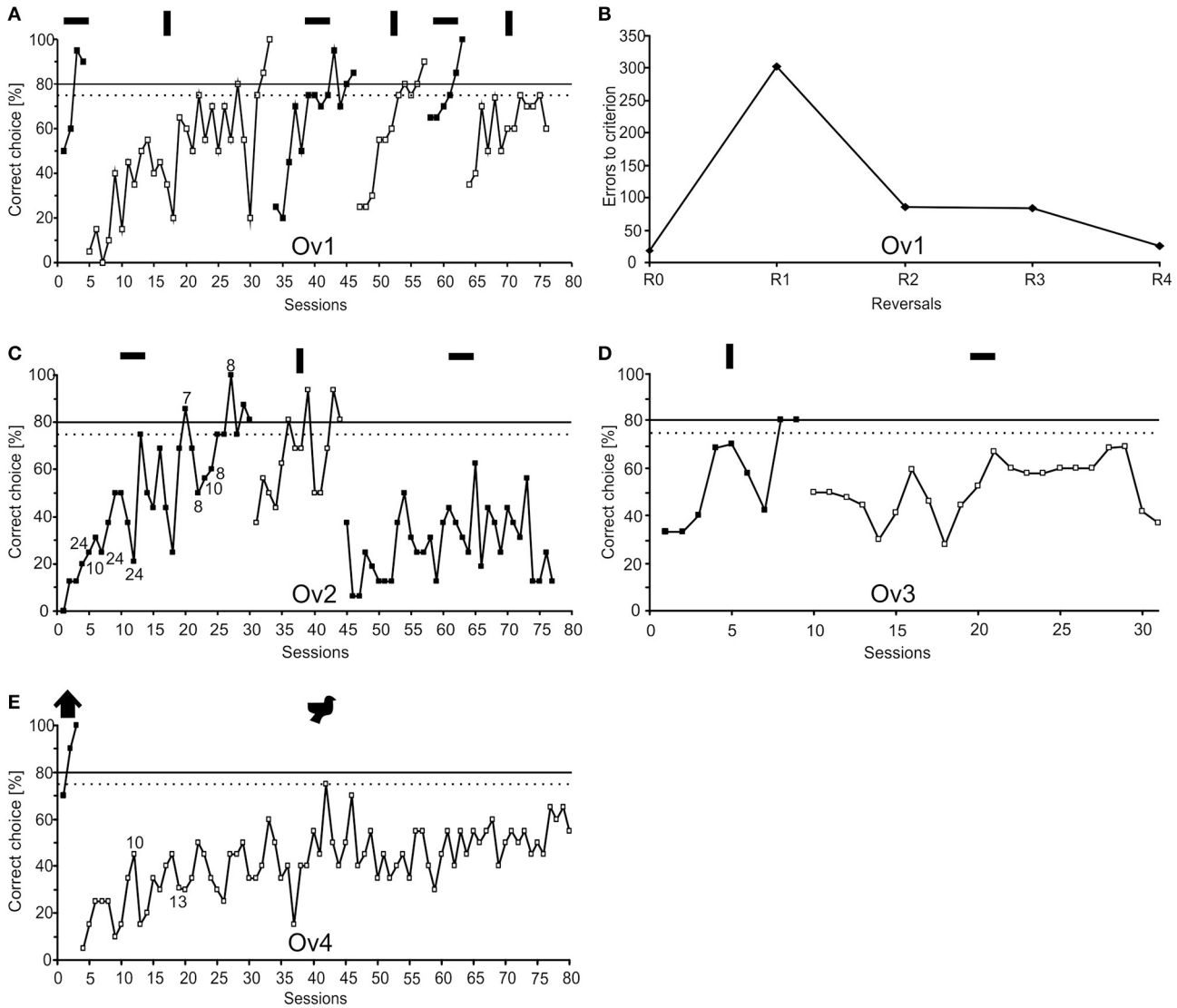

FIGURE 2 | Learning performance of the four subjects (Ov1–Ov4) together with the stimuli presented to each animal. The dotted line indicates statistical significance at 75% correct choices ($p < 0.05$). The continuous line indicates the learning criterion of 80% correct choices (for a session of 16 trials: $p < 0.05$, for a session of 20 trials: $p < 0.01$, χ^2-test) that needed to be met in the sessions of two consecutive days or in 2 sessions of 20 trials. Symbols above the reversals indicate the S+ of the respective phase. **(A)** Learning performance of animal Ov1 during R0 and the five consecutive reversals R1–R5. The number of trials needed to reach the learning criterion decreases with subsequent reversals. **(B)** Number of errors to criterion during R0 and the following completed four reversals in animal Ov1. After an increase of errors in R1 compared to R0, there is a continuously decreasing number of errors to criterion with subsequent reversals. This compares favorably to the performance found in reptiles, birds and mammals. **(C)** Animal Ov2 showed fewer errors in learning R1 compared to R0, but failed to learn R2. With Ov2, a daily session usually consisted of 16 trials; if the session length differed from 16 trials, the number of trials is indicated at the data points. **(D,E)** Animals Ov3 and Ov4 both succeeded in learning R0 but failed to learn R1. Numbers indicate sessions with less than 20 trials.

able to reach the learning criterion not only in R1 but also in the following three reversals, thus, it successfully finished four consecutive reversals (**Figure 2A**). Results revealed an increase in errors to criterion in R1 from 21 errors in R0 to 305 errors in R1 (**Figure 2B**). In contrast, the animal showed a decrease in errors to criterion throughout the reversals following R1 (**Figure 2B**). However, this animal ceased cooperation during training in R5, most likely due to senescence, and training had to be stopped as a consequence. Ov2 (**Figure 2C**) finished R1 successfully but in contrast to Ov1, there was a decreasing number of errors during R1 as compared to R0, as only 13 sessions were required to complete R1. In R2, however, the animal did not succeed and training had to be stopped after 33 sessions. Ov3 and Ov4 (**Figures 2C,D**) reached the learning criterion in R0 within at least 9 sessions, but both animals failed in reaching the learning criterion during Rl. Ov3 failed to rereach the learning criterion in 22 sessions, and training with Ov4 was stopped after 77 sessions.

TABLE 4 | Overview of the performance of the four octopus individuals during the phases of the reversal learning experiment including the stimuli used during reversal training with the S+ of R0 indicated in brackets, the outcome of the preference test as number of trials, in which the S+ of R0 was chosen out of the total number of preference test trials as well as the number of correct responses per total number of trials to criterion per phase of the reversal training (R0-Rn).

Ov	Stimuli	Preference test	R0	R1	R2	R3	R4	R5
1	Rectangle (horizontal)	3/7	59/80	275/580	167/260	130/220	92/120	157/259*
2	Rectangle (horizontal)	0/10	217/459	145/224	156/544*			
3	Rectangle (vertical)	2/8	98/180	219/440*				
4	Bird-House (house)	3/7	51/60	616/1463*				

**Stop of reversal training before learning criterion had been reached.*

DISCUSSION

In this study, four octopus individuals were trained on a serial visual reversal learning experiment as a first proof of concept of the new methodology. From a methodological perspective, this serial reversal learning study stands out from previous discrimination experiments and previous reversal learning experiments in octopus (Boycott and Young, 1957; Mackintosh, 1964; Mackintosh and Mackintosh, 1963; 1964). As a methodological advancement in cephalopod research, a secondary reinforcer, as routinely applied in behavioral experiments with e.g., vertebrates, was introduced in this study to signal the correctness of a response and to announce the subsequent primary reinforcement, food. Our training revealed that the octopus individuals of this and follow-up studies (unpublished data) seem to readily and easily learn the association between food and the secondary reinforcer, they learnt the experimental procedure within a few days, and all individuals acquired the original task. Generally, the use of a secondary reinforcer offers many advantages. First, it allows perfect timing of the feedback after a response as it can instantly signal the correctness which is impossible with food under most circumstances. In previous discrimination experiments, experimenters sometimes attached reinforcement directly to the stimuli in order to avoid a time delay between response and reinforcement (Boal, 1996). However, adopting this procedure most likely enabled the animals to use chemical traces of the food in the water to make their decisions and to improve their performance over time (Boal, 1996). Second, the secondary reinforcer can also function to guide the experimental animal to specific locations such as the starting position, thereby also speeding up experimental procedures as e.g., the animal readily detach from the stimuli upon perceiving the secondary reinforcer. The secondary reinforcer thus substitutes previous handling methods such as chasing the animals. In conclusion, a secondary reinforcer proved to be a useful method for training our octopods in behavioral experiments.

Stimulus presentation was automatized as computer controlled stimuli were presented on monitors (see also Papini and Bitterman, 1991), thus stimulus presentation and movement were very standardized. Moreover, the current type of stimulus presentation allowed shielding the aquarium from all sides prohibiting secondary cueing by the experimenter. In previous octopus discrimination experiments with only a few exceptions (see e.g., Boal, 1993, 1996), stimuli had been manipulated by the experimenter (see e.g., Young, 1956; Muntz et al., 1962; Messenger and Sanders, 1971) and thus, secondary cueing might have affected the results. Generally, secondary cueing is thought to facilitate learning. However, as octopus is easily distracted by extraneous cues, the experimental animals were significantly less successful if the stimuli were submerged and moved by the experimenter (Boal, 1996). In conclusion, the presence of secondary cues is undesirable (Boycott and Young, 1956; reviewed in Boal, 1996). In this study, we provide clear evidence that octopus is able to show learning when stimuli are presented simultaneously and in an automatized fashion without the presence of experimenter given secondary cues.

Unlike previous discrimination experiments involving reversal learning experiments, this study did so without pre-training. Previous reversal learning studies (Mackintosh, 1964; Mackintosh and Mackintosh, 1963) except for Mackintosh and Mackintosh (1964), pre-trained on the new S+ after each reversal. This meant that the animal was presented only with the new S+ and was rewarded upon choosing it for a specific number of sessions or trials (Mackintosh, 1964) or the new S+ was presented until the animal reached a specific performance level (Mackintosh and Mackintosh, 1963). This procedure was adopted as the experimental animal was punished with an electric shock for each incorrect response as well as being reinforced for each correct response. Provided the experimental animal would continue responding to the old S+ although a reversal had taken place, the probability of a mistake in the first trial after a reversal would have been high. As a consequence, many experimental animals directly stopped working. Pre-training seemed to be an appropriate method to overcome this issue. However, already during pre-training, the animal learns about the new S+ which is most likely affecting the results during the subsequent reversal. Moreover, after pre-training on the S+, the animals might only choose on the basis of stimulus familiarity (Boal, 1996). In this study, the experimental subjects were trained with positive reinforcement alone. Therefore, pre-training on the new S+ after a reversal had taken place was unnecessary. Thus, our refined method allowed determining reversal learning abilities in octopus in the classical way without pre-training, which forms the basis for the assessment of learning abilities in octopus and allows interspecific comparison.

Assessing reversal learning abilities with this refined methodology, our results show that at least some octopus

individuals can solve a serial visual reversal learning task and can even show progressive improvement. However, the performance was highly individual. Individual performances have already been highlighted for octopus (see e.g., Mather, 1995), even in reversal learning studies (Mackintosh, 1964; Mackintosh and Mackintosh, 1963; 1964). There are many possible reasons that might account for the apparent individuality. First, in line with Young (1956), differences in behavior might be hereditary or due to different experiences in the past. These differences might indeed be pronounced as, due to the fact that it is still not possible to rear octopus in aquaria, wild caught animals have to be taken for experiments. Moreover, cephalopods seem to vary in personality (Mather and Anderson, 1995; Sinn et al., 2006). The personal variability of behaviors along the dimensions activity, reactivity, and avoidance, defined for *Octopus rubescens* (Mather and Anderson, 1995), could, if also applicable for *O. vulgaris*, also lead to different learning performance. In general, a multitude of factors including sex, size, home tank size, or the experimental history (**Table 2**) might additionally influence the training outcome, this could be a topic for future research.

Secondly, stimulus preferences might affect the individual experimental outcome. The results of Ov1, Ov2, and Ov3 were obtained with a vertical and a horizontal rectangle as the stimuli, which were shown to be easily discriminable by octopus (Boycott and Young, 1956; Sutherland, 1957). The experimental animals of this study showed very strong stimulus preferences as previously reported for a diverse set of stimuli (reviewed in Boal, 1996 and Wells, 1978). Ov1 and Ov2 preferred the vertical rectangle whereas Ov3 mostly responded to the horizontal rectangle during training. The preference for the vertical rectangle could result from the documented preference of octopus to preferably pick the stimulus that is moved along its long axis (Young, 1958, 1965; Sutherland and Muntz, 1959; Sutherland, 1960, 1964; Sutherland and Carr, 1963; Messenger and Sanders, 1972). Strong stimulus preferences could ultimately lead to problems during reversal learning as it might be particularly difficult to learn against a stimulus preference. Whereas, stimulus preferences might thus account for the failure of Ov2 and Ov3 during reversal training, it can, however, not explain why Ov1 was very successful in reversing its response behavior despite its initial strong stimulus preference. A further test was used to elucidate on the effect of the stimuli and of stimulus preferences on reversal learning outcome. Ov4 had shown a high preference for the vertical rectangle and was thus asked to discriminate between a completely different set of stimuli, a house- and bird-like stimulus. With these arbitrarily chosen stimuli, Ov4 almost equally often chose both stimuli in the preference test trials. After a very quick acquisition phase in R0, the experimental animal failed during R1. It is possible that Ov4 had an untrained preference for the house-like stimulus, which was the S+ in R0, which did not become apparent during the few preference test trials, and upon reinforcing in line with the preference, it persisted on responding on the preferred stimulus. Consequently, as already generally discussed in Boal (1996), the performance Ov4 showed in R0 might not have indicated learning as preferences can increase over time in octopus even in the absence of rewards (Fiorito and Scotto, 1992). In conclusion, stimulus preferences might be a factor that strongly influences discrimination experiments. Despite large efforts, stimulus preferences, stimulus processing and discrimination processes, in general, are still poorly understood in octopus.

Thirdly, it is possible that the individual outcome of this study is partially due to the reinforcement type. The animals of the study at hand were only trained using food as positive reinforcement in contrast to previous discrimination experiments in octopus that also used electric shocks as negative reinforcement besides food (see e.g., Sutherland, 1957; exceptions reviewed in Boal, 1996). Food might not be the major factor controlling octopus behavior in its natural environment as octopuses are specializing generalists (Anderson et al., 2008) with an access of available prey (Mather, 1991a). In contrast, octopus is exposed to strong interspecific competition and predator pressure (Alves et al., 2008). Thus, aversive elements might primarily drive decisions in octopus. Indeed one study showed abrupt learning when electric shocks were finally introduced (Sutherland et al., 1963). Electric shocks are very strong aversive elements, however, it is also conceivable to apply mild aversion such as pushing the animal. The role of negative reinforcement in learning discrimination experiments needs further examination.

Fourthly, the experimental design might account for some of the individual variation. We asked the octopus individuals participating in this study to perform in a visual reversal learning experiment. A visual task was chosen due to the octopus' well-developed eyes, its large optic lobes, previous successful visual discrimination experiments including visual reversal learning experiments and its good memory capabilities (Wells, 1978; Mather and Kuba, 2013). An alternative could be to train octopus for a spatial reversal learning task. A more consistent outcome in a spatial task might be expected as spatial orientation is crucial for octopus that occupies dens (Mather, 1991a). They leave their dens for foraging but return later probably navigating via landmarks (Mather, 1991b). From time to time, octopuses also change dens (Mather and O'Dor, 1991), which requires relearning of the spatial layout. There is laboratory evidence from different octopus species that octopuses are capable of spatial learning in detour experiments (Wells, 1964, 1967, 1970), arenas (Boal et al., 2000), and mazes (Walker et al., 1970). Walker et al. (1970) even successfully trained *Octopus maya* to reverse a spatial preference at least once. Good spatial reversal learning abilities have also been demonstrated in a different cephalopod species, the cuttlefish (Karson et al., 2003). Widening the view to other species, most animals tested in visual and spatial reversal learning experiments (see e.g., Holmes and Bitterman, 1966) showed better reversal learning performance with spatial tasks, which further strengthens the hypothesis of better spatial reversal learning abilities, compared to a visual alternative. Current experiments on spatial reversal learning in octopus in our lab will provide deeper insight into reversal learning in octopus.

At least one of the individual octopuses trained in this study with the refined methodology showed good reversal learning performance. Despite our methodology differing from previous studies, Ov1 showed similar performance to the octopus individuals trained in previous reversal learning studies (**Table 1**; Boycott and Young, 1957; Mackintosh, 1964; Mackintosh and Mackintosh, 1963, 1964). Indeed, Ov1 showed progressive

improvement, and it took the animal longer to learn the first reversals than to learn the original task. In contrast, Ov1 made substantially more errors in R1-R3 and stopped cooperating at an earlier stage, during R4. Animals in Mackintosh and Mackintosh (1964) could complete up to 14 reversals, but this was variable between individuals. In the just mentioned study, even one octopus achieved the best possible reversal performance of one error to criterion. In our opinion, these differences in performance can most likely be attributed to methodological differences and individual differences. Generally, the performance Ov1 showed is also comparable to many other organisms including invertebrates and vertebrates. Indeed, many animals such as rats (Mackintosh et al., 1968) and chicken (Bacon et al., 1962) also perform worse during the first reversals as compared to R0. Furthermore, the reversal learning curves suggest that the octopus performance can be explained by proactive interference (Gonzalez et al., 1967; Shettleworth, 1998). At the beginning of R1-R3, Ov1 showed a performance far below chance level, it continued to respond to the S+ as defined during the previous reversal training phase. After a short period, the animal, however, learnt to respond to the new S+. Finally, during R4, Ov1 showed an initial performance at chance level which might indicate that it could no longer remember which stimulus was currently defined as the S+. During R4, the learning curve was very steep before Ov1 stopped cooperating during the fifth reversal, and training was ended. Thus, the best performance of Ov1, 28 errors to criterion, was achieved during R4. Ov1 did not reach the maximum performance possible of one error to criterion seen in other invertebrates such as bumblebees (Chittka, 1998) and cockroaches (Balderrama, 1980). Nevertheless, some octopus individuals seem indeed able to learn to reverse even when the individual is trained to reverse in the "classical" way without pre-training and experimenter given cues. Thus, these octopuses learn more than just stimulus specific responses. Additionally, the results obtained with the octopus individuals in this study provide first evidence that there is no clear separation in reversal learning performance between vertebrates and invertebrates as previously suggested (see e.g., Bitterman, 1965; Warren, 1965) as animals being able to solve reversal tasks even showing progressive improvement can be found in both classes.

The results of Ov1, that showed good reversal learning abilities and even progressive improvement during reversal training, are in line with what we had expected from the octopus biology, adopting an ecological, adaptive approach to learning (Kamil and Mauldin, 1988). Already Young (1961) assumed that long learning phases might be perilous for an octopus when foraging or avoiding predators or conspecifics. Our expectation is based on the fact that the cognitive abilities underlying reversal learning might be generally important for an animal that needs to be behaviorally flexible (Bond et al., 2007). Behavioral flexibility is likely to be important for octopus, living in complex environments that require the animal to respond and adapt quickly to changes in the environment. Furthermore, various features of octopus biology, such as its short life span, active foraging, competition of niches and predator pressure (Packard, 1972; Alves et al., 2008) probably also require the individual to be behaviorally flexible (Mather, 1995; Shettleworth, 1998; Day et al., 1999). An example of a flexible behavior or adaptation to changes in the environment was given by Meisel et al. (2013) who showed that, if a predator is present, octopus switched its activity phase. However, as mentioned, it remains to be answered why only one out of four individuals showed reversal learning abilities consistent with this hypothesis derived from the octopus biology.

In conclusion, with this study, we provide a proof of concept of the new experimental design as all animals learnt the original task and even one individual was able to perform successfully in a reversal learning experiment showing progressive improvement.

AUTHOR CONTRIBUTIONS

All authors designed the study; AB, SW, and SS trained the octopus; all authors analyzed the data; AB and FH wrote the manuscript; all authors edited the manuscript and approved the final version.

FUNDING

This experiment was supported by a grant of the Landesgraduiertenstiftung Mecklenburg-Vorpommern to AB and a grant of the VolkswagenStiftung to GD.

ACKNOWLEDGMENTS

The authors acknowledge the continuous technical help by Lars Miersch, Marine Science Center Rostock, as well as the support of the "Cost action FA1301-CephsInAction."

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 24 August 2017
doi: 10.3389/fphys.2017.00613

Eye Development in *Sepia officinalis* Embryo: What the Uncommon Gene Expression Profiles Tell Us about Eye Evolution

*Boudjema Imarazene[1], Aude Andouche[1], Yann Bassaglia[1,2], Pascal-Jean Lopez[1] and Laure Bonnaud-Ponticelli[1]**

[1] UMR Biologie des Organismes et Ecosystèmes Aquatiques, Museum National d'Histoire Naturelle, Sorbonne Universités, Centre National de la Recherche Scientifique (CNRS 7208), Université Pierre et Marie Curie (UPMC), Université de Caen Normandie, Institut de Recherche Pour le Développement (IRD207), Université des Antilles, Paris, France, [2] Université Paris Est Créteil-Val de Marne, Paris, France

In metazoans, there is a remarkable diversity of photosensitive structures; their shapes, physiology, optical properties, and development are different. To approach the evolution of photosensitive structures and visual function, cephalopods are particularly interesting organisms due to their most highly centralized nervous system and their camerular eyes which constitute a convergence with those of vertebrates. The eye morphogenesis in numerous metazoans is controlled mainly by a conserved Retinal Determination Gene Network (RDGN) including *pax, six, eya,* and *dac* playing also key developmental roles in non-retinal structures and tissues of vertebrates and *Drosophila*. Here we have identified and explored the role of *Sof-dac, Sof-six1/2, Sof-eya* in eye morphogenesis, and nervous structures controlling the visual function in *Sepia officinalis*. We compare that with the already shown expressions in eye development of *Sof-otx* and *Sof-pax* genes. *Rhodopsin* is the pigment responsible for light sensitivity in metazoan, which correlate to correlate visual function and eye development. We studied *Sof-rhodopsin* expression during retina differentiation. By *in situ* hybridization, we show that (1) all of the RDGN genes, including *Sof-pax6,* are expressed in the eye area during the early developmental stages but they are not expressed in the retina, unlike *Sof-otx*, which could have a role in retina differentiation; (2) *Sof-rhodopsin* is expressed in the retina just before vision gets functional, from stage 23 to hatching. Our results evidence a role of *Sof-six1/2, Sof-eya*, and *Sof-dac* in eye development. However, the gene network involved in the retinal photoreceptor differentiation remains to be determined. Moreover, for the first time, *Sof-rhodopsin* expression is shown in the embryonic retina of cuttlefish suggesting the evolutionary conservation of the role of *rhodopsin* in visual phototransduction within metazoans. These findings are correlated with the physiological and behavioral observations suggesting that *S. officinalis* is able to react to light stimuli from stage 25 of organogenesis on, as soon as the first retinal pigments appear.

Keywords: eye development, *Sepia officinalis, dac, six, eya, rhodopsin*

OPEN ACCESS

Edited by:
Frederike Diana Hanke, University of Rostock, Germany

Reviewed by:
Shuichi Shigeno, Stazione Zoologica Anton Dohrn, Italy
Oleg Simakov, University of Vienna, Austria

****Correspondence:***
Laure Bonnaud-Ponticelli
laure.bonnaud@mnhn.fr

Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *07 November 2016*
Accepted: *09 August 2017*
Published: *24 August 2017*

Citation:
Imarazene B, Andouche A, Bassaglia Y, Lopez P-J and Bonnaud-Ponticelli L (2017) Eye Development in Sepia officinalis Embryo: What the Uncommon Gene Expression Profiles Tell Us about Eye Evolution. Front. Physiol. 8:613. doi: 10.3389/fphys.2017.00613

INTRODUCTION

In metazoans, the evolution of photosensitives structures is difficult to establish as there are a high diversity of shapes, at histological and cellular level, and functioning at physiological and optical level, and analogous "eyes" appeared during evolution several times in different lineages (Land, 1988). Nevertheless, "eye" morphogenesis is controlled by a conserved genetic network of transcription factors (Gehring, 2002). Among these genes, *pax6* is a member of the highly conserved paired-box family of transcription factors (Burri et al., 1989; Noll, 1993). *Pax6* is considered as a universal master gene controlling eye morphogenesis, and its expression is reported in developing photoreceptors (Echelard et al., 1993; Chi and Epstein, 2002; Pichaud and Desplan, 2002; Gehring, 2005; for review see Kumar, 2009).

In vertebrates as in *Drosophila*, genes that govern eye specification are numerous. Indeed, eye formation is known to be controlled particularly by the Retinal Determination Gene Network (RDGN). It includes *pax6, eya (eyes absent), six (sine oculis*), and *dac (dachshund)* which act as a regulatory network of eye formation and retinal differentiation (Kumar and Moses, 2001; Donner and Maas, 2004). More studies indicate that these genes are also involved in the proliferation of progenitor cells, differentiation of retinal precursors, specification and/or maintenance of photoreceptor neurons and finally in the development of many other non-retinal tissues and organs (Bessa et al., 2002; Brodbeck and Englert, 2004; Christensen et al., 2008; Lopes and Casares, 2009; Peng et al., 2009). *Pax6, six3, six6, eya1, eya2, eya3,* and *Dach1* are known to play crucial roles in eye development in vertebrates. Furthermore, it has been shown that *pax6* is an upstream regulator in the RDGN in *Drosophila* (Czerny et al., 1999). Besides this network, *otx (Orthodenticle homeobox 2)* and *Notch* play a key role in photoreceptor cell differentiation and retinal organization (for review see Boyl et al., 2001; Buresi et al., 2012; Koenig et al., 2016). *In fine*, photoreception is allowed by the presence of pigments of the *opsin* family, present in all groups whatever the structure of the photoreceptor cells (Gehring, 2002). *Opsin* proteins are known to be involved both in visual and extraocular phototransduction (Porter et al., 2011). The signal cascade of visual phototransduction is initiated in the retinal photoreceptors when a photon is absorbed by a G protein-coupled receptor that is attached to a vitamin A-derived chromophore, 11-cis-retinal. The activated visual pigment molecule (*opsin*) induces a transduction cascade that results in the opening or closing of cation cGMP-gated channels in the photoreceptors (Hargrave, 2001).

Among metazoans, researchers are beginning to study the RDGN in lophotrochozoans. It has been shown that this regulatory network is involved in morphogenesis of the pigment-cup eyes of *Terebratalia transversa* (Passamaneck et al., 2011) and *Platynereis dumerilii* (Arendt et al., 2002), of the eyespot of *Lineus sanguineus* and *Leptochiton asellus* (Loosli et al., 1996; Vöcking et al., 2015), and of the cup eye of *Dugesia japonica* (Dong et al., 2012; Kamijyo et al., 2015). Within lophotrochozoans, cephalopods are good model species in the context of research of evolution and development (Evo-Devo) due to their highly centralized nervous system that is more centralized than in any group of invertebrates (Zullo and Hochner, 2011) and their specific "complex" camerular eyes, which constitute a convergence with those of vertebrates.

The cephalopod eye consists from the inside to the outside of: a retina covering the deepest part of the optic vesicle, a lens "closing" the vesicle, an iris and a cornea covering the eye (**Figure 1**). The retina is composed of rhabdomeric photoreceptor cells supported by a layer of support cells. Each photoreceptor consists of an outer (posterior) segment containing the nuclei and an inner (anterior) segment. The two segments are limited by a basement membrane. The development of the eye has been described in *Sepiella japonica*, in *Sepioteuthis australis*, in *Loligo vulgaris,* and recently in *Doryteuthis pealeii* (Marthy, 1973; Yamamoto, 1985; Bozzano et al., 2009; Koenig et al., 2016). The iris and cornea derive from two layers (respectively inner and outer) of ectodermal and mesodermal tissues growing around the optic vesicle (Lemaire and Richard, 1978; Tomarev et al., 1997); the circular lens is produced by lentigenic cells (West et al., 1995), and the retina, is formed during invagination of the primary optic vesicle (Lemaire, 1971; Lemaire and Richard, 1978).

Studies about the cephalopod's photosensitivity during embryogenesis have suggested that embryos become photosensitive early before hatching before the final differentiation of the retina (*S. japonica,* Yamamoto et al., 1985; *S. australis*, Bozzano et al., 2009). Unlike any other cephalopod, *Sepia officinalis* embryos develop in a dark visual environment because of the black capsule surrounding the egg, which attenuates the light reaching the embryo. Nevertheless, *S. officinalis* is able to react to light stimulus from stage 25 of

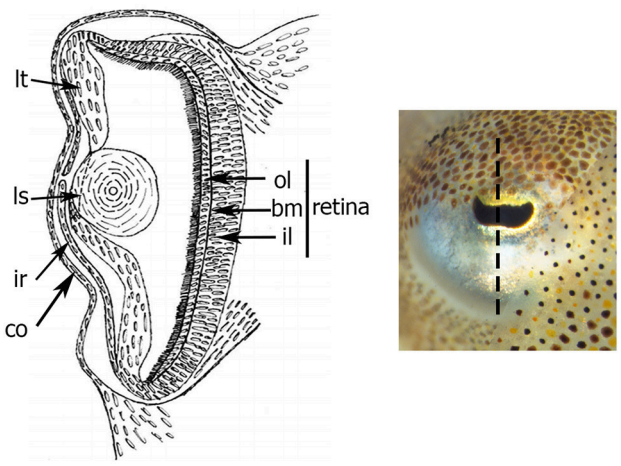

FIGURE 1 | Schema of transversal section of the eye of *S. officinalis* at stage 30. Black dotted-line shows the level and the orientation of the section. bm: basement membrane; co: cornea; il: inner layer; ir: iris; ls: lens; lt: lentigenic tissue; ol: outer layer.

organogenesis, i.e., as soon as the first retinal pigments appear (Lemaire, 1971; Lemaire and Richard, 1978; Romagny et al., 2012). Maturation of the visual system occurs in the last stages of photoreceptor differentiation before hatching: the main elements for photosensitive function and the cuttlefish's eyes are entirely functional at hatching, as the juvenile immediately adopts the visual-guided behavior of predation. It must be noted that cephalopods are known to have a remarkable capacity to transform their appearance by changing their dermal coloration, patterning and shape using chromatophores, iridophores, leucophores, and papillae (Cloney and Brocco, 1983; Allen et al., 2009). The skin pattern is controlled by the eye and probably by an extraocular or non-visual photoreception, as shown in some cephalopod species (Kingston et al., 2015a). All of these extraocular photoreceptors described are known to use many phototransduction components including *retinochrome, visual arrestin, rhodopsin kinase,* and *rhodopsin* identical to the isoform expressed in the eyes (Tong et al., 2009).

The development of the cephalopod eye is investigated in numerous molecular and genomic studies (Tomarev et al., 1997; Hartmann et al., 2003; Bozzano et al., 2009; Ogura et al., 2013; Peyer et al., 2014; Yoshida et al., 2014; Koenig et al., 2016). To complete the behavioral approaches and to understand mechanisms of eye maturation and visual function appearance, we have chosen to explore the molecular pathways underlying the developmental processes in an evolutionary perspective in *S. officinalis*. *Pax6* expression has been already determined in numerous cephalopods. During development, *pax6* is expressed in eyes particularly in the ocular primordia, optic ganglia, and light organ (a photosensitive structure of bobtail squid) (Tomarev et al., 1997: *Loligo opalescens*; Hartmann et al., 2003: *Euprymna scolopes*; Navet et al., 2009: *S. officinalis*; Peyer et al., 2014: *E. scolopes*; Yoshida et al., 2014: *Idiosepius paradoxus*; Koenig et al., 2016: *D. pealeii*). Expression of other genes of the RDGN, *six*, *eya,* and *dac* has been described in the central nervous system, optic area and light organ of *E. scolopes* and during eye morphogenesis of the *D. pealeii* embryo (Peyer et al., 2014; Koenig et al., 2016). Finally, *Sof-otx* expression has been characterized in *S. officinalis* embryo in early to late organogenesis of the eye (Buresi et al., 2012). Nonetheless, most of these studies have been performed on wholemount embryo, without considering specifically the retina morphogenesis and its function.

Our goal was to understand the evolutionary mechanisms, the complexity and the emergence of photosensitive structures and visual phototransduction in a cephalopod group. Thus, we described the morphological differentiation of the retina, and complement the identification and description by spatio-temporal expression patterns of the *Sof-six1/2*, *Sof-eya*, *Sof-dac* genes. Then, we highlight and discuss the role of these genes during eye specification in *S. officinalis* including the *pax genes* and *otx* expressions in the eyes. Furthermore, in order to link the RDG network with the visual phototransduction components and the appearance of photosensitivity during the development, *Sof-rhodopsin* expression patterns were explored in the developing retina.

MATERIALS AND METHODS

Collection, *S. officinalis* Eggs Incubation, and Staging

In France, cuttlefish experiment and maintenance are covered under European Union guidelines (Directive 86/609) and the French law (decree 87/848) regulating animal experimentation that does not concerns embryos before hatching. Nonetheless, all experiments were performed according to France and European ethical guidelines in the treatment and handling of all animals used within this study. Fertilized eggs of *S. officinalis* used in this study come from SMEL (Synergie MEr et Littoral, Blainville, France). The protocols for the staging and the fixation of the embryos are detailed in Buresi et al. (2012).

Phylogenetic Analysis, Characterization, and Sequencing of *S. officinalis* Genes

mRNA fragments of *Sof-six1/2*, *Sof-eya*, *Sof-dac,* and *Sof-opsin* were characterized in an embryonic EST library of *S. officinalis* (ADY0AAA48YE16CM1, tc_01401, ADY0AAA73Y015CM1, and tc_01048 respectively; Bassaglia et al., 2012). For the phylogenetic analyzes all alignments were performed using MAFFT and the G-INS-I iterative refinement method (Katoh and Standley, 2013). The best maximum-likelihood trees were inferred using PhyML with the WAG evolutionary model and 100 bootstrap replicates. Each of the genes has been tested in a phylogenetic work including genes from other metazoans. Six is identified as a six1/2, and our *opsin* sequence shows exactly the same sequence as that of AY450853 and that of O16005, identified as a true Gq-coupled/rhabdomeric photoreceptor *opsin* in the phylogenetic tree of Yoshida et al. (2015). From these characterized sequences, specific primers were designed for PCR amplification: *Sof-rhodopsinF3* (5′-GTACAACCCC ACCATGGAGG-3′) and *Sof-rhodopsinR3* (5′-CGCCGAT GAAGCCGTATACT-3′), *Sof-six1/2F3* (5′-CCTCCCATGCT TCCATCGTT-3′) and *Sof-six1/2R3* (5′-GAAATTTTCGGC GGACCCTG-3′), *Sof-eyaF1* (5′-ACCTACACGAGGTGGTC GTC-3′), and *Sof-eyaR1* (5′-CCACGGACTCCAGTTGCTAT -3′), *Sof-dacF1*: (5′-CGGCCAGAAGCACCAGTTAT-3′) and *Sof-dacR1* (5′-CAGTGCTTCACCATTGGGGACT-3′). They were used to amplify respectively a 311, 346, 372, and 400 bp fragment. *Sof-dac* was cloned as described in the protocol of Buresi et al. (2012). Concerning *Sof-six1/2*, *Sof-eya,* and *Sof-rhodo*psin genes, each of the genes has been synthetized and cloned by Genecust (Dudelange, Luxembourg). The probes for *in situ* hybridization were synthetized from theses plasmids.

In situ Hybridization

Whole-Mount *In situ* Hybridization

RNA probes were synthetized with the digoxigenin (DIG) RNA labeling mix from Roche (Mannheim, Germany). According to the sense of PCR product insertion into the vector, sense and antisense probe were obtained with T3 and T7 polymerase (Roche). Spatio-temporal expression patterns of *Sof-six1/2, Sof-eya, Sof-d*ac, and *Sof-opsin* gene transcripts during

early embryogenesis of *S. officinalis* (from stage 18 to stage 22) were examined by whole-mount *in situ* hybridization (ISH) according to the protocol detailed in Buresi et al. (2012).

Cryo-Sections *In situ* Hybridization

From stage 23 on, it is necessary to observe the expression on sections to localize the expressing cells. Embryos used for cryo-sections *in situ* hybridization were impregnated in 0.12M phosphate buffered (0.08M di-sodium hydrogen orthophosphate, 0.02M sodium dihydrogen phosphate dehydrate) plus 30% sucrose treated with 0.1% DEPC (Diethyl pyrocarbonate) for 48 h at 4°C. Then, they were included in Tissue-Tek and blocks were frozen in isopentane cooled at −80°C for 1 min. Sections of 20 μm were performed with cryostat Leica and used for ISH experiments. From stage 23 to stage 30, the expression patterns of those genes were stained by a cryo-sections *in situ* hybridization. Note that control negatives were used for each slide as a test for the same embryo selected and each gene studied. Unless otherwise specified, all steps of the experiments were performed in a humid chamber at room temperature. After 30 min at room temperature, the sections were rehydrated 2 times in 1X phosphate buffered saline (1X PBS) with 0.1% DEPC and treated 1 time in standard 5X saline citrate (75 mM tri-sodium citrate, 0.75 M NaCl) each time for 15 min. A prehybridization step was done in hybridization solution (HS) for 2 h at 65°C (50% deionized formamide, 5X standard saline citrate, 40 μg/ml salmon sperm DNA, 5X Denhardt's, 10% Dextran sulfate). Sections were next incubated overnight at 65°C in HS containing 300 ng/ml of probes. Excess probe was removed by 2 rinses in standard 2X saline citrate (30 mM tri-sodium citrate, 0.3 M NaCl) (respectively 30 min then 1 h, 65°C). Slides were washed for 1 h at 65°C in standard 0.1X saline citrate (0.015 mM tri-sodium citrate, 15 mM NaCl). Sections were then treated twice (15 min each) with MABT (100 mM Maleic acid, 150 mM NaCl, 1% Tween20, pH 7.5). Saturation was performed for 1 h in blocking solution (MABT, 4% Blocking powder (Roche), 15% fetal bovine serum), followed by incubation for 1 h at 4°C with anti-digoxigenin antibodies (Roche) coupled to alkaline phosphatase (AP) and diluted at 1:500 in blocking solution (MABT, 1% Blocking powder, 5% fetal bovine serum). Excess antibody was eliminated by 3 rinses (10 min each) in MABT then 3 rinses (5 min each) in PTW (PBS plus 0.10% Tween20). Sections were impregnated for 20 min in AP solution (100 mM tris hydrochloride, 50 mM Magnesium chloride, 0.1% tween20) with 100 μM levamisole hydrochloride (Sigma, France). The revelation of AP activity was conducted in AP solution (100 mM tris hydrochloride, 50 mM Magnesium chloride, 0.1% tween20 plus 1 mM levamisole hydrochloride) containing 165 μg/ml BCIP (5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt) and 330 μg/ml NBT (nitro-blue tetrazolium chloride) (Roche). The reaction was stopped by washing 3 times (10 min each) in 1X PBS. The slides were treated with DAPI (4′,6-diamidino-2-phenylindole; 25 μg/ml).

For fluorescent *in situ* hybridization, a POD-coupled anti-digoxigenin antibody (Roche) diluted 1:500 was used and bound antibodies were revealed using FITC-tyramide diluted 1:200 in PTW containing 0.001% of hydrogen peroxide, at room temperature for thrice 45 min, in the dark. After washing, the sections were mounted in Mowiol.

Microscopy Observation and Image Processing

A Leica M16 2F binocular stereomicroscope was used to observe the embryos labeled by *in situ* hybridizations. For cryo-sections ISH, the sections were observed under a Leica DMLB compound microscope. All images were taken by a camera color CoolSnapPro and treated using Adobe Photoshop Elements 9 (Adobe, CA, USA) for contrast and brightness.

RESULTS

Eye Development and Retina Differentiation

The embryonic development of *S. officinalis* has long been the subject of morphological research (e.g., Naef, 1928; Lemaire, 1971; Boletzky, 1989, 2003; Boletzky et al., 2006; for review Boletzky et al., 2016). The authors have described 30 stages along three main periods. The cephalopod eye is composed of numerous structures, analogous to those of vertebrates (**Figure 1**) and its development has been described for long time. In *S. japonica,* the development has been described in 40 stages with four phases of retinal differentiation (Yamamoto, 1985). Here we described the development of the *S. officinalis* retina based on that of *S. japonica*. The eye morphogenesis of cuttlefish is characterized by four successive ectodermal folds and begins at stage 15 when the ocular primordium is visible. At stage 16, the invagination of the ocular primordium yields the primary optic vesicle that becomes completely closed at stage 18 (**Figure 2**). Development of the retina of *S. officinalis* begins at stage 18, when the primary optic vesicle is closed and appears as a single layer with uniform columnar cells (**Figure 2**). At stage 21, the primary cornea develops from an outer fold surrounding the retinal thickening and the iris develops from an external second ectodermal fold (**Figure 2**). The lens starts to form at this stage, first teardrop shaped and becomes subspherical at stage 25 (**Figure 2**). Between stages 21 to 25 (corresponding to stages 24–29 in *S. japonica*), differentiation of two cell types begins. At stage 24, the retina begins to be slightly colored with orange in the periphery of the middle retina. From stage 25 on, the eyes are entirely orange and darken until hatching. Further in the development (stages 25 and 26), a third ectodermal fold covering the eye appears to form a secondary cornea (**Figure 2**). During the process of establishment of the secondary cornea, the photoreceptor cells and supporting cells begin to differentiate. From stage 28 on, the photoreceptors continue to grow and complete their specific differentiation, forming rhabdomeric cells. The end of the embryonic period at stage 29 is marked by the formation of the eyelid (**Figure 2**). At hatching, the eye is completely functional (Gilbert et al., 1990).

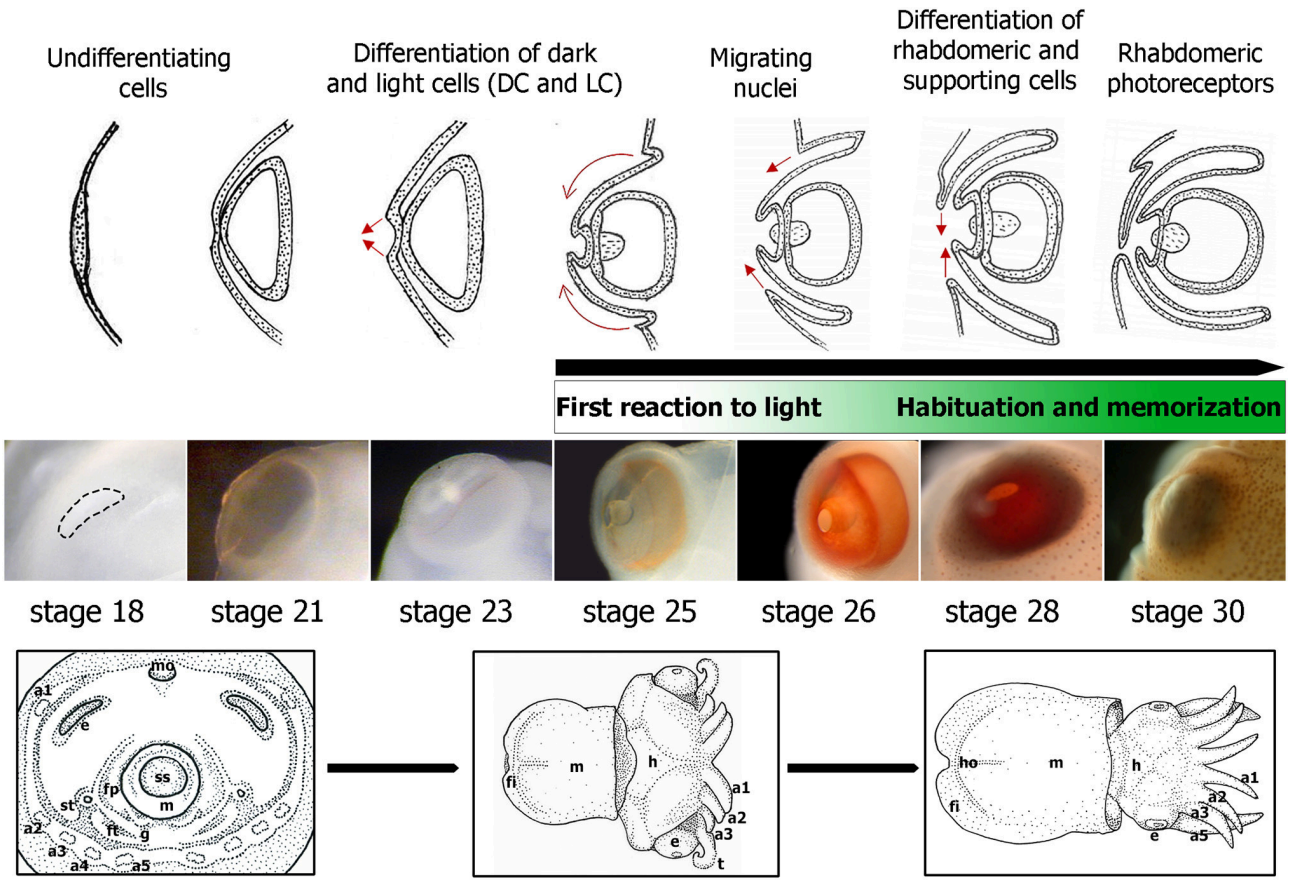

FIGURE 2 | Embryonic eye development in *S. officinalis*. From top to bottom: schematic drawings summarizing the eye morphogenesis between stages 18 and 30 (from Grassé, 1989) with indication of the differentiation of retina cell types based on *Sepiella* (Yamamoto, 1985). Green rectangle: first reaction and habituation to light from stage 25 and the memory process at late stages of development. Eye embryos' photographs between stages 18 and 30. Down: drawings of three major phases of *S. officinalis* organogenesis. a1, a2, a3, a4, a5: arms 1 to 5; e: eye; fi: fin; fp: funnel pouch; ft: funnel tube; g: gill; h: head; ho: hoyle organ; m: mantle; mo: mouth; ss: shell sac; st: statocyste; t: tentacle.

Phylogenetic Analyses of the *Sof-six1/2*, *Sof-eya*, *Sof-dac,* and *Sof-rhodopsin* ESTs

The eye morphogenesis in numerous metazoans is controlled by several important genes that includes *pax, six, eya,* and *dac*. They were shown to play key developmental roles in non-retinal structures and tissues of vertebrates and *Drosophila*. Blast analyses from our *S. officinalis* EST library revealed the existence of ESTs putatively encoding for *Sof-dac, Sof-six, Sof-eya* and Sof-*opsin*. However, since it was previously shown that cephalopods contain several opsins (Yoshida et al., 2015) or more than one six gene (Koenig et al., 2016), we performed Maximum-likelihood tree based analyses to confirm the identity of ESTs studied here. Using a set of chordate, lophotrochozoan, and ecdysozoan published sequences we obtained a phylogenetic position for the eyes absent (EYA) (**Figure 3**), sine oculis homeobox (SIX) family (**Figure 4**), dachshund (**Figure 5**), and opsins (**Figure 6**) ESTs. Moreover, to strengthen our phylogenetic analyses, we also used unpublished data corresponding to a recent draft assembly of several transcriptomes of juveniles from *S. officinalis*. Our data demonstrate that our six EST belong to the Six1-Six2 Clade (**Figure 4**), and that the opsin EST is identical to a previous opsin of the Clade II Gq-coupled/rhabdomeric opsin Yoshida et al., 2015 (**Figure 6**).

Sof-otx, Sof-six1/2 and *Sof-eya* Expressions in the Developing Eyes of *Sepia officinalis*

In the whole-mount embryo, *Sof-eya* transcript appears expressed at stages 20 and 21 in the eye area (**Figures 7A,C**). *In situ* hybridization on cryo-sections, allowing a more precise tissue localization shows a staining localized only in the tissue surrounding the eyes at stage 20 and 21 (**Figures 7B–D**). In contrast, *Sof-six1/2* expression is not detected in the surrounding tissue (data not shown). For both genes (*Sof-six1/2* and *Sof-eya),* no expression is observed in the retina during these stages and from stage 23 to hatching, no expression is detected in any

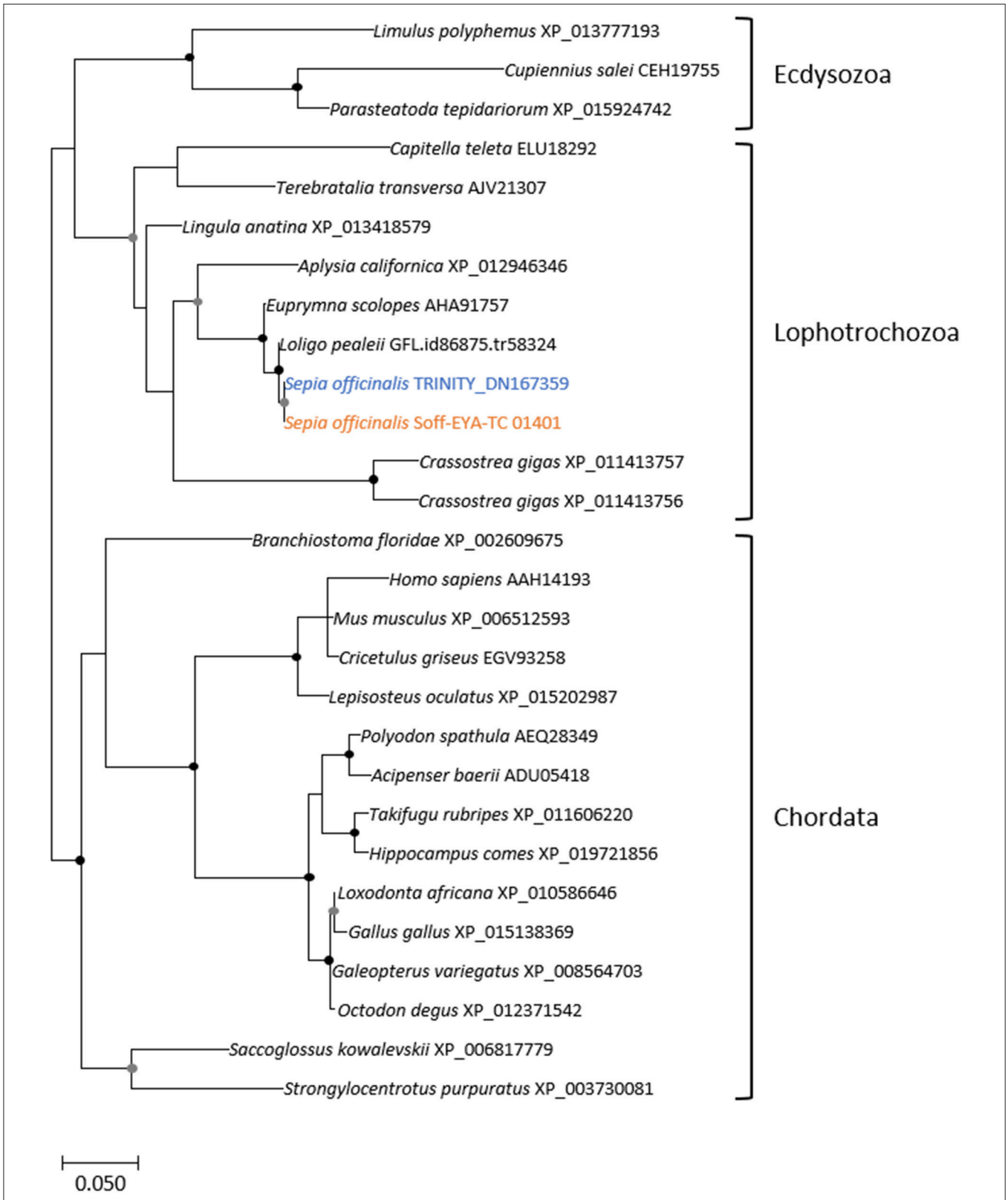

FIGURE 3 | Diversity of eyes absent (EYA) homolog. Maximum-likelihood tree based on 287 aligned amino-acids. The specific *S. officinalis* EST is shown in orange, and the *S. officinalis* contig/gene is in blue. Bootstrap support values are shown in the circles at the nodes (Black, 100–80%; gray, 80–50%).

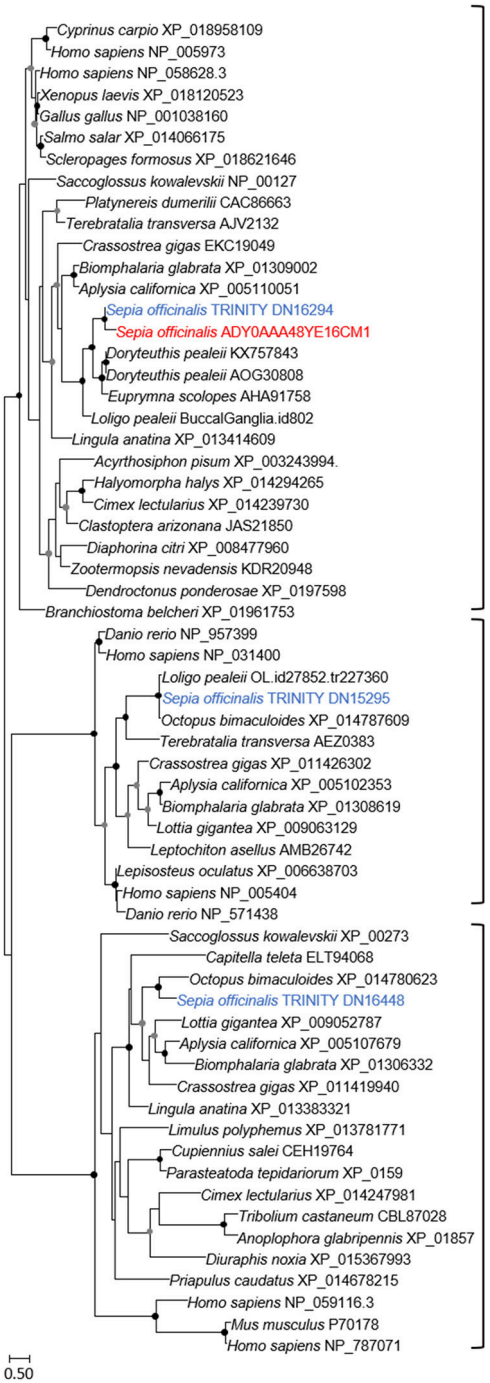

FIGURE 4 | Diversity of sine oculis homeobox (SIX) family. Maximum-likelihood tree based on 183 aligned amino-acids. The specific *S. officinalis* EST is shown in orange, and the *S. officinalis* contigs/genes are shown in blue. For the color code used for the bootstraps refer to **Figure 3** legend.

other tissues. By contrast, *Sof-otx* is expressed in the eye area as shown in whole-mount embryo ISH and this expression is located precisely in the retina at stage 20 and 21 (**Figures 7I–L**). The expression in the retina stops after stage 26 (Buresi et al., 2012).

Sof-dac Expression during Organogenesis

In all studied stages (from 18 to 30), *Sof-dac* appears expressed in the eye area. *Sof-dac* expression begins at stage 18 when the primary optic vesicles are not yet closed. The expression is observed in the eye area, in tissues surrounding the primary optic vesicle and in peripheral structures such as the arms (**Figures 7E–H**). By ISH on sections, allowing tissue localization, we show that there is expression neither in the retina nor in other tissues surrounding the eyes at early organogenesis (**Figures 7F–H**). No expression of *Sof-dac* is observed in the retina in later stages, from 24 to hatching. Nevertheless, *Sof-dac* is expressed, in the arms, cerebroid ganglia, visceral ganglia, and gills (**Figure 8**). *Sof-dac* expression is maintained in the arms until late stages of development (**Figures 8C,E**). At stage 24, when the ganglia merge and begin to differentiate into lobes, *in situ* hybridization on cryo-sections shows that *Sof-dac* expression is located in the plexiform area and medullar zone of the optic lobes, in some cells of the arm cord, in tissue layer surrounding the nerve cord, supraesophageal (SPM) and subesophageal (SBM) masses (**Figures 8B,C**). From stage 25 to 30, *Sof-dac* expression is restricted to the developing central nervous system, especially SPM and SBM, in some cells of the arm cord and in tissue layer surrounding the nerve cord, in the inner and outer plexiform layers and central medulla of the optic lobes (**Figures 8D–F**).

Sof-rhodopsin Expression in the Differentiating Retina

From stage 23, *Sof-rhodopsin* expression is detected only in the undifferentiated retina (**Figure 9A**). *Sof-rhodopsin* expression in the retina continued through stage 30 before hatching (**Figure 9**). This expression is variable; from stage 23 to 25, *Sof-rhodopsin* is expressed weakly and is observed only in the outer portion of the retina, corresponding to the area where the nuclei of the undifferentiated receptor cells and support cells are localized (**Figures 9A,B**). From stages 28 to 30, *Sof-rhodopsin* appears strongly expressed in the entire retina (**Figures 9C,D**). Actually, *rhodopsin* is normally restricted to the photoreceptor cells and is not present in all cells. Our observation is probably due to the numerous and juxtaposed rhabdomeric photoreceptors at the end of the development. *Sof-rhodopsin* expression should be limited to the outer segments of the differentiated retina cells.

DISCUSSION

Eye Specification Gene Expression in *S. officinalis* Embryo

Our expression data show that *Sof-eya* expression is restricted to the eye area and surrounding tissues at stages 20 and 21. *Sof-six1/2* is also expressed in the eye area during the early stages of organogenesis (from stage 20 to stage 21) in *S. officinalis*. Surprisingly, no *Sof-six1/2* and *Sof-eya* expression is shown in the eye area before stage 20 and after stage 22. *Eya* and Six have been strongly proposed to have an ancient role in eye development and their orthologs are involved in visual system development in both "invertebrates" and vertebrates (Vopalensky and Kozmik, 2009). The analysis of *Eya* genes expression in vertebrate eye

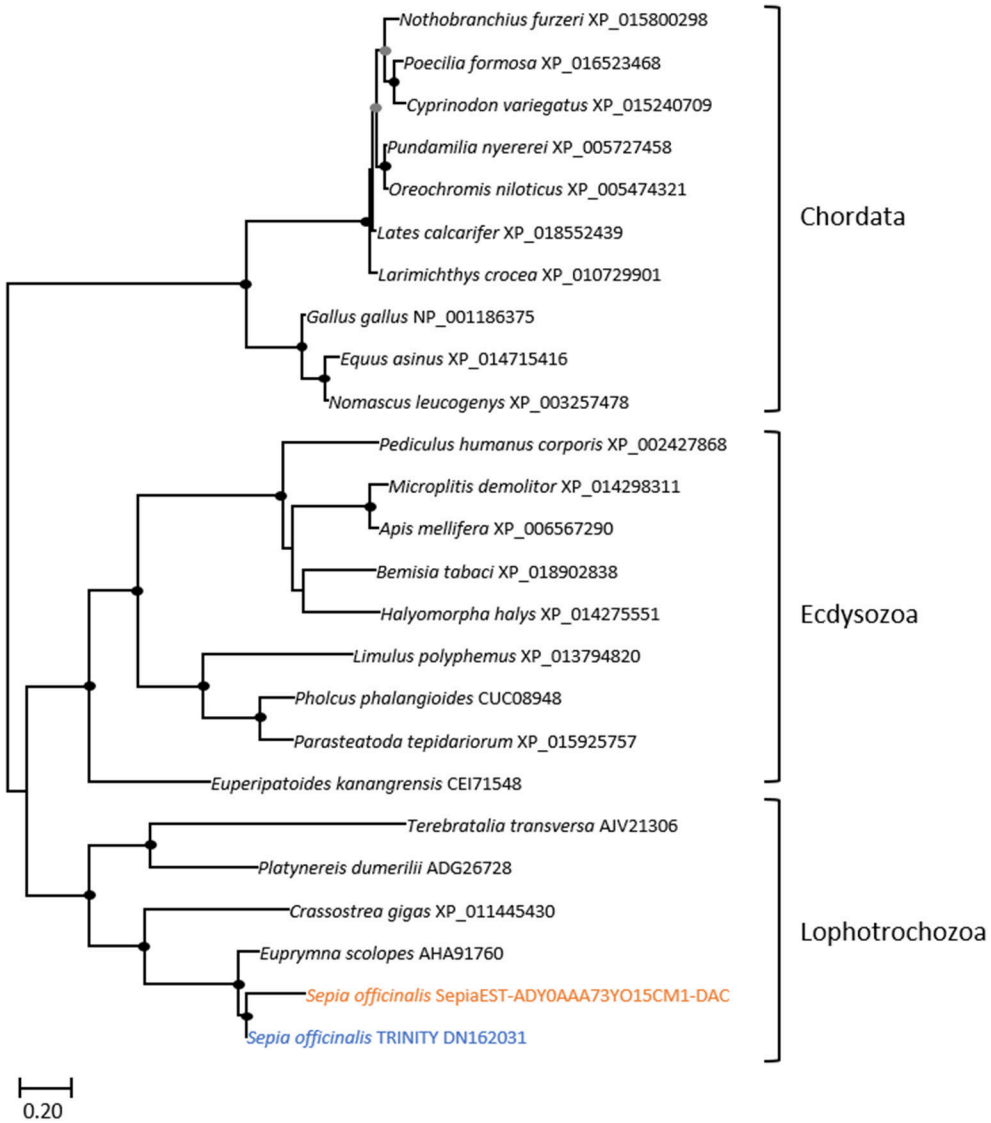

FIGURE 5 | Diversity of dachshund. Maximum-likelihood tree based on 170 aligned amino-acids. The specific *S. officinalis* EST is shown in orange, and the *S. officinalis* contig/gene is shown in blue. The EST and the contig protein sequences are slightly divergent in the N-terminal part of the sequences, probably because of EST sequence errors but importantly only the central and conserved region was used to generate the probe for *in situ* hybridization. For the color code used for the bootstraps refer to **Figure 3** legend.

development has shown that the three *Eya* genes, *Eya1, 2, 3* are differentially expressed in the developing eye (Xu and Saunders, 1997). Indeed, *Eya1* is expressed in the lens, optic stalk, and neural retina (Xu et al., 1997). *Eya2* is expressed in the neural retina only. Eya3 is present in the optic vesicle and the periocular mesenchyme, but both genes Eya2 and Eya3 are absent in the lens (Xu et al., 1997). In *Drosophila*, *sine oculis* has been shown to be required for the development of both, the compound eyes and the ocelli (Cheyette et al., 1994). Indeed, it has a critical role for the development of the entire visual system (Gehring, 2002). In mouse, *sine oculis* orthologs *six1/six2* are known to be expressed in the adult differentiating cells of the retina (Oliver et al., 1995). In protostome, *six1/2* homologs are known to be important for the early specification of the visual system (Cheyette et al., 1994; Arendt et al., 2002). The role of *six1/2* homologs in early visual system specification has been proposed to be evolutionary conserved outside Bilateria (Stierwald et al., 2004). Recent studies in cephalopods indicate that *six2, six3,* and *eya* expressions have been observed in the eye area, the optic lobes and often, at very early stages: in the lip of the placode and edges of the lid in *D. pealeii* embryo until stage 27. This suggests that six genes and *eya* are involved in lens formation as it is observed in vertebrates (Koenig et al., 2016). In *E. scolopes, six* and *eya* expressions were detected in numerous tissues including, until stage 26, the ventral light organ, with no known "visual function" (Peyer et al., 2014). However, in the results shown in these

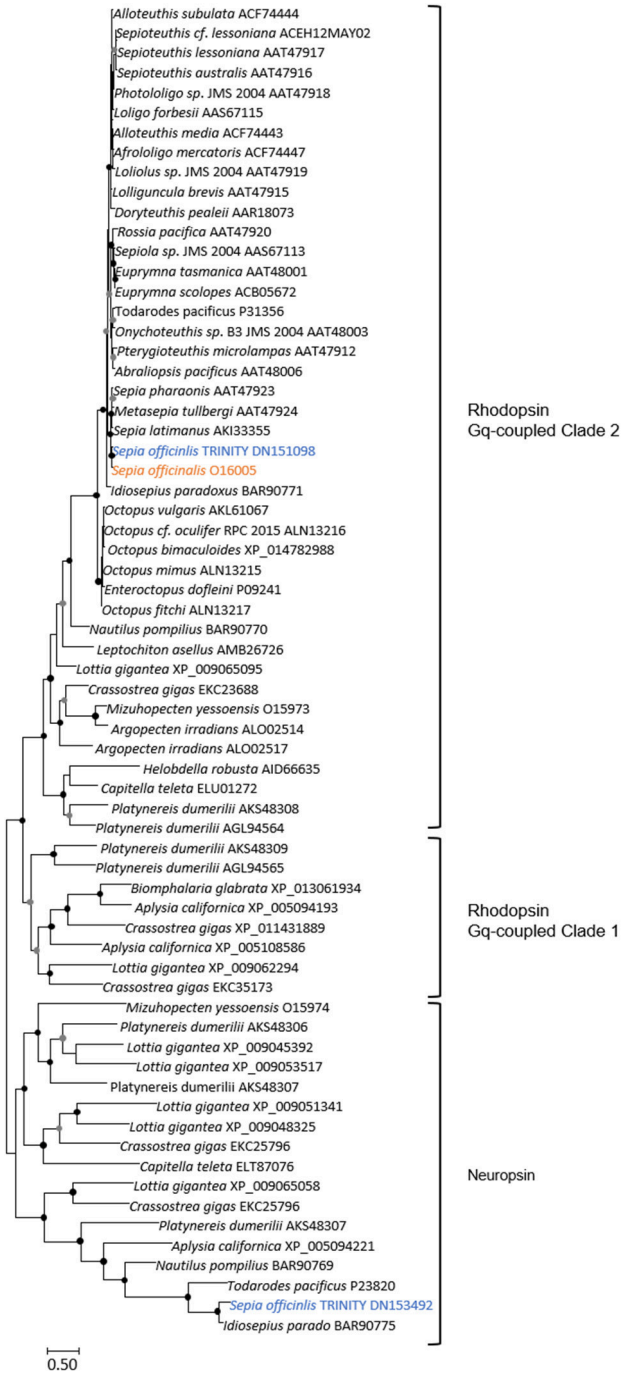

FIGURE 6 | Diversity of *Lophotrochozoa* opsins. This unrooted tree corresponds only to Gq-coupled/rhabdomeric opsins and to Neuropsin types. Maximum-likelihood tree based on 170 aligned amino-acids. The opsin-like protein which identical (100% conserved at the protein level, and 99% (717/718) identities at the nucleotide level); not shown) to the EST used in our study is shown in orange, and the *S. officinalis* contigs/genes are shown in blue. For the color code used for the bootstraps refer to **Figure 3** legend.

studies, expressions are presented by whole-mount ISH without showing precisely the staining in the eye fields with convincing histological data. According to our results both on sections and whole-mount, we point out that the expression shown in whole mount ISH in these studies must be confirmed by sections in the areas supposed to be stained. Furthermore, Ogura et al. (2013) have shown that *six3/6* is involved in the lens formation and its expression is localized in the lentigenic cells. Studies from vertebrates and *Drosophila* and even cnidarian, report *six3/6* expression in the developing eye (Oliver et al., 1995; Loosli et al., 1999; Zuber et al., 1999). Although we encountered problems and were not able to repeat staining of *six1/2* expression on sections, our expression data do not suggest any evolutionary conserved role of *Sof-six1/2* in the retinal specification and the eye formation. However, we cannot exclude the expression of another *six*-subfamily gene such as *six3/6* proposed to have an evolutionary conserved role. *Sof-dac* transcript is expressed from stage 18 to stage 30 in eyes and the optic lobes (**Figure 8**) but surprisingly its expression is never observed in the retina of cuttlefish. In vertebrates and *Drosophila, dac* expression is observed in the central nervous system, optic cup and also in the entire neural retina as it is shown on sections (Hammond et al., 1998; Caubit et al., 1999; Heanue et al., 1999; Loosli et al., 2002; Martín-Durán et al., 2012). In molluscs, *dac* is expressed in eye photoreceptor cell's development in larval chiton (Vöcking et al., 2015). In a cephalopod, *E. scolopes*, *dac* transcript expression is observed in the eyes, arms, mantle and light organs by whole mount *in situ* hybridization (Peyer et al., 2014). Strikingly, this expression is shown only on sections in the light organs but not in the retina. We point out that *dac* expression at the cellular level of eyes and other tissues must be confirmed by sections. In addition to being expressed in eye-associated tissues, *Sof-dac* has been also stained in supra-esophageal and sub-esophageal masses, gills, arms, and statocysts, all tissues playing important roles in sensory-motors functions. Our results show that the role of *dac* transcript is conserved in the central nervous system of metazoans. Nevertheless, *Sof-dac* seems expressed in optic lobes, precisely in the inner, the outer plexiform layers and also in the medullar zone at late stages until stage 30 before hatching. The plexiform area is also called the "deep retina" due to its similarity with the ganglionic layer of the vertebrate retina (Young, 1962). It is constituted by an inner and outer plexiform layer separated by a complex neuropil zone (Young, 1974). In cephalopods, the plexiform area contains several cell types (amacrine neurons, centrifugal, centrally, and centripetal cells). Furthermore, the retinal photoreceptors connect with the optic lobes by the photoreceptor axons in the plexiform area (Hartline and Lange, 1974). Indeed, the photoreceptors make synaptic contact with the amacrine neurons located in the inner and outer plexiform layers (Young, 1971; Case et al., 1972). *Sof-dac* expression in these areas could be an indication of the presence of photoreceptive cells in the optic lobes. These results suggest that *Sof-dac* is involved in the morphogenesis of the eye and visual control structures such as optic lobes in developing *S. officinalis*. In addition, we evidenced unexpected *Sof-dac* expression, for the first time: in some cells of the arm cord and in tissue layer surrounding the nerve cord. The histological character of this tissue is not yet identified, and additional studies must be done to determine if it is nervous or muscular cells as shown in some species (Heanue et al., 1999).

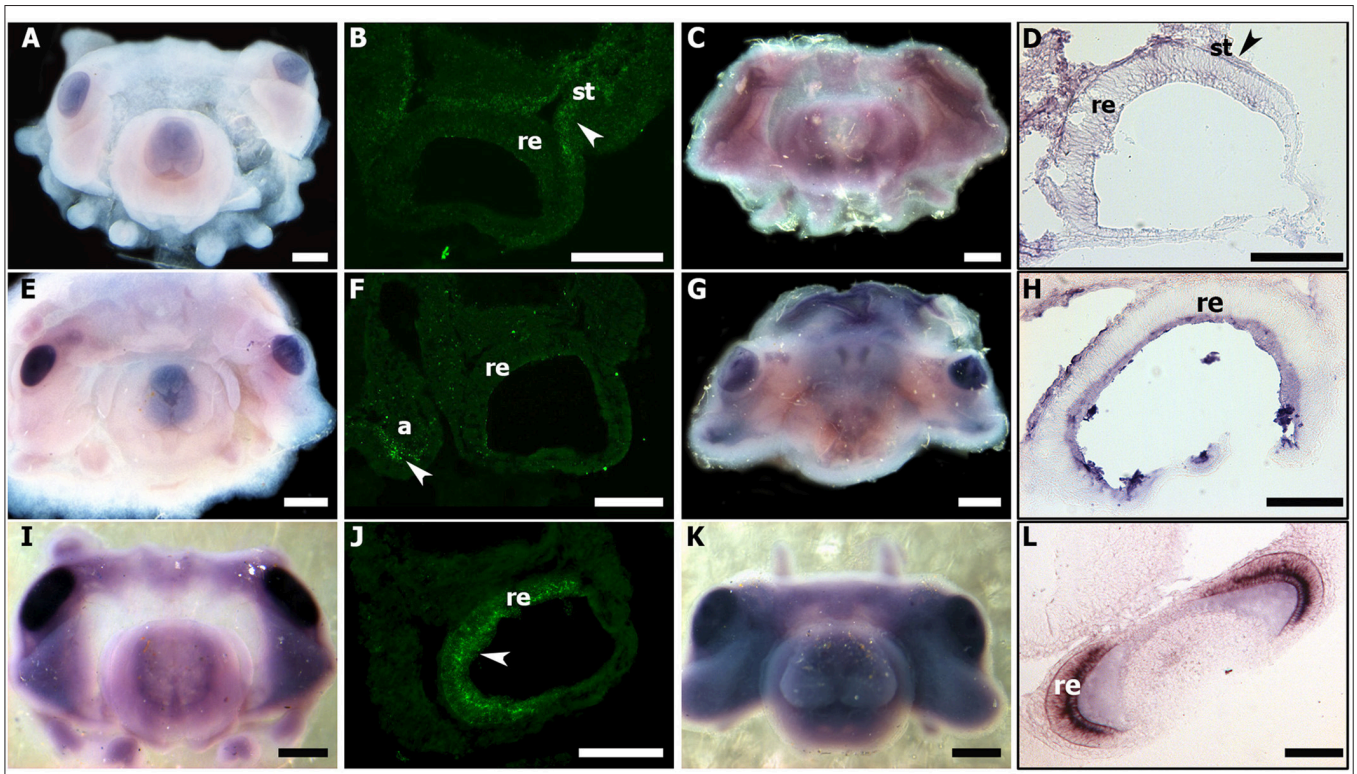

FIGURE 7 | *Sof-eya, Sof-dac,* and *Sof-otx* expressions during stage 20 and 21 of *S. officinalis*. **(A–D)**: *Sof-eya*. **(E–H)**: *Sof-dac*. **(I–L)**: *Sof-otx*. **(A,E,I)**: embryos stage 20. **(B,F,J)**: thin sections of stage 20. White head arrows point expressions of genes. **(C,G,K)**: embryos stage 21. The pink color in eyes and around the eyes is background: no staining is visible through sections **(D,H,L)** [thin sections of embryos **(C,G,K)**, respectively]. a: arms; re: retina; st: surrounding tissue. **(A,C,E,G,I,K)**: scale bar is 500 μm. **(B,D,F,H,J,L)**: scale bar is 150 μm.

In *S. officinalis* we evidence that the gene network, *six1/2*, *eya,* and *dac* is involved in the early eye area formation but not in the differentiation of the retina. Similar results were found with *Pax6,* not involved in the retina formation (Navet et al., 2017). *Pax6* is considered as a universal master control gene of eye formation and developing photoreceptors in metazoans (Gehring, 2005; see review in Kumar, 2009); it regulates upstream the RDGN, which instructs the formation of the adult eye in *Drosophila*. In numerous cephalopods (*L. opalescens*: Tomarev et al., 1997; *E. scolopes*: Hartmann et al., 2003; Peyer et al., 2014; *S. officinalis*: Navet et al., 2009, 2017; *I. paradoxus*: Yoshida et al., 2014; *D. pealeii*: Koenig et al., 2016), *Pax6* expression has been shown in optical areas, eye and optic lobes. In *S. officinalis*, *Pax6* presents a large ectodermic and mesodermic expression in the optic area but never in the retina (Buresi et al., 2016; Navet et al., 2017) and no clear expression in retina cells has been finally shown in the other species. These findings suggest the non-conservation of *pax6* in the differentiation of the retinal photoreceptors by the loss of the conserved RDGN upstream regulation: this could explain the loss of expression of *six, eya,* and *dac* in the retina. No other *pax* genes (*pax3/7* and *pax2/5/8*) is expressed in *S. officinalis* embryo all along the development in the retina cells from the first steps of specification until the rhabdomeric photoreceptors are fully differentiated. Nevertheless, other genes appears to have a role in retina differentiation, such as *Sof-otx*, which is expressed in the retina as already shown (Buresi et al., 2012).

Indeed, *otx2* transcript factor is known to play a role equally in the development of the eye and the photoreceptor cells. In vertebrates, *otx* orthologs could control all retinal cell types (Chen et al., 1997; Viczian et al., 2003). *Otx2* and *pax6* are expressed upstream of the opsin: they could act to enhance r-opsin expression for the rhabdomeric photoreceptor; but only *otx2* (and not *pax6*) with Rx activate the expression of c-opsin, characteristic of ciliary photoreceptors (Vopalensky and Kozmik, 2009). *Otx2* transcription factor expression is reported in the photoreceptors of the fly ommatidia, in the photoreceptor precursors of larval eyes of *T. transversa* and the planarian pigment-cup eyes (Umesono et al., 1999; Passamaneck et al., 2011). In *Sepia* embryos, *otx* is expressed in the eyes particularly in the retina from early to late developmental stages (from stage 19 to stage 26) when the photoreceptor's differentiation started but its expression is not found from stage 26 when the retinal cell type organization is being achieved (Buresi et al., 2012). Thus, the mystery of the final differentiation of retinal photoreceptors and the genes that control this process remains to be characterized in order to understand what the underlying molecular mechanisms are.

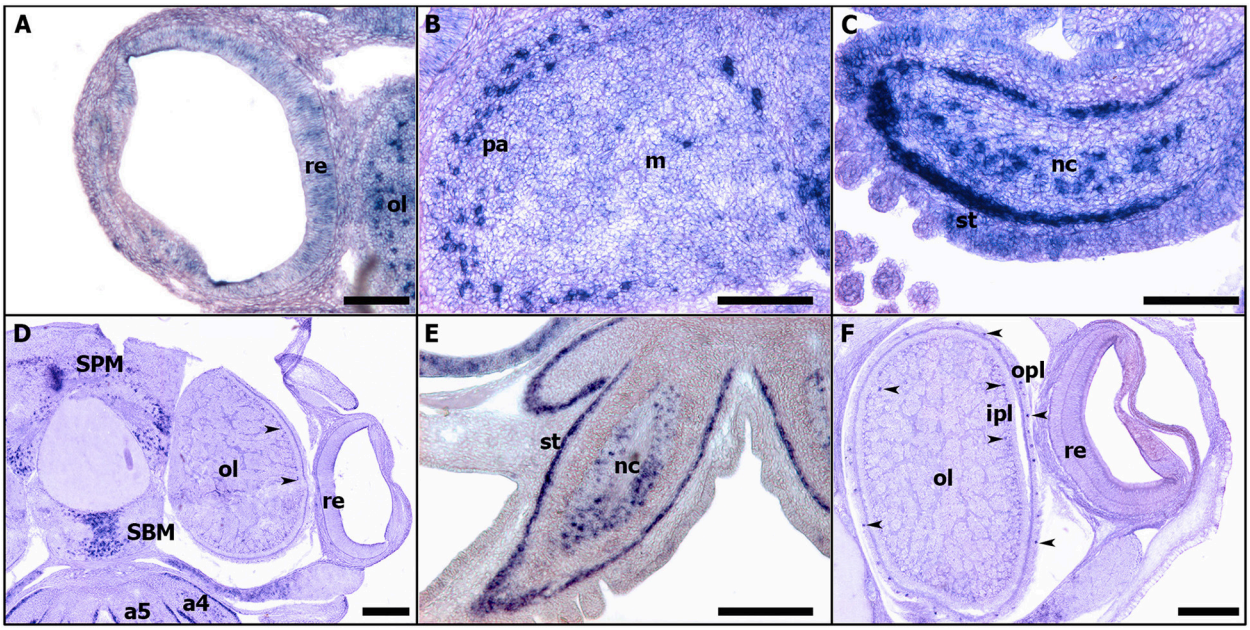

FIGURE 8 | Expressions of *Sof-dac* during *S. officinalis* organogenesis on transversal cryo-sections. Dorsal side is up and ventral side is down. **(A–C)**: stage 24. **(A)**: eye. **(B)**: optic lobe. **(C)**: arm 5. **(D,E)**: stage 26. **(D)**: expressions in the supraesophageal and subesophageal masses, in inner and outer plexiform layers (black head arrow) and medullar zone of the optic lobes. **(E)**: arm 5. **(F)**: stage 28, optic lobe and eye. Black head arrows point out the expressions of the respective genes in the eyes. a4, a5: arms; ipl: inner plexiform layer; m: medulla; nc: nerve cord; ol: optic lobes; opl: outer plexiform layer; pa: plexiform area; re: retina; SBM: subesophageal mass; SPM: supraesophageal mass; st: surrounding tissue. **(A–C)**: scale bar is 150 μm. **(D–F)**: scale bar is 300 μm.

Sof-rhodopsin Expression in the Differentiating Rhabdomeric Photoreceptors

Our results reveal for the first time in *S. officinalis* embryo the expression patterns of *rhodopsin* transcript in the developing retina from stage 23 to hatching (**Figure 9**). Several cephalopod species have both retinal and extraocular photoreceptors located in the light organ, skin, paraolfactory vesicles, epistellar body, and nervous system where a great diversity of *opsin* proteins can be found (Cobb and Williamson, 1998; Tong et al., 2009). In coleoid cephalopods, the retina only has a single layer containing rhabdomeric photoreceptors, supporting cells and blood vessels (Messenger, 1981). Additionally, the retinal rhabdomeric photoreceptors are known to express a single type of *rhodopsin* in coleoid cephalopods (Bellingham et al., 1998). *Rhodopsin* transcript has been evidenced by RT-PCR and immunolabeling not only in the retinas of several adult species of cephalopods such as squid *D. pealeii*, cuttlefish *S. officinalis* and *Sepia latimanus* but also in the skin of the same species (Kingston et al., 2015a,b). These authors indicate that the *rhodopsin* detected both in the skin and retina of adult cuttlefish is the same. But interestingly, in *S. officinalis* embryo, unlike adults, we have evidenced *rhodopsin* expression only in the retina, not in other tissues or structures, such as the skin. As the *rhodopsin* sequence used is exactly the same described in Kingston et al. (2015a) and Yoshida et al. (2015), and as no other *rhodopsin* has been found in our EST-library, we propose that *rhodopsin* is expressed in the skin after hatching, in the juveniles, when the patterns are useful for camouflage. A differential expression of rhodopsin during development has been shown in annelid suggesting a control of the "visual function" in accordance with developmental stage (Randel et al., 2013). Finally, *rhodopsin* transcript can be expressed so weakly that it cannot be detected by *in situ* hybridization. The weak expression of *Sof-rhodopsin* observed between stages 23 and 25 can be explained by the first differentiation step of cells at stage 23, and the beginning of the differentiation into photoreceptor cells and supporting cells from stage 25 on, as observed in *Sepiella*. This expression might be localized in the cells which differentiate into rhabdomeric photoreceptors at late stages as it is observed in other cephalopod adult species. But, in order to link *sof-rhodopsin* expression to the retinal cell differentiation process, further studies should be conducted during cuttlefish embryogenesis. Moreover, the expression of *Sof-otx* in cuttlefish's retina at stage 19, long before the expression of *rhodopsin* (stage 23) is congruent with the upstream regulation of *rhodopsin* in vertebrates (Vopalensky and Kozmik, 2009), and the timing of retina cone differentiation in mouse preceding the *rhodopsin* expression (Rodgers et al., 2016). Nevertheless, as *Sof-otx* expression stopped at stage 26 (Buresi et al., 2012), we question the control of *rhodopsin* expression in late developmental stages, i.e., in rhabdomeric photoreceptors. This expression and the production of *visual rhodopsin* combined with the observation of reactivity of the eye from stage 25 on show that *rhodopsin* is present before the setting up of the rhabdomeric photoreceptors (Yamamoto et al., 1985). These data strengthen the hypothesis of Romagny et al. (2012) suggesting that *S. officinalis* is able to react to

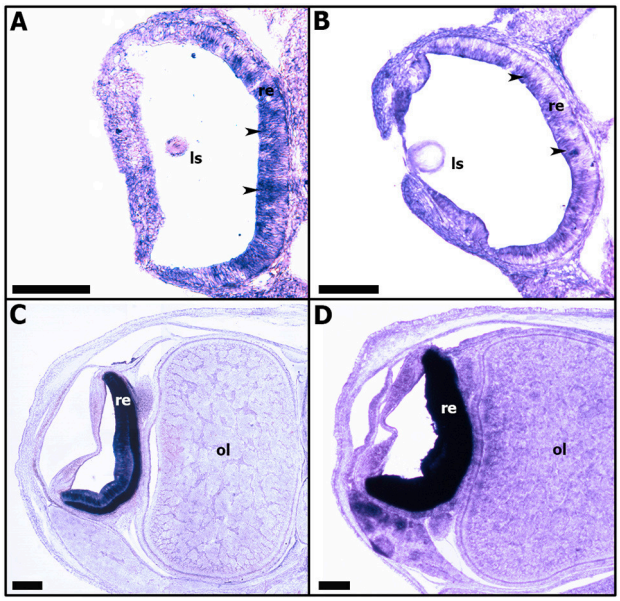

FIGURE 9 | Expressions of *Sof-rhodopsin* during *S. officinalis* organogenesis on transversal cryo-sections. Dorsal side is up and ventral side is down. **(A,B)**: expressions in the developing retina (black head arrows) respectively at stages 23 and 25. **(C,D)**: expressions in differentiating retina, respectively at stages 28 and 30. ls: lens; ol: optic lobe; re: retina. Scale bar is 200 μm.

light stimuli from stage 25 of organogenesis on, when the first retinal pigments appear, a stage when the rhabdomes are not totally differentiated. As a consequence, a fully differentiated rhabdomeric photoreceptor is not necessary to have a "basic" answer to light stimulus. Nevertheless, Romagny et al. (2012) have shown, by behavioral experiments of answer to light, that the habituation to light, the memory process is evidenced only at late stages of development, when the retinal rhabdomeric photoreceptors are totally differentiated and when the *rhodopsin* expression is very high (**Figure 9**). It is linked to the final maturation of the analysis centers (brain and optic lobes).

CONCLUSION

The aim of this study was to lay the molecular basis of eye formation, differentiation, and specification of retinal photoreceptors in *S. officinalis*. The results obtained indicate that three genes important for eye morphogenesis and photoreceptors differentiation in numerous groups of metazoans are involved in eye formation but never in retina cell differentiation during *S. officinalis* embryonic stages studied (17 to 30). We cannot exclude that they are expressed before stage 17 but as there are undifferentiated cells until stage 21, their role in the retina cell differentiation would be questioned. These findings reveal or reinforce the divergence and the broad complexity in the genetic network underlying the cephalopod retinal differentiation. *Sof-six1/2, Sof-eya*, and *Sof-dac* are localized only in the eye area questioning about the gene network involved in the differentiation of rhabdomeric photoreceptors in cephalopods. Actually, all of the previous studies in eye development in cephalopods have been performed by whole mount *in situ* hybridization. However, according to our results, we point out that gene expressions must be studied on sections to make it possible to exactly locate the expression of these genes at the cellular level. Nevertheless, cephalopods are an interesting model to study the evolution of the nervous system, the eye development complexity and the diversity of photosensitive structures. Besides the fact that RDGN expression levels could be outside the threshold of detection, the *Sepia's* retina development and the final differentiation of rhabdomeric photoreceptors is probably controlled by other genes than those identified until now. Thus, it will be necessary to explore the role of other genes such as *Notch* that is known to intervene in the retina and lens formation in vertebrates and *Drosophila* where it regulates the cell cycle progression within retina and lens (Livesey and Cepko, 2001; Charlton-Perkins et al., 2011).

Our study opens up other opportunities to investigate the evolution of functions complexity within metazoans. *Sof-rhodopsin* expression in the retina during *S. officinalis* embryogenesis correlates with the behavioral observation and the light sensitivity of cuttlefish embryos before the final differentiation of rhabdomes. Nevertheless, it seems necessary to investigate the diversity of photoreception molecules, the character of tissues and cells that expressed these molecules, in skin, optic lobes, and brain, to build an understanding about the evolution of photosensitive structures and phototransduction function in the retina and in the extraocular photoreceptor tissues. As it is described above, other genes involved in the phototransduction cascade and/or signaling pathways must be explored such as *arrestin* already identified in *E. scolopes* adult eye (Tong et al., 2009).

ETHICS STATEMENT

We use cephalopod embryos before hatching. In this case, according to the European law, our protocol is not in the field of animal experiment and there is no need to obtain an authorization from our local committee (MNHN Commitee Cuvier N) which has been consulted. LB-P is a member of Cuvier Commitee.

AUTHOR CONTRIBUTIONS

Conceived of project: LB-P; Designed experiments: LB-P, BI, and AA; Executed experiments: BI and AA; Analyzed data: BI; Phylogenetic analysis: PL; Edited manuscript: BI, LB-P, PL, AA and YB.

FUNDING

This work was funded by MNHN-CNRS.

ACKNOWLEDGMENTS

The authors are deeply grateful to Basuyaux Olivier from "Synergie MEr et Littoral" for providing us *Sepia* eggs. We thank also Auxane Buresi for *Sof-otx* pictures (**Figures 7I,K,L**) and reviewer who has underlined the necessity to obtain section from early stages.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 13 June 2017
doi: 10.3389/fphys.2017.00402

Visual Ecology and the Development of Visually Guided Behavior in the Cuttlefish

*Anne-Sophie Darmaillacq *, Nawel Mezrai, Caitlin E. O'Brien and Ludovic Dickel*

UMR Centre National de la Recherche Scientifique Université de Caen-Université de Rennes 1, Normandie Université, Université de Caen Normandie, Team NECC, Caen, France

Cuttlefish are highly visual animals, a fact reflected in the large size of their eyes and visual-processing centers of their brain. Adults detect their prey visually, navigate using visual cues such as landmarks or the *e*-vector of polarized light and display intense visual patterns during mating and agonistic encounters. Although much is known about the visual system in adult cuttlefish, few studies have investigated its development and that of visually-guided behavior in juveniles. This review summarizes the results of studies of visual development in embryos and young juveniles. The visual system is the last to develop, as in vertebrates, and is functional before hatching. Indeed, embryonic exposure to prey, shelters or complex background alters postembryonic behavior. Visual acuity and lateralization, and polarization sensitivity improve throughout the first months after hatching. The production of body patterning in juveniles is not the simple stimulus-response process commonly presented in the literature. Rather, it likely requires the complex integration of visual information, and is subject to inter-individual differences. Though the focus of this review is vision in cuttlefish, it is important to note that other senses, particularly sensitivity to vibration and to waterborne chemical signals, also play a role in behavior. Considering the multimodal sensory dimensions of natural stimuli and their integration and processing by individuals offer new exciting avenues of future inquiry.

Keywords: cephalopod, vision, embryo, brain, polarization, camouflage, behavioral plasticity

OPEN ACCESS

Edited by:
Daniel Osorio,
University of Sussex, United Kingdom

Reviewed by:
John R. Gray,
University of Saskatchewan, Canada
Chuan-Chin Chiao,
National Tsing Hua University, Taiwan

****Correspondence:***
Anne-Sophie Darmaillacq
anne-sophie.darmaillacq@unicaen.fr

Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *31 October 2016*
Accepted: *29 May 2017*
Published: *13 June 2017*

Citation:
Darmaillacq A-S, Mezrai N, O'Brien CE and Dickel L (2017) Visual Ecology and the Development of Visually Guided Behavior in the Cuttlefish. Front. Physiol. 8:402. doi: 10.3389/fphys.2017.00402

INTRODUCTION

One of the most remarkable experiences one can have as a SCUBA diver is an encounter with a cuttlefish. Not only is it unexpected (during daytime, cuttlefish are mostly camouflaged, and only an experienced eye is likely to spot one), but you have a strange feeling of being observed! Indeed, the eyes of the cuttlefish are large and captivating (**Figure 1**). They are single-chambered camera-type eyes whose structure strikingly resembles that of vertebrates. This convergence is unique among invertebrates and was probably driven by shared ecology and competition with fish (Packard, 1972). Another indication of the importance of vision to cuttlefish, though other senses are important, is the size of the optic lobes. These two bean-shaped lateral nervous structures process visual information and occupy 140% of the whole central nervous system (Nixon and Young, 2003; **Figure 2**). The primary purpose of the visual system is to recognize objects so that individuals may interact with them appropriately and execute the behaviors necessary for survival. Vision plays a crucial role in the early life stages, as functional vision is essential for perception of prey, predator

FIGURE 1 | Eyes of the cuttlefish *Sepia elongata* caught off the coast of Eilat (Gulf of Aqaba, Israel; photo AS Darmaillacq).

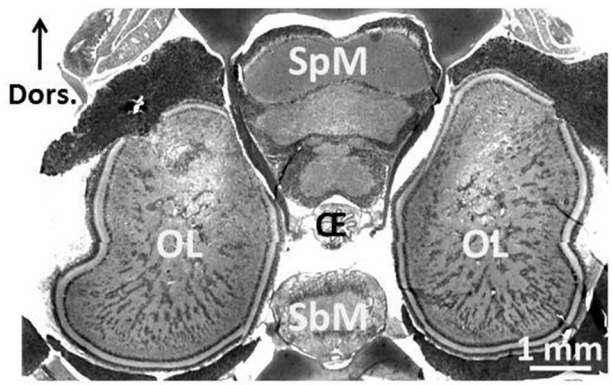

FIGURE 2 | Central nervous system of 3-month-old *Sepia officinalis* cuttlefish. Frontal section. Prenant-Gabe trichrome stain. Abbreviations: OL, optic lobe; SpM, supra-esophageal mass; SbM, sub-esophageal mass; Oe, esophagus. Modified from Jozet-Alves et al. (2012a).

avoidance and visually-guided behavior (e.g., predation, Darmaillacq et al., 2004; camouflage, Zylinski et al., 2012; navigation, Cartron et al., 2012). Consequently, the early development of functional vision is critical because it enhances the chances of survival. Although the visual capacities of cephalopods have been studied extensively in adults, few studies have investigated their development. Indeed, embryos were traditionally considered to possess only limited abilities because of the immaturity of their developing brains. In this review, we will describe how the visual system develops in embryos and how it allows embryonic visual learning. We will also summarize our knowledge of some of the interesting particularities of cephalopods: polarization sensitivity (PS) and contrast perception (Shashar et al., 2002), and that of visual lateralization. Lastly, more recent data regarding the development and plasticity of defensive behavior in juveniles will be presented.

EMBRYONIC DEVELOPMENT OF THE VISUAL SYSTEM AND EMBRYOS' RESPONSES TO VISUAL STIMULI

Development of Sensory Systems

Sepia officinalis eggs are laid in clusters on various kinds of rigid support such as algae, tubeworms, ropes or nets. Unlike other species of *Sepia*, the eggs are usually darkened with maternal ink but become more translucent due to the expansion of the capsule during embryonic development (Boletzky, 2003). *S. pharaonis* eggs are completely translucent.

During the final phase of embryonic development (stages 23–30; Boletzky et al., 2016), rhythmic mantle contractions are visible through the egg capsule after removal of the outer darker envelopes. These can be measured to assess embryonic responses to various external stimuli. Like this, Romagny et al. (2012) showed that in cuttlefish embryos, the order of the onset of function of chemosensitivity, touch and vision follows the same sequence as that of birds and mammals, with the visual system being the last to develop. Neurobiological data illustrating the early development of sensory neurons in embryos support these behavioral observations (Baratte and Bonnaud, 2009). This is another evidence of convergent evolution between cephalopods and vertebrates, perhaps instigated by similar environmental pressures and direct competition (Packard, 1972). Because embryonic development takes place outside of the mother and in the absence of direct parental care, there is strong evolutionary pressure for the rapid development of functional sensory systems, so that predators can be avoided and feeding can begin. Unlike some vertebrate species, in which the visual system is still immature at birth (Bremner et al., 2012), indirect evidence suggests that cuttlefish embryos can discriminate objects outside the egg. However, to date, no systematic study has been conducted on the development of retina morphology and physiology in the embryo (but see Imarazene et al., in press).

Embryonic Visual Responses

There is increasing empirical evidence that prenatal experience influences postnatal perception, cognitive performance and behavior. Embryonic perceptual learning, (tested in neonates) has been demonstrated across many taxa, including insects (Caubet et al., 1992), amphibians (Mathis et al., 2008), rats (Hepper, 1988), dogs (Wells and Hepper, 2006), precocial birds (Sneddon et al., 1998), altricial birds (Colombelli-Négrel et al., 2012, 2014), and humans (Moon et al., 2013).

Studies showed that embryonic visual experience affects both feeding and defensive behaviors. Cuttlefish embryos visually exposed to juvenile crabs for the last week before hatching will prefer crabs to their innately preferred shrimp prey (Darmaillacq et al., 2008). Likewise, cuttlefish innately prefer black crabs to white crabs but will preferentially select white crabs following embryonic exposure to them (Guibé et al., 2012; **Figure 3A**). Thus, it seems that not only do the cuttlefish pay attention to the shape of the prey (crab vs. shrimp) but also to its brightness. The relative importance of shape and brightness can be inferred from the fact that cuttlefish select black

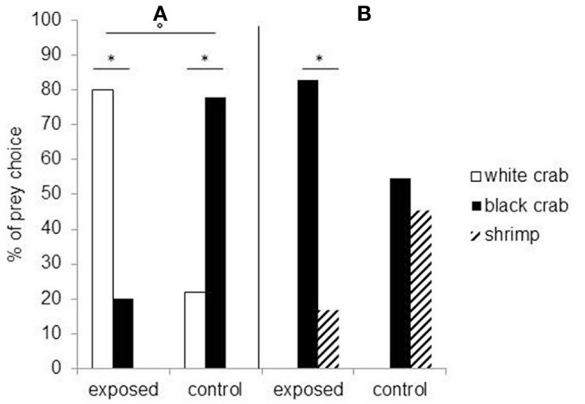

FIGURE 3 | Seven-day-old cuttlefish's prey choice depending on whether they have been exposed to white crabs during embryonic development ("exposed") or not ("control"). **(A)** To the left of the vertical: when they are presented a choice between white and black crabs. **(B)** To the right: when they have a choice between black crabs and shrimp. *Significant prey preference within groups (chi-square exact test: $p < 0.05$) and °significant difference in prey choice between groups (Fisher's exact test: $P < 0.05$). Modified from Guibé et al. (2012).

crabs over shrimp after embryonic exposure to white crabs, suggesting that they are generalizing the characteristics of a learned preference (crab shape) to the closest alternative (black crab) if the preferred item is not present (Guibé et al., 2012; **Figure 3B**).

Juvenile cuttlefish, that spontaneously prefer dark shelters, lose this bias when they have been exposed embryonically to white ones (Guibé and Dickel, 2011). Lee et al. (2012) also showed that cuttlefish raised prenatally in a visually enriched have a preference for high-contrast backgrounds whereas control cuttlefish have no substrate preference. More experiments are needed to study the direct response of the embryo to visual stimuli and the development of related brain structures.

These preferences for certain visual characteristics such as shape and brightness following embryonic exposure are relatively straight-forward. In contrast, chemical exposure to waterborne cues from shrimp or crab alters visual preferences after hatching in a less explicable fashion. Embryonic exposure to crab odor and blank seawater had no effect on the normal preference for shrimp; exposure to shrimp cue however resulted in a reversal of the normal shrimp preference (Guibé et al., 2010). The authors suggested that this is possibly due to cross-modal effects, in which odor cue modulates a primarily-visual preference. Alternatively, it could be that because embryos in this experiment were exposed to the odors of adult shrimp and crabs and they were somehow able to determine the size of the animal by its odor cue, perceiving them as a danger rather than as prey. Repeating these experiments with shrimps and crabs of various sizes could determine whether age causes differences in odor cues that are distinguished by cuttlefish.

DEVELOPMENT OF PS, CONTRAST SENSITIVITY, VISUAL ACUITY AND VISUAL LATERALIZATION

The cephalopod rhabdomeric-type eye has only one type of photoreceptor. The microvilli of neighboring photoreceptors are arranged orthogonally in the retina which confers sensitivity to the linear polarization of light (Shashar et al., 2002), one of the main properties of light in shallow water (Cronin and Shashar, 2001). Cephalopod eyes are positioned laterally on the head allowing both a monocular and a binocular vision.

Spatial Resolution and Polarization Sensitivity

Spatial resolution (or visual acuity), is the ability to discriminate fine detail (Tansley, 1965), and plays an extremely important role in the lives of animals, as it allows them to navigate in space, evade predators, catch prey, and in some species differentiate between males and females. Using an optomotor apparatus and stripes of different width, Groeger et al. (2005) showed that visual acuity improves as cuttlefish grow, ranging from a minimum separable angle of 2.5–0.57° (a decrease in this angle value means a better spatial resolution). A decrease in light intensity affects visual acuity whatever the age of the individual.

Polarization sensitivity (PS) improves the visibility of objects by enhancing the contrast between them and the background. In cephalopods, PS increases the success of predation on transparent prey or silvery fish (Shashar et al., 1998, 2000); in cuttlefish, it may also play a role in communication between adults (Shashar et al., 1996; Boal et al., 2004) and in navigation (Cartron et al., 2012). PS matures gradually after hatching. Cartron et al. (2013a) found that only 20% of cuttlefish hatchlings showed an OMR to a polarized striped pattern when it was rotated slowly. The proportion of cuttlefish responding increased throughout the first month of life (100% by the age of 30 days; **Figure 4**). However, a choice test with fully polarized or depolarized mysids (transparent shrimps) showed that 1 week-old cuttlefish detect polarized shrimp faster than non-polarized, suggesting an earlier maturation of PS (Cartron et al., 2013a). These apparently contradictory results could be explained by the motion of the rotating pattern in the OMR apparatus compared with the more stationary prey. It is possible that polarization contrast is more useful in assessing the shape of prey and that motion can interfere somewhat with this ability. This deficiency could be mitigated by the fact that polarization is not the only quality of light to which cuttlefish are sensitive. Though colorblind (Mäthger et al., 2006; but see Stubbs and Stubbs, 2016), cuttlefish are sensitive to contrast. Indeed, most hatchling cuttlefish (75%) showed an OMR to the black, white and gray striped pattern rotating at the lowest velocity, with the proportion reaching 100% by the age of 1 month. Thus, it can be hypothesized that polarization and luminance signals are processed separately and may play different roles in vision as observed in insects (Pfeiffer et al., 2005). In the desert locust *Schistocerca gregaria* for instance, a group of neurons in the central complex (a neuropil in the center of the brain), has been found to be

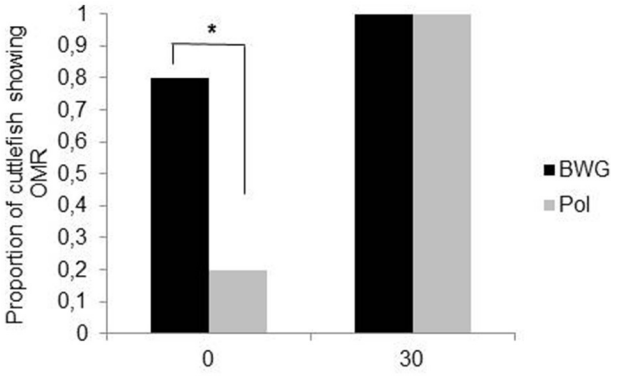

FIGURE 4 | Proportion of the cuttlefish ($N = 10$ per group) that showed an optomotor response (OMR) to BWG (luminance only; black) or Pol (polarization; gray) patterns rotating at a velocity of 30 deg s^{-1}, at hatching (0) and at the age of 30 days. Asterisks indicate a significant difference in the percentage of cuttlefish showing an OMR between the BWG and Pol patterns McNemar's test, ($P < 0.05$). Modified from Cartron et al. (2013a).

sensitive to polarized light while neighboring neurons are not (although all neurons responded to unpolarized light). More experiments, notably electrophysiological and immunochemistry investigations, are needed in order to determine the neural pathways for polarization and luminance information processing in cuttlefish.

Ontogenesis of Visual Lateralization

Cerebral lateralization, a trait that is widespread in animal kingdom (Vallortigara and Rogers, 2005; Frasnelli et al., 2012), is often revealed behaviorally by motor and perceptual asymmetries. In cuttlefish, adults have a preference for turning right or left (side-turning preference) in a T-maze (Alves et al., 2007), which can be the result of an eye use preference as in octopus (Byrne et al., 2002, 2004). In juveniles, Jozet-Alves et al. (2012b) showed that although cuttlefish do not show any side-turning preference in a basic T-maze, they do develop a left-turning bias when shelters are available at the end of the maze's arms from the age of 3 to 60 days. Interestingly, when cuttlefish have been exposed to a predator odor before hatching, they preferentially turn to the left in the simple T-maze (Jozet-Alves and Hebert, 2013); this suggests an influence of environmental factors on the ontogenesis of visual lateralization in cuttlefish. This may be adaptive for young cuttlefish to decide rapidly which shelter to choose specially in a risky situation where predators are potentially present around.

Influence of Environmental Constraints on PS and Visual Lateralization

S. officinalis, the European cuttlefish, is widespread in the English Channel, the Atlantic Ocean and the Mediterranean Sea where the turbidity can be high. On the other hand, *S. pharaonis* and *S. prashadi* are found in the Red Sea, on coral reefs, where the water is clearer. All these species are able to detect a polarized stimulus at higher turbidity levels than an unpolarized one (Cartron et al., 2013b,c), indicating that PS can improve the capacity for object detection through turbid waters when intensity information alone is insufficient. *S. officinalis* can detect objects, whether polarized or unpolarized, at higher turbidity levels than the other two (Cartron et al., 2013b). It is thus likely that PS, which is present in most cuttlefish species (but see Darmaillacq and Shashar, 2008), is a product of natural selection driven by visual features of the species' environment. This hypothesis is supported by the fact that the *S. officinalis* used in this experiment were lab-reared individuals that had never encountered turbidity, yet were still better-equipped to discriminate objects under these conditions.

DEFENSIVE BEHAVIOR

Cephalopods are known for their skills in quickly changing skin patterns in response to environmental change, a property referred to as "dynamic camouflage" (Hanlon and Messenger, 1996; Hanlon, 2007). This dramatic behavior is made possible by their unique skin structure that comprises three layers of cells: the chromatophores (containing dark-brown, reddish-orange or yellow pigments), within the most superficial dermis of the dorsal part of the mantle and arms, under the direct control of the brain; the iridophores, underneath, that reflect environmental light to create iridescence (particularly prominent on the ventral part); and the leucophores, the deepest, that reflect mainly white. Together with textural, postural and locomotor components, these chromatic elements constitute the "body pattern" of cuttlefish (Hanlon and Messenger, 1988). Body patterns displayed in a chronic fashion are mainly used for crypsis in juveniles as a primary defense strategy to avoid detection. Cuttlefish adopt a brightness similar to the substrate (general color resemblance), or a display disruptive colorations that breaks up the outline of the body so that the overall form of the animal is lost (Hanlon et al., 2009). The disruptive pattern has been the most studied. In the lab, it has been shown that artificial backgrounds such as 2d checkerboards can elicit this pattern (Chiao and Hanlon, 2001; Chiao et al., 2007). More, several authors (Chiao and Hanlon, 2001; Barbosa et al., 2007, 2008) showed that both check size and achromatic contrast affected the body patterns. Other characteristics of the objects present in the vicinity of cuttlefish are taken into account by juveniles such as the presence of egdes, the spatial phase and the three dimensionality (Chiao et al., 2005; Zylinski et al., 2009; Ulmer et al., 2013).

Other body patterns (such as the deimatic and flamboyant displays) are shown in a more acute manner (only for a few seconds) and are used mainly as "secondary" defense strategies after a cuttlefish has been detected. Cuttlefish can also adopt a deceptive resemblance to natural objects in the environment (e.g., floating algae) to deceive potential predators or prey. In juvenile cuttlefish, uniform and mottle patterning are generally displayed on uniform/fine sandy backgrounds (**Figure 5A**) while disruptive coloration occurs on more patchy/contrasted substrates (**Figures 5B,D**). Uniform, mottle and disruptive patterns are usually mixed to varying degrees (Hanlon et al., 2009;

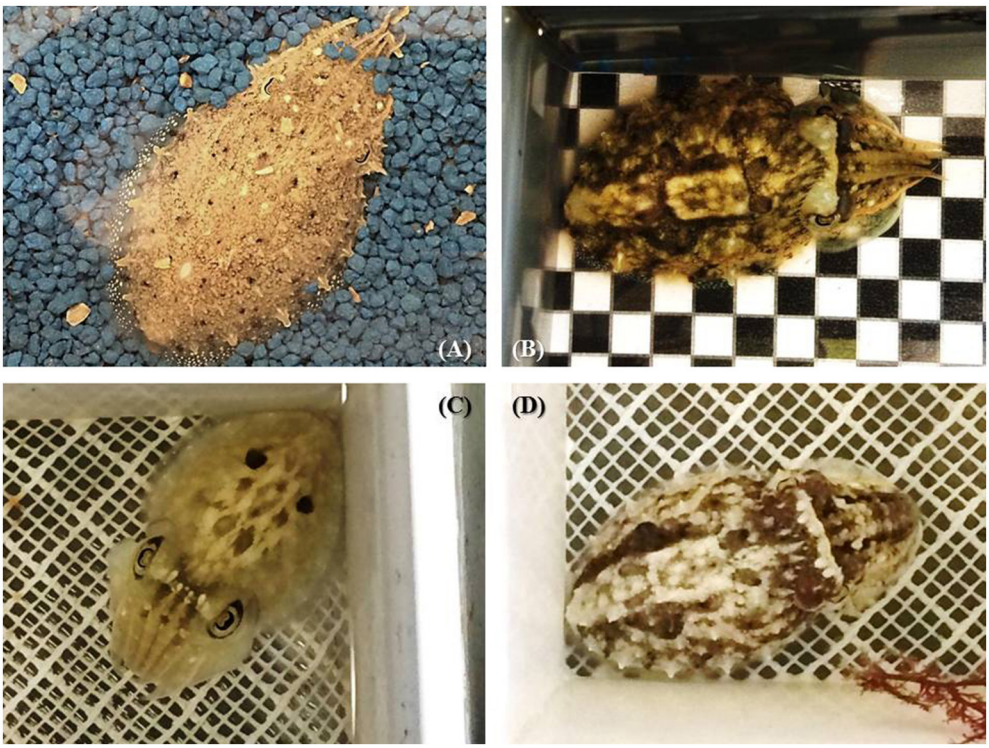

FIGURE 5 | The diversity of body patterns displayed by 2-month-old cuttlefish (ca. 3–4 cm dorsal mantle length). **(A)** stipple-uniform pattern elicited on uniform blue gravel; **(B)** disruptive pattern elicited on a black and white checkerboard combined with mottle pattern; **(C)** deimatic pattern following exposure to a "threat" **(D)** mottle coloration with some components of the disruptive pattern (i.e., white square, white head bar, and paired black dots). Note that patterns are not always fully expressed but exist in combination with others and may or may not directly reflect the visual background.

Figures 5B,C,D), making camouflage "efficiency" very difficult to define or measure (see discussion in Hanlon et al., 2009). Last, in adults, body patterning plays a large role in intra-specific signaling, especially in agonistic and courtship behavior (Hanlon and Messenger, 1988). While social interaction between hatchlings appears to be non-existent (see Holmes, 1940; Hanlon and Messenger, 1996), it is still possible that body patterning also plays a role in signaling between young cuttlefish. This remains unclear as inter-individual communication has never carefully investigated in juvenile cuttlefish, and scarcely even in adults (see Boal et al., 2004).

Functional chromatophores first appear *in ovo* during stage 25 of embryonic development, when the dorsal mantle length of the animal is about 2 mm (Bonnaud-Ponticelli and Boletzky, 2016). While the total number of chromatophores increases with age, their density progressively decreases from 400 to 500/mm^2 at hatching to 35 to 50/mm^2 in adults (Hanlon and Messenger, 1988). Nevertheless, both juveniles and adults possess a high density of cells that allow them to express an infinite range of gradations of various components of their body patterns, depending on background and lighting (Hanlon and Messenger, 1988). Thirteen "typical" body patterns have been identified in adults, but since the body patterning related to sexual behavior is absent in juveniles, the number of color, postural-kinetic, and structural components is lower—only nine distinct patterns (Hanlon and Messenger, 1988). Qualitative changes in body patterning also occur in juveniles. For example, when a late juvenile (about > 6 weeks) or adult is threatened by a small predator, it often displays a "deimatic pattern" in an attempt at intimidation: it flattens its body and flashes two big spots against a white dorsal mantle in a manner resembling eyes (**Figure 5C**). In younger animals, this pattern appears very rarely (Thorpe, 1963; Hanlon and Messenger, 1988), and though the postural components are the same as in adults they flash not two but six dark spots (Hanlon and Messenger, 1988; Mangold, 1989) until about 2 weeks of age. While this version of the deimatic display is used sometimes, newly-hatched cuttlefish are more likely to respond to potential danger with a general darkening or blanching of its body or a cryptic flamboyant display (Hanlon and Messenger, 1988).

One wonders whether body patterning development in juvenile cuttlefish is rigidly fixed or is more influenced by prior individual experience. Simple observations of body patterning in early juveniles speak to this question: when placed on the same background different individuals display different body patterns, suggesting that the response is partially determined by previous experience. Other anecdotal and experimental evidence has the opposite implication however. Hanlon and Messenger (1988) released young cuttlefish (from <1 to 17 weeks of age) previously reared in captivity into the field

and observed that they concealed themselves effectively against every substrate encountered and were extremely difficult to see by human observers. Unfortunately, the personal histories of individuals were not described (i.e., whether they were reared in groups or in isolation, the amount of time spent in the wild before the behavioral observations, etc.), so we cannot make any definitive conclusions. Still, this observation suggests that body patterning development could be hard-wired since the impoverished artificial conditions of rearing do not seem to have any deleterious effects on the concealment skills in juveniles.

More controlled experiments also support an innate origin. Cuttlefish were reared in either "impoverished" conditions (housed individual tanks on a dark uniform background) or in "enriched" conditions (housed in groups in a variegated environment with sand, stones, shells, and artificial seaweeds) for 2 months (Poirier et al., 2005). Later, individuals from each group were tested on either a uniform gray substrate or checkered black and white background. In juveniles, a uniform background should elicit a uniform or slightly mottled body pattern (but see discussion in Hanlon et al., 2009), while a disruptive color pattern seems most adaptive against a contrasted background. The authors then assessed camouflage efficiency of by measuring the hue and intensity of various components of body patterning, on both uniform and contrasted substrates. At hatching, many cuttlefish display disruptive patterning regardless of background type. But starting at 15 days of age, cuttlefish previously reared in enriched conditions were better able to match both background types. Cuttlefish raised in enriched conditions also had greater cell proliferation in the optic lobes than those of cuttlefish from impoverished conditions. This makes sense, as the optic lobes are key structures controlling body patterning in cephalopods (Nixon and Young, 2003). Further evidence for greater innate or "hard-wired" control of body patterning comes from experiments with potential predators, in which *S. officinalis* was found to show the deimatic pattern toward small, low-threat teleost fish but not toward larger more dangerous predators such as sea bass or small sharks (Langridge et al., 2007; Langridge, 2009). Moreover, these reactions occur the first time such threats are encountered, suggesting innate recognition of threat type.

While the preponderance of evidence suggests that body patterning is preprogrammed the fact that different individuals may use a different concealment strategies when placed in the same environment (Poirier et al., 2004), suggest some amount of experience-dependence, potentially through learning and phenotypic plasticity, although we cannot rule out the possibility that these inter-individual differences are the result of genetic history or parental experience. These data lead us to conclude that body patterning in cuttlefish is definitely not a simple stimulus-response process, as it is commonly presented in the literature. It probably involves a complex integration of visual information, genetic history and individual experience (West-Eberhardt, 1989), possibly even before hatching (**Figure 6**). Thus, further investigation of body pattern development could lead to insight not only about camouflage and defense, but also to a better understanding of learning, plasticity, decision making and higher-order cognitive processes in cephalopods (Vitti, 2012; Skelhorn and Rowe, 2016).

FIGURE 6 | Stage 30 embryo (less than 1 cm) showing a mottle-disruptive coloration inside the egg. It has also squirted ink; note the cloud of ink in the perivitellin fluid. Note that the embryo is seen from under through a peeled *S. officinalis* egg (photo C.E. O'Brien).

CONCLUSION: EMBRYONIC ECOLOGY

In this review, we discussed the fact that the visual system is functional well before hatching, as indicated by indirect evidence from embryonic visual learning. By stage 25, the embryo's eyes are mature enough to perceive light and also to discriminate stimulus shape, movement and brightness. Unfortunately, little is known about the direct response of embryos to such stimulations and about the development of the brain structures that process visual information in cuttlefish, namely the optic lobes. The fact that cuttlefish are able to attend to and learn from their biotic and abiotic environment during the final stages of their embryonic development from the relative safety of their egg suggests that prenatal learning plays a large facilitative role in finding food and shelter after hatching. This ability may also enable prenatal social learning. Eggs are laid in clusters, and as a consequence, embryos are likely to be able see each other during development. Social rearing conditions after birth are known to have strong effects on growth and memory (Dickel et al., 2000), so the possibility of prenatal effects exists. No studies have yet addressed this, and experiments to test the effect of embryonic development in isolation on postembryonic behavior are needed.

Many questions about the development of vision in cuttlefish remain to be explored. For instance, do females actively choose their egg-laying site in order to increase offspring learning and survival (i.e., non genetic maternal effects)? Cuttlefish reproduce only once in their lifetime and hence, have only a single opportunity to produce offspring. This, combined with the potential for juvenile behavior to be shaped by embryonic learning, implies that strong selection pressure (based on the presence of predators, shelters or prey for juveniles) is exerted on females' decision. Since it has long been assumed that invertebrate behaviors are mostly genetically programmed, attention should be paid to such previously-neglected effects.

This synthesis highlights the importance of vision in embryo and juvenile cuttlefish behaviors. However, like other animals, cuttlefish live in a multisensory world, and even if vision appears predominant, their behaviors may be influenced by other senses. In most animals, the senses are not equal in their ability to provide accurate information about the environment (Bremner et al., 2012). For example, in a turbid environment, relying only on vision may be risky, and other senses may play a greater role. Komak et al. (2005) have demonstrated that young cuttlefish are sensitive to local water movements thanks to specialized cells on the arms and the head that are analogous to the lateral lines of fish. Water movement detected by these cells could alert cuttlefish to the presence of prey or predators before it is possible to see them. The importance of particular senses may also vary throughout the life of an individual. In cuttlefish, given the opacity of the egg capsule, the sensory world of embryos is probably dominated by chemosensory information. This likely changes as soon as the cuttlefish leaves the egg. Assessing the relative importance of vision and its interactions with the other senses through multimodal perception in different situations and at different ages offers exciting new tracks of research such as prey and predator recognition through visual and/or chemical information.

AUTHOR CONTRIBUTIONS

All authors read and approved this version of the ms; ASD, wrote the main part of the article; NM, wrote the section about embryonic responses; LD, wrote the section about body patterns; CEO, co-wrote the section about embryonic behavioral response and copy-edited the ms.

FUNDING

This review is funded by the French national agency for research (ANR), grant Presto'cog #ANR-13-BSV7-0002-01.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 12 December 2016
doi: 10.3389/fphys.2016.00620

Lateralization of Eye Use in Cuttlefish: Opposite Direction for Anti-Predatory and Predatory Behaviors

Alexandra K. Schnell[1]*, *Roger T. Hanlon*[2], *Aïcha Benkada*[1] and *Christelle Jozet-Alves*[1]

[1] Normandie University (UNICAEN), Caen, France, [2] Program in Sensory Physiology and Behavior, Marine Biological Laboratory (MBL), Woods Hole, MA, USA

Vertebrates with laterally placed eyes typically exhibit preferential eye use for ecological activities such as scanning for predators or prey. Processing visual information predominately through the left or right visual field has been associated with specialized function of the left and right brain. Lateralized vertebrates often share a general pattern of lateralized brain function at the population level, whereby the left hemisphere controls routine behaviors and the right hemisphere controls emergency responses. Recent studies have shown evidence of preferential eye use in some invertebrates, but whether the visual fields are predominately associated with specific ecological activities remains untested. We used the European common cuttlefish, *Sepia officinalis*, to investigate whether the visual field they use is the same, or different, during anti-predatory, and predatory behavior. To test for lateralization of anti-predatory behavior, individual cuttlefish were placed in a new environment with opaque walls, thereby obliging them to choose which eye to orient away from the opaque wall to scan for potential predators (i.e., vigilant scanning). To test for lateralization of predatory behavior, individual cuttlefish were placed in the apex of an isosceles triangular arena and presented with two shrimp in opposite vertexes, thus requiring the cuttlefish to choose between attacking a prey item to the left or to the right of them. Cuttlefish were significantly more likely to favor the left visual field to scan for potential predators and the right visual field for prey attack. Moreover, individual cuttlefish that were leftward directed for vigilant scanning were predominately rightward directed for prey attack. Lateralized individuals also showed faster decision-making when presented with prey simultaneously. Cuttlefish appear to have opposite directions of lateralization for anti-predatory and predatory behavior, suggesting that there is functional specialization of each optic lobe (i.e., brain structures implicated in visual processing). These results are discussed in relation to the role of lateralized brain function and the evolution of population level lateralization.

Keywords: eye use, functional lateralization, cephalopod, invertebrate, brain specialization, vision

OPEN ACCESS

Edited by:
Daniel Osorio,
University of Sussex, UK

Reviewed by:
Richard Payne,
University of Maryland, USA
Andrew C. Mason,
University of Toronto Scarborough, Canada

***Correspondence:**
Alexandra K. Schnell
alex.k.schnell@gmail.com

Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *12 September 2016*
Accepted: *28 November 2016*
Published: *12 December 2016*

Citation:
Schnell AK, Hanlon RT, Benkada A and Jozet-Alves C (2016) Lateralization of Eye Use in Cuttlefish: Opposite Direction for Anti-Predatory and Predatory Behaviors. Front. Physiol. 7:620. doi: 10.3389/fphys.2016.00620

INTRODUCTION

Vertebrates with laterally placed eyes typically show preferential eye use for ecological activities including scanning for potential predators (Franklin and Lima, 2001; Koboroff et al., 2008; Lustig et al., 2010; Martin et al., 2010) or searching for prey (Mench and Andrew, 1983; Robins and Rogers, 2004; Ventolini et al., 2005; Bonati et al., 2008). Processing visual information predominately through the left or right visual field has been associated with specialized function of the left and right brain (i.e., lateralized brain function; Rogers et al., 2013). Many lateralized vertebrates share a general pattern at the population level, whereby the left-brain hemisphere attends to routine behaviors (i.e., processing relevant stimuli), while the right-brain hemisphere attends to emergency responses (i.e., flight or escape responses; MacNeilage et al., 2009).

Lateralization of brain function has been associated with several cognitive advantages, including increasing neural capacity, by avoiding the duplication of functions in the two brain hemispheres (Levy, 1977). Lateralized individuals can also process information in parallel (Rogers, 2002; Rogers et al., 2004), by utilizing one hemisphere to control specific functions (Andrew, 1991; Vallortigara, 2000) and leaving the other hemisphere free to control different functions. Moreover, controlling different functions through separate hemispheres may prevent interference between conflicting responses (i.e., functional incompatibility). That is, responses evoked by stimuli that have been perceived simultaneously, whereby each stimulus demands a different response (Ingle, 1973; Vallortigara et al., 1999; Güntürkün et al., 2000; Vallortigara, 2000).

Recent studies have provided evidence of preferential eye use in invertebrate taxa, including molluscs and insects (reviewed in Frasnelli, 2013). For example, individual common octopuses, *Octopus vulgaris,* showed a significant eye preference when inspecting potential prey items (Byrne et al., 2002) and when exploring novel objects (Byrne et al., 2006). A study on European common cuttlefish, *Sepia officinalis*, showed significant left eye preference when looking for shelter (Jozet-Alves et al., 2012a). The strength of this eye preference was correlated with asymmetries in the optic lobes and vertical lobe, the primary visual processing center and multi-sensory integrative center, respectively (Jozet-Alves et al., 2012b). These studies suggest that invertebrates may predominantly use the left or right visual field to process information for specific ecological activities.

Like most coleoids (i.e., soft-bodied cephalopods), cuttlefish have laterally placed eyes and keen visual acuity. They are voracious visual predators that feed on a range of prey items (i.e., fish and crustaceans) using multiple predatory tactics including ambush predation and active hunting (Neill and Cullen, 1974; Hanlon and Messenger, 1996). Actively hunting for prey makes these soft-bodied invertebrates vulnerable to predators including dolphins, seals, sharks, and many teleost fishes as well as diving seabirds. The active predatory lifestyle of cuttlefish combined with the need to maintain a constant vigilance against predators requires effective information processing from multiple stimuli. Processing information in this way might be more efficient if each visual field is predominately used for specific functional roles, as seen in lateralized domestic chicks, *Gallus gallus*. Lateralized chicks are able to search for grain on a mixed substrate using their right eye (i.e., left brain hemisphere; Rogers, 1990), while simultaneously monitoring overhead for aerial predators using their left eye (i.e., right brain hemisphere; Rogers, 2000). This ability to search for food and monitor predators simultaneously may contribute to biological fitness. Indeed, previous studies have shown that lateralized individuals can outperform non-lateralized conspecifics in some biological circumstances (McGrew and Marchant, 1999; Güntürkün et al., 2000; Rogers et al., 2004). However, whether lateralization of brain function is also associated with cognitive advantages in invertebrate species is yet to be investigated.

In the present study, we conducted lateralization experiments on laboratory-reared European common cuttlefish to test whether they shared similar attributes of lateralization with vertebrates. As cuttlefish have been shown to favor the left eye when searching for shelter (i.e., a defensive behavior; Jozet-Alves et al., 2012a), we hypothesized that the left eye is implicated in emergency responses, while the right eye may be implicated with routine behaviors. To determine whether the left visual field is indeed associated with emergency responses, we tested whether the left eye was predominately used for scanning for potential predators (i.e., vigilant scanning). To determine whether the right visual field is associated with routine behaviors, we tested whether the right eye was predominately used for scanning for potential prey (i.e., prey attack). To investigate vigilant scanning we conducted a laboratory experiment in which individuals were introduced into a new environment and required to choose between the left or right visual field to use for scanning for potential predators. To investigate prey attack we presented individuals with a prey item in each visual field and required them to choose between attacking one prey item to the left or to the right of them. A further aim of the study was to determine whether lateralized individuals exhibited faster decision-making compared to non-lateralized individuals when they were simultaneously presented with two shrimp. We posed three main questions (1) Do cuttlefish show lateralization of vigilant scanning and prey attack? (2) If individuals exhibit visual lateralization, do cuttlefish have opposite directions of lateralization for vigilant scanning and prey attack? (3) Do lateralized cuttlefish show faster decision-making when presented with prey simultaneously?

MATERIALS AND METHODS

Animals

Ninety-three sub-adult European common cuttlefish were used in this study, ranging in age from 7 to 10 months. For experiment 1, two populations of cuttlefish were used, the first population ($N = 10$) was reared from eggs in the Grand Aquarium de Saint Malo, France (48°38′N, 2°00′W), and the second population ($N = 83$) was reared from eggs in the Marine Biological Laboratory (MBL), Marine Resources Center, Woods Hole, USA (41°31′N, 70°39′W). All the eggs were collected from the English Channel; eggs for Saint Malo were gathered along the coast of Brittany,

while eggs for Woods Hole were gathered along the southern coast of England. For experiment 2, cuttlefish from experiment 1 ($N = 72$) in the MBL Marine Resources Center were re-used. Dorsal mantle lengths were measured (mean mantle length ± SEM = 44.16 ± 1.08 mm; range = 31–60 mm). Throughout these experiments, subjects were housed in groups in tanks at their respective facilities (i.e., Grand Aquarium and MBL Marine Resources Center). Tanks were supplied with a constant flow of filtered seawater (∼10 L min^{-1}) and maintained at a temperature of 15–17°C. Cuttlefish were maintained under daylight conditions and were fed a mixed diet of food items *ad libitum* including, thawed frozen prawn, smelt, *Osmerus eperlanus*, live eastern grass shrimp, *Palaemonetes paludosus*, and live gammarid shrimp, *Platorchestia platensis* (Krøyer, 1845). Subjects were used in several non-invasive experiments and were housed for the remainder of their life cycle (i.e., ∼1 year) until they died following senescence. All applicable, international, national, and/or institutional guidelines for care and use of animals were followed. Procedures undertaken in France were approved by the regional ethical committee (Comité d'Ethique Normandie et Matiére d'Expérimentation Animale, CENOMEXA; agreement number 54). Ethical approval was not required for the experiments conducted at MBL as there are currently no ethical regulations in place for research on cephalopods in the USA.

Test Apparatus

For experiment 1, for the Saint Malo population we used a rectangular arena 800 × 300 × 400 mm ($l \times w \times h$), while for the Woods Hole population we used a circular arena, 260 × 90 mm (*diameter* × *h*) constructed from gray PVC (**Figure 1A**). A digital video camera (Sony VX-1000) was placed directly over each arena to record the vigilant scanning behavior of cuttlefish over a period of 120 min. For experiment 2, we used an isosceles triangular arena, 287 × 400 mm (*h* × *base*) constructed from gray PVC (**Figure 1B**). A semi-circular gray PVC barrier was placed at the apex of the arena to visually isolate subjects from the prey items and allow the cuttlefish to settle into the apex during the first phase of the test. In the two opposite vertexes, we placed a dead shrimp within a glass vial 20 × 100 mm (*diameter* × *h*). The top of each glass vial surpassed the water level in the arena, preventing chemical exchange between the prey items, and the subject. Each shrimp was supported by a metal rod, which was attached to a horizontal pole controlled by a Boekel rocker (Rocker II, model 260350, Boekel Scientific) to simulate the movement of live shrimp.

Each apparatus was illuminated by a LED strip light (I Daylight White 3528 Double Row LED, 240/m, 15 mm wide), which was placed within plastic tubing and positioned 500 mm above the center of the arena. The arenas were surrounded by black plastic walls to eliminate external cues and were supplied with a constant flow of fresh filtered seawater (95 mm deep).

Test Procedure

Experiment 1 was carried out in April 2015 in France and April 2016 in the USA. Subjects were placed individually in the arena and allowed to move freely around the apparatus. The set-up aided in determining where cuttlefish vigilance was being directed. Previous research on cuttlefish in laboratory tanks has demonstrated that they avoid open environments when they cannot bury themselves and typically align their body against an opaque surface or object (i.e., wall or rock; Alves et al., 2007). This requires them to choose which eye to orient away from the opaque arena to scan for potential predators. The panoramic field of vision of most cephalopods ensures that a large volume of water can be searched using a single visual field (Hanlon and Messenger, 1996). We are confident that this behavior is driven by the desire to scan for predators because it is typically performed when cuttlefish are introduced to open environments with predatory fish odor (i.e., gray mullet, *Mugil cephaus*; unpublished data). The orientation of individuals relative to the arena wall provided an indication of eye preference. For example, cuttlefish with a left eye preference would typically orientate the right side of their body against the arena wall and use their left eye for vigilant scanning (**Figure 1A**). The opposite situation held for individuals with a right eye preference. Each individual was video recorded for a 120 min period and eye preference was documented every 5 min within that period (i.e., 24 trials per

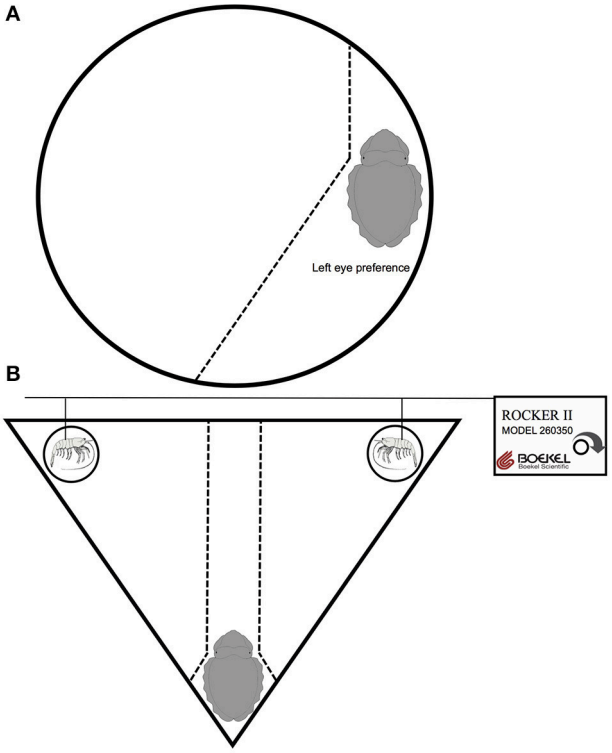

FIGURE 1 | Diagrammatic representation of the experimental apparatus for investigating (A) anti-predatory behavior and **(B)** predatory behavior in European common cuttlefish, *Sepia officinalis*. **(A)** Depicts a cuttlefish with a left eye preference for vigilant scanning. The idealized field of view is represented by the dotted line. **(B)** Depicts a cuttlefish settled in the apex of the isosceles triangle arena and two dead shrimp, in glass vials, in opposite vertexes. Prey movement was controlled by singular metal rods attached to a horizontal pole controlled by a Boekel rocker to simulate the movement of a live shrimp. Objects are not drawn to scale.

individual). When individuals were not aligned against the arena wall the trial was omitted because clear eye preference was not detectable, hence some individuals participated in less than 24 trials (range: 12–24 trials).

Experiment 2 was carried out in May–June 2016. Dead eastern grass shrimp of similar size were placed within the glass vials at each vertex and the Boekel rocker was turned to the highest motion level. Cuttlefish were placed individually in the apex of the triangular arena with the semi-circular gray barrier in place to visually isolate them from the prey items. Each cuttlefish was allowed to acclimate to the new arena for a minimum of 15 min. The barrier was removed once the cuttlefish settled with the posterior end of its mantle in the point of the apex and its head facing the barrier, a position that most animals assumed within 5–25 min. Once the barrier was removed the left and right visual field of the cuttlefish were simultaneously exposed to a dead shrimp. Cuttlefish with a right eye preference would attack the dead shrimp in the right vertex and cuttlefish with a left eye preference would attack the dead shrimp in the left vertex. As soon as the subject attacked one of the glass vials, the cuttlefish was gently lifted out of the water using a small glass beaker and placed back in its home tank. If an individual did not attack either prey item within 5 min, it would be returned to its home tank and tested again the following day. This procedure was repeated once per day for each individual until they reached 10 choices. Dead eastern grass shrimp were replaced each day. We attempted to reduce uncontrolled external cues as a source of bias by rotating the arena 90° between each day of experimentation (i.e., *four* possible orientations of the apparatus, with the same number of subjects tested with each possible orientation).

Data Analysis

To determine the direction of eye preference for experiments 1 and 2, we converted the eye use data for each individual to a laterality index (LI; Bisazza et al., 2000). To calculate the LI, we used the following formula: (Number of trials where the individual used the right eye – Number of trials where the individual used the left eye)/(Total number of trials). LI is a continuous variable that ranges from −1 to +1. A left eye preference was indicated by a significantly negative value; a right eye preference was indicated by a significantly positive value. To analyse the strength of the eye preference, regardless of the direction, we also calculated the absolute value of LI. A value of 0 meant that an individual used its left and right eye equally; a value of 1 meant that an individual consistently used the same eye.

All statistical analyses were completed using R (version 2.9.0, http://www.r-project.org). We used parametric tests as well as non-parametric tests, when data did not meet the assumption of normality and homoscedasticity. To test for eye preference in each individual for both ecological activities (i.e., vigilant scanning, and prey attack), we used binomial tests. We then calculated the percentage of cuttlefish showing a left eye preference, right eye preference, or no preference for both vigilant scanning and prey attack. To compare the number of cuttlefish with a left and a right eye preference, we used a Chi-square test. To determine whether cuttlefish showed an eye preference at the population level for each ecological activity, we tested the overall LI values using a one-sample Wilcoxon test on the Saint Malo population and one-sample *t*-tests on the Woods Hole population. To test whether the mean LI differed between the two populations for vigilant scanning (i.e., Saint Malo and Woods Hole), we used exact permutation tests for independent samples. To test whether left biased cuttlefish were more strongly lateralized than right biased cuttlefish, we also used exact permutation test for independent samples for the Woods Hole population for both ecological activities. We also used one-sample Wilcoxon tests to determine eye preference for prey attack in individuals that were categorized previously as left, right or no preference for vigilant scanning. To test whether decision-making latencies during prey attack differed between lateralized and non-lateralized individuals, we used a generalized linear mixed model (GLMM). Lateralization was the predictor variable and latency (log transformed) was the dependent variable with subject as a random factor.

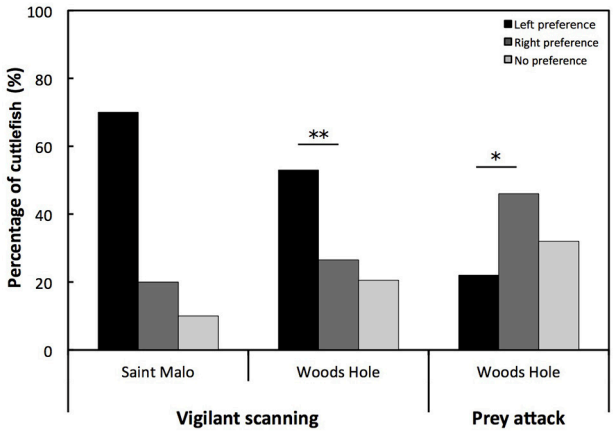

FIGURE 2 | Eye preference for vigilant scanning and prey attack. Percentage of European common cuttlefish, *Sepia officinalis* that exhibited left eye preference, right eye preference, and no preference for vigilant scanning and prey attack. Bars marked with a solid line and accompanying asterisks represent results of a Chi-square test and signify a population level eye preference for lateralized cuttlefish. *$p < 0.05$; **$p < 0.01$.

RESULTS

Cuttlefish were categorized as left, right, or no preference (**Figure 2**). The number of cuttlefish with a left eye preference for vigilant scanning was higher than the number of cuttlefish with a right eye preference in the Woods Hole population [Chi-square: $\chi^2_{(1,\,N=66)} = 7.333$; $p < 0.01$; **Figure 2**]. This was only conducted for the Woods Hole population as we were prevented from applying a Chi-square analysis on the Saint Malo population due to a low sample size. By contrast, the number of cuttlefish with a right eye preference for prey attack was higher than the number of cuttlefish with a left eye preference [Chi-square: $\chi^2_{(1,\,N=49)} = 5.898$; $p < 0.05$; **Figure 2**].

For the Saint Malo population, there was a statistical tendency for a population level left eye preference for vigilant scanning

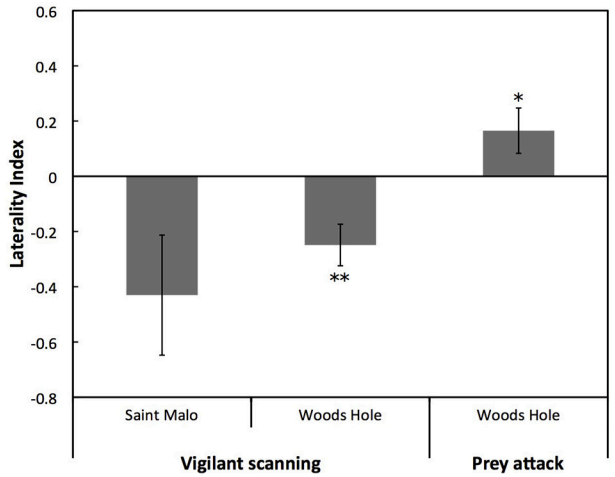

FIGURE 3 | Lateralization for vigilant scanning and prey attack. Mean ± *SE* laterality index for vigilant scanning and prey attack in laboratory reared European common cuttlefish, *Sepia officinalis*. Negative results represent left eye preference and positive results represent right eye preference. Bars marked with asterisks represent results of one-sample *t*-tests and indicate population level eye preferences that differed significantly from 0. Significant differences between groups are indicated by a solid line and accompanying asterisks. $^{*}p < 0.05$; $^{**}p < 0.01$.

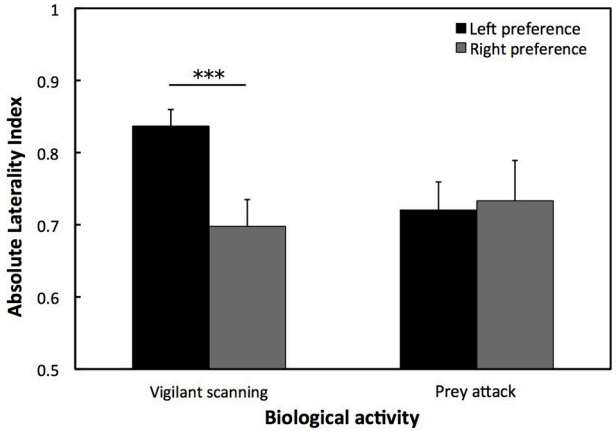

FIGURE 4 | Strength of lateralization for vigilant scanning and prey attack. Mean ± *SE* absolute laterality index for lateralized European common cuttlefish, *Sepia officinalis* in Woods Hole. A solid line and accompanying asterisks represent results of exact permutation tests and indicates a significant difference between left and right eye preference. $^{***}p < 0.001$.

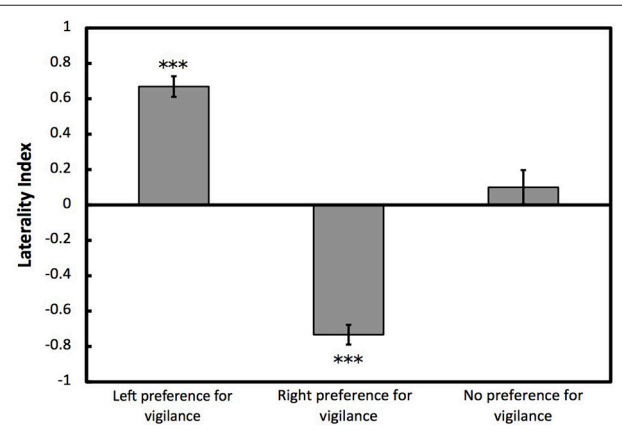

FIGURE 5 | Lateralization for prey attack for categorized individuals. Mean ± *SE* laterality index for prey attack for European common cuttlefish, *Sepia officinalis* that had previously been categorized as left, right, or no preference for vigilant scanning. Negative results represent left eye preference for prey attack and positive results represent right eye preference prey attack. Bars marked with asterisks represent results from one-sample Wilcoxon tests. $^{***}p < 0.001$.

($W = 10$, $p = 0.083$). Moreover, for the Woods Hole population, there was a significant population level left eye preference for vigilant scanning [$t_{(82)} = 14.136$, $p < 0.001$; **Figure 3**]. By contrast, there was a significant population level right eye preference for prey attack [$t_{(71)} = 2.156$, $p < 0.05$; **Figure 3**]. There was no significant difference of the overall LI for vigilant scanning between the two populations (exact permutation: $T = -21.135$, $p = 0.446$; **Figure 3**). For the Woods Hole population, comparison of absolute LI values of cuttlefish with a left and a right eye preference for vigilant scanning showed that the bias was stronger in cuttlefish displaying a left eye preference (exact permutation: $T = 2237$, $p < 0.001$; **Figure 4**). However, a stronger bias was not shown for prey attack: comparisons of absolute LI values of cuttlefish with a left and a right eye preference showed no significant difference (exact permutation: $T = 1620$, $p = 0.907$; **Figure 4**).

To determine whether cuttlefish showed opposite directions of lateralization for vigilant scanning and prey attack, we used one-sample Wilcoxon tests on overall LI for prey attack for individuals that had previously been categorized as left, right or no preference for vigilant scanning. Cuttlefish categorized previously as left preference for vigilant scanning showed a significant right bias for prey attack ($W = 1.5$, $p < 0.001$; **Figure 5**). Cuttlefish categorized previously as right preference for vigilant scanning showed a significant left bias for prey attack ($W = 780$, $p < 0.001$). Cuttlefish that were categorized previously as having no preference for vigilant scanning did not show any eye preference for prey attack ($W = 15$, $p = 0.395$; **Figure 5**). Lateralized cuttlefish (i.e., individuals exhibiting either left or right preference for prey attack) attacked shrimp faster than non-lateralized cuttlefish (GLMM: $\chi^2 = 5.859$, $p < 0.05$; **Figure 6**).

DISCUSSION

Our study provides behavioral evidence of lateralization of brain function in the European common cuttlefish. We found that most cuttlefish exhibited lateralization for vigilant scanning and prey attack. Cuttlefish were lateralized at the population level; that is, most cuttlefish were significantly more likely to favor the left visual field to scan for potential predators. This pattern is comparable to previous studies on lateralization of

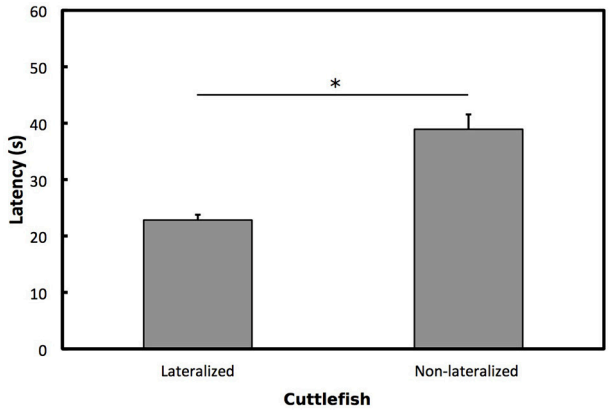

FIGURE 6 | Prey attack latencies. Mean ± *SE* latencies for lateralized and non-lateralized European common cuttlefish, *Sepia officinalis.* Bars marked with a solid line and accompanying asterisks represent results from a GLMM and indicate a significant difference between groups. *$p < 0.05$.

anti-predatory behavior, revealing that cuttlefish exhibit a left eye preference when seeking shelter (Jozet-Alves et al., 2012b). Our data also demonstrate that cuttlefish were significantly more likely to favor the right visual field for prey attack. Furthermore, cuttlefish that were leftward directed for vigilant scanning were predominately rightward directed for attacking prey. The opposite situation held for individuals with a rightward preference for vigilant scanning. This indicates that cuttlefish have opposite directions of lateralization for vigilant scanning and prey attack. Lateralized individuals also showed faster decision-making when presented with prey simultaneously, suggesting that lateralized cuttlefish may have a cognitive advantage over non-lateralized conspecifics.

The behavioral evidence for lateralization of eye use in cuttlefish suggests that there is associated specialized brain function. Our results demonstrate that cuttlefish have opposite directions of lateralization for emergency responses (i.e., vigilant scanning and shelter) and routine behaviors (i.e., prey attack). Behavioral lateralization in vertebrate taxa is considered to be a consequence of specialized function of the left and right brain hemisphere. However, cephalopods do not have obvious left and right brain hemispheres, but they do exhibit paired structures of the central nervous system. These paired structures include the optic lobes, which are implicated *inter alia* in visual processing and located behind the eyes (Nixon and Young, 2003). Previous research has shown that individual cuttlefish exhibit anatomical asymmetries in the size of the left and right optic lobes and these asymmetries are correlated with behavioral lateralization (Jozet-Alves et al., 2012b). Interestingly, a correlation was also found between an unpaired structure, the vertical lobe, and behavioral lateralization. Cuttlefish with a larger right optic lobe and a vertical lobe with an engorged right side showed a stronger left-turning bias when seeking shelter (Jozet-Alves et al., 2012b). Furthermore, only one side of the cortex of the vertical lobe was activated when the corresponding eye was exposed to light (unpublished data). There are two plausible explanations for these correlations between behavioral and brain asymmetries. First, one part of the brain may be more dominate than its counterpart, which may explain why cuttlefish favored their left eye when searching for shelter (Jozet-Alves et al., 2012a). Second, the brain is specialized, whereby each side of the brain predominately processes information for specific ecological activities. Our results support the latter notion as cuttlefish in our study used specific visual fields for vigilant scanning and prey attack. The use of both left and right visual fields for particular ecological activities suggests that one part of the brain is not dominate over the other, rather there appears to be functional specialization of each optic lobe. Anatomical brain asymmetry has also been observed in another cephalopod species, the deep-sea squid, *Histioteuthis* (Wentworth and Muntz, 1989). In this species, individuals possess a large left optic lobe, used to look upwards in the water column to potentially detect predators. Conversely, the right optic lobe is considerably smaller and orients downwards to potentially search for prey.

In our study, most individuals showed a similar direction of bias, significantly favoring the left visual field for vigilant scanning and the right visual field for prey attack. This bias indicates that cuttlefish exhibit population level lateralization for these ecological activities. Although brain lateralization is thought to provide benefits such as performing simultaneous tasks more efficiently (i.e., vigilance and foraging; Dadda and Bisazza, 2006), lateralization does not need to be expressed at the population level to attain such benefits. In fact, lateralization at the population level may have some drawbacks, because it makes the behavior of each individual more predictable to other animals (i.e., potential predators or prey; Ghirlanda and Vallortigara, 2004). For example, if cuttlefish predominately used the left visual field to scan for predators, a predator could learn to exploit this bias and always attack from the right. This disadvantage would not occur if the direction of lateralization varied from one individual cuttlefish to another. The social constraint hypothesis has been proposed as a framework for understanding the reason animals exhibit population level biases (Ghirlanda and Vallortigara, 2004). In a prey-predator context, the hypothesis suggests that population level lateralization may have evolved due to social pressures that require individuals to align the direction of their bias with the direction of the other individuals of the group (Vallortigara and Rogers, 2005). This hypothesis has been supported by studies on turning biases of fish when escaping from predators. For example, many shoaling species of fish exhibit population level lateralization for turning behavior, whereas most non-shoaling fish species only show lateralization at the individual level (Bisazza et al., 2000).

Social constraints may influence whether biases occur at the individual level or the population level in cephalopods. Octopus and cuttlefish vary in their degree of sociality, ranging from solitary to aggregating species. Interestingly, solitary common octopuses show significant eye preference when presented with a crab, yet show no population level bias (Byrne et al., 2002, 2004). However, European common cuttlefish, which form loose aggregations (i.e., 3–8 individuals) briefly during reproduction, show a weak population level eye preference (e.g., 55–60%). These lateralization differences across various cephalopod species

deserve further exploration, particularly in studies of brain function.

Comparisons between lateralized cuttlefish that exhibited a left or right eye preference for vigilant scanning showed that the strength of lateralization was stronger in leftward directed cuttlefish. Despite the prevalence of brain lateralization across taxa, there is considerable intraspecific variation in the strength of lateralization. Previous research has shown that in humans, right-handers are more consistent in their hand preference for various tasks compared to left-handers (Oldfield, 1971). In these cases, the bias of the more strongly lateralized individuals is consistent with the population level bias. However, the results obtained from humans are difficult to interpret, as there are potential cultural factors that influence handedness. For this reason, cuttlefish may be a useful model to explore why individuals that exhibit population level biases are more strongly lateralized than their counterparts that have an opposite pattern of specialization.

Our study also showed that when cuttlefish were simultaneously presented with two shrimp, one visible in the left visual field and the other in the right visual field, lateralized individuals exhibited faster decision-making compared to non-lateralized individuals. That is, lateralized cuttlefish showed shorter latencies to prey attack than non-lateralized conspecifics. Lateralized cuttlefish may have an advantage because information is prioritized by one visual field when searching for prey. This is one of the few examples showing that lateralized individuals could have a cognitive advantage over non-lateralized individuals in an invertebrate species (but see also Pascual et al., 2004). Our results provide further evidence that brain lateralization plays an important role in cognitive function and suggests that laterality may lead to fitness consequences for organisms in their natural environments. However, further exploration is needed to determine whether lateralized cuttlefish are more efficient at performing two tasks simultaneously than non-lateralized conspecifics. This can be tested using a dual-task design, to determine whether strength of lateralization is associated with the ability to scan for predators and search for prey simultaneously.

In conclusion, this study demonstrates that cuttlefish share similar attributes of lateralization with many vertebrate species. In fact, the pattern observed in cuttlefish is comparable to the pattern observed in most vertebrate taxa, whereby the left visual field plays a predominate role in emergency responses and the right visual field plays a predominate role in routine behaviors. This suggests that there are strong selective pressures driving general patterns of lateralization across diverse groups of animals. To our knowledge, our study provides the first evidence of lateralization homology between invertebrates and vertebrates (i.e., emergency responses and routine behaviors processed by different parts of the brain).

AUTHOR CONTRIBUTIONS

AS contributed to the conception of the study, the experimental design, the data collection, the statistical analysis, and wrote the manuscript. RH contributed to the conception of the study, the experimental design, the data collection, the analysis and interpretation of the results, and assisted with the writing of the manuscript. AB contributed to the conception of the study, the experimental design, the data collection, the presentation of the results, and assisted with the writing of the manuscript. CJ-A contributed to the conception of the study, the experimental design, the data collection, the analysis and interpretation of the results, and assisted with the writing of the manuscript.

FUNDING

This work was supported by a post-doctoral study grant from the Fyssen Foundation to AS, and by a research grant "Sélavie" from the Fyssen Foundation to CJ-A. The Sholley Foundation provided partial support for the research in Woods Hole.

ACKNOWLEDGMENTS

We thank Yann Guillouzo and the technical staff of the Grand Aquarium de Saint Malo. We also thank the technical staff at the MBL Marine Resources Center.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

PERSPECTIVE
published: 28 March 2017
doi: 10.3389/fphys.2017.00164

Embodied Organization of *Octopus vulgaris* Morphology, Vision, and Locomotion

Guy Levy † *and Binyamin Hochner* *

Department of Neurobiology, Silberman Institute of Life Sciences, Hebrew University, Jerusalem, Israel

Keywords: octopus vulgaris, motor control, soft bodied animal, embodiemnt, Mollusca

OPEN ACCESS

Edited by:
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***Correspondence:**
Binyamin Hochner
benny.hochner@mail.huji.ac.il

†Present Address:
Guy Levy,
Biology Department, Tufts University, Medford, MA, USA

Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *29 November 2016*
Accepted: *06 March 2017*
Published: *28 March 2017*

Citation:
Levy G and Hochner B (2017) Embodied Organization of Octopus vulgaris Morphology, Vision, and Locomotion. Front. Physiol. 8:164. doi: 10.3389/fphys.2017.00164

The rich motor behavior of *Octopus vulgaris* is an outstanding biological example of motor control in a soft-bodied animal. The flexible hyper-redundant arms of the octopus endow it with high maneuverability but also place a great burden on its control system. The main difficulty in using the arms for precise goal-directed movements and coordinated locomotion is the problems of interfacing the incoming sensory information with the issuing of the proper motor commands. Skeletal animals evolved a solution for this interfacing difficulty by employing central "representation maps" that represent the sensory and the motor information in an organization that maintains the spatial relationships of the body morphology (somatotopic representation), yet the relative size of each body part reflects the number of sensory receptors and the number of muscle groups in each of the body parts. Therefore, in our brain, this brain organization resembles a topography of a "little man"—homunculus in Latin. The implication of such topological organization is that in the central brain, (e.g., in our motor and sensory cortices) the sensory and motor activities are represented in "body parts coordinates." This representation format likely serves as a useful "reference table" for the brain to compute feedforward motor commands for motor interaction with the external world. This computational mechanism is feasible because the number of body parts and their dynamic locations with respect to each other is constrained by the limited number of joints and the fixed configuration of the skeleton which limits the number of controlled parameters (i.e., degrees of freedom, DOFs) needed to be computed for the execution of specific movements. Implementing in the octopus a motor control mechanism that is similarly based on body parts representation would be ineffective because of the lack of fixed spatial relationships between the flexible body parts that would require an enormous computational power to calculate the feedforward commands that are needed to control the enormous number of DOFs that are required for computing the coordinated interaction of eight long and flexible arms with the external world. Indeed, the body of the octopus is not represented somatotopically in the higher motor centers (the basal lobes) in the octopus brain (Zullo et al., 2009) and as we describe below, the evolved control algorithms of the arms in goal directed movement and locomotion highlights control strategies that seem to overcome the need for central representation of the body.

Previous and more recent results suggest that the solution for this difficulty has evolved through "embodied evolution" of the octopus unique morphology to enable the nervous system to employ special motor-control strategies that alleviate the need to rely on central body parts representation (reviews: Zullo and Hochner, 2011; Hochner, 2012, 2013).

Here, we first give a short account of the unique mechanisms that have evolved to simplify the control in goal-directed movements, and then present new surprising results that suggest a control

mechanism for coordinating the flexible appendages during locomotion and show how vision of *Octopus vulgaris* is embodied in this novel locomotion control mechanism.

CONTROL OF GOAL-DIRECTED MOVEMENTS

The reaching movement, as first example, is controlled by a motor program that does not depend on body-part coordinates and is essentially a stereotypical movement combination of several motor primitives; the arm is extended toward the target by propagating a bend along it and independently controlling elongation of the arm segment that is proximal to the propagating bend (Gutfreund et al., 1996, 1998; Hanassy et al., 2015). This control strategy reduces the number of DOFs involved in the central control of reaching to only three or four: two DOFs are needed for controlling the direction of the base of the arm, one for the propagation of the stiffening wave that pushes the passive bend forward, and possibly another DOF for controlling the elongation and straightening of the arm. Amputated arms can generate the same typical arm extension when the exposed axonal tract of the arm nerve cord is given a short train of electrical stimulations, indicating that the motor program for generating arm extension is embedded in the peripheral neuromuscular system of the arms (Sumbre et al., 2001).

In goal-directed fetching movements the octopus brings food precisely to its mouth. To do this, the long, flexible arm is "reshaped" into an "articulated," skeletal-like structure of three segments with the proximal and medial segments having similar length and the food is held by the distal segment using a group of suckers (serving as a hand). The food is brought to the mouth by rotating the pseudo-elbow situated between the proximal and media segments. As in our arms, the equal segment lengths simplify the precise reaching of the distal segment to the mouth that is located, in the octopus, at the center of a circle created by the bases of the arms. But, in sharp contrast to articulated skeletal appendages, pseudo-articulations in the octopus are dynamic and are reshaped for each fetching movement and are adjusted according to the holding position of the target along the arm. At first, this seems somewhat puzzling, as it is hard to perceive a simple way for the central nervous system to coordinate such a dynamic structure but remarkably, the octopus uses the arm itself for calculating the site of the pseudo elbow. After contact with the target is made, two waves of muscle activation start traveling toward each other—one propagates from the site of contact with the target proximally along the arm, and the other propagates from the base of the arm distally along it. The elbow is formed where the two waves collide (Sumbre et al., 2006).

The motor programs for these two goal-directed movements are embedded in the neuromuscular system of the arm (Sumbre et al., 2001, 2006). This notion is supported by newer findings showing that the motor programs are represented in the higher motor centers of the octopus brain (Zullo et al., 2009). So at least for some movements, the higher motor centers in the octopus central brain, in contrast to skeletal animals, are involved only in the activation and scaling of peripheral programs and in adjusting the movements according to relevant visual and tactile information by controlling only few DOFs that are involved in their execution.

These results show very clearly that special evolutionary solutions have evolved to cope with the complex motor control problems of goal directed movements in hyper-redundant appendages.

CONTROL OF ARM COORDINATION IN LOCOMOTION

For all of us the way *Octopus vulgaris* is moving swiftly around in the aquarium or in nature seems very elegant and effortless. This should amaze us because it is not simple to perceive a control system that can mediate such locomotion capabilities in a hyper-redundant body that lacks a skeleton. Indeed, *Octopus vulgaris* appears to have evolved unique control mechanisms that enable it to coordinate its eight arms efficiently during various forms of locomotion. The main difficulty in controlling locomotion with the long and flexible appendages of the octopus arises from the fact that they lack any structural constrain. Thus, no type of feedforward control mechanism can be easily implemented in its locomotion (unless, in theory, a supercomputational power could have been integrated in the control). This sharply contrasts the requirements of computational power necessary to control skeletal appendages, where a small number of joints limit the interactions with the environment to a small number of DOFs, making the control of locomotion feasible with repeated rhythmical patterns of motor output generated by rather simple central pattern generators (CPGs). This is a universal control mechanism found in all types of locomotion throughout the animal kingdom.

The first indication that the octopus is a unique exception and lacks CPGs in locomotion control was found by studying arm coordination during crawling (Levy et al., 2015). The octopus crawls by making moment-to-moment *ad hoc* decisions; essentially choosing which of its arm(s) to recruit for pushing the body. A group of suckers on the chosen arm(s) adheres to the substrate, giving an anchoring point for a stereotypical elongation of a proximal segment to generate the thrust. The moment-to-moment direction of crawling is determined by a vectorial summation of the pushing directions of the active arms, where each arm has a single predefined pushing direction that is determined by its position around the body. This calculation is simple because the arms are organized in a radial symmetry around the body and the active arms at each moment in time apply virtually equal pushing forces. As shown in **Figure 1** there is no apparent order in the octopus arm stepping records (C) and Fast Fourier Transform (FFT) analysis of instantaneous crawling velocity did not reveal any characteristic frequencies that would indicate of the presence of a rhythmical CPG, as clearly evident in a similar analysis of insect walking (**Figures 1A,B**, originally adapted from Mendes et al., 2013 and from Graham, 1972, respectively).

We are now investigating the mechanism of arm coordination during several octopus locomotion maneuvers. Again, the results

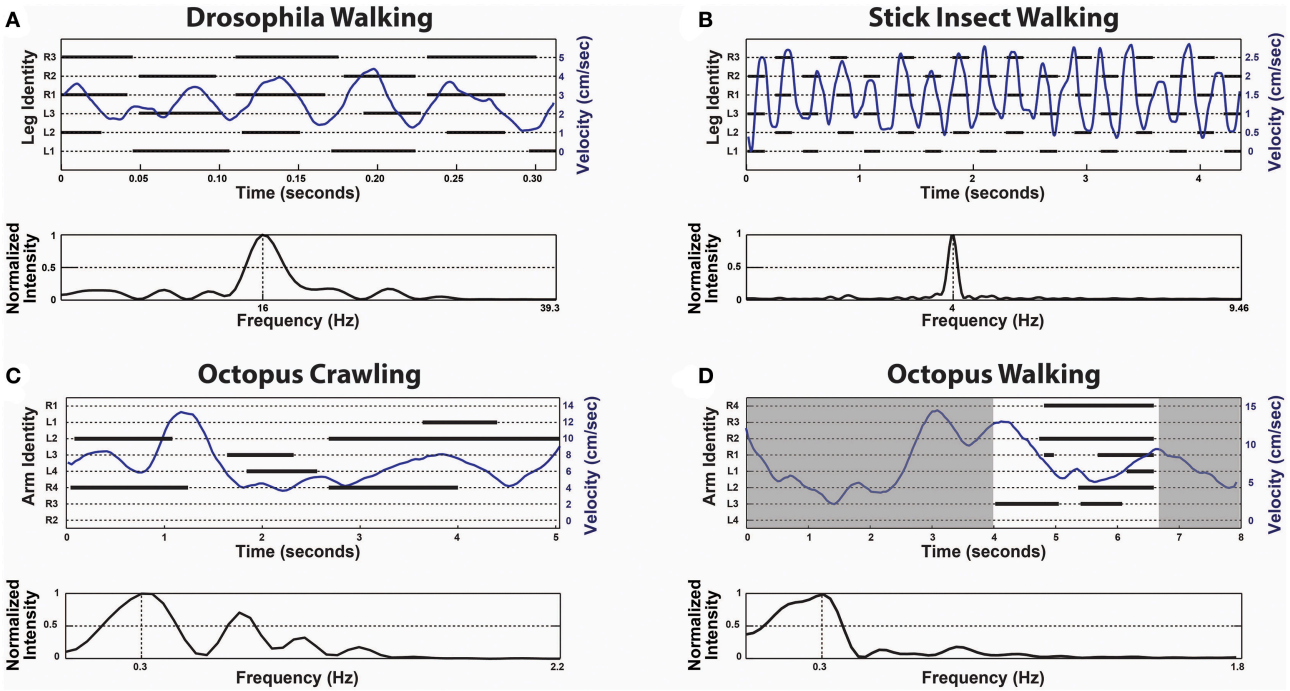

FIGURE 1 | In contrast to the universal role of CPGs in locomotion, octopus locomotion involves *ad hoc* recruitment of the arms interacting with the environment. Upper panels are Stepping Records (black) with the body's instantaneous velocity superimposed (blue) and lower panels give the spectrum of frequencies of the respective velocity extracted by Fast Fourier Transform (FFT). In the upper panel of **(D)** only the time interval between 4 s and about 6.6 s was analyzed (white area) because in the rest of the time there was obscuring of some of the arms. **(A)** Drosophila Walking, originally adapted from Mendes et al. (2013). **(B)** Stick insect (*Carausius morosus*) Walking, originally adapted from Graham (1972). **(C)** Octopus Crawling. **(D)** Octopus Walking. Note the lack of temporal pattern in **(C,D)**. The extracted frequencies of octopus crawling and walking merely reflect the window sizes (for example, the frequency of 0.3 Hz in **(C)** means a cycle every 3.3 s, but the entire movement lasts only 6 s). In contrast, the extracted frequencies of Drosophila and stick insect walking each shows a single prominent characteristic frequency (reflecting the underlying CPGs rhythmicity).

are surprising and further indicative of the existence of unique locomotion control mechanisms.

During various forms of locomotion, octopuses keep their head constantly horizontal (**Figure 2** and see video). This is not surprising because, as in many animals, especially those living outside water, keeping the head in fixed reference to the external world simplifies interfacing the visual information with movement commands that drive the interaction with the external world. Indeed, even the simplest creatures have mechanisms for sensing gravity and cephalopods are known for their highly evolved vestibular system, with a pair of statocysts embedded within the rigid cartilaginous brain capsule (Barber, 1966; Young, 1971; Wells, 1978). This location of the statocysts enables them to gauge directly only the orientation of the head and thus, out of the whole soft body, to keep the head at a fixed orientation to the external world (**Figure 3**). This arrangement simplifies the control because the head and eyes are in a fixed reference to the external world thereby reducing the complexity involved in the interfacing of the external sensory information with the generation of motor commands needed for the interaction with the surrounding.

On the other hand, while keeping the body in a stable posture relative to the force of gravity seems fundamental and simple for animals with a rigid skeleton, it is a much more difficult challenge for an animal with flexible appendages. We find that, as in fetching, the evolved solution is based on "shaping" the soft body instead of controlling joint angles as in skeletal animals. As indicated by the name of their class "Cephalopoda," octopus arms emerge directly from the base of the head around which they are radially distributed. During locomotion, the imaginary axis that runs between the eyes remains close to horizontal (**Figure 2** and Video), implying of an active adjustment of the eyes' height by controlling the distance between the contact points of the active arms with the environment and the base of the head (**Figure 3B**, straight blue lines). This simplifies the controlling of the head's orientation because it is achieved by a straightforward mechanism that only controls the stiffness of the arms (**Figure 3B**). Such stiffens control may involve only one DOF per interacting arm. Because the octopus almost doesn't have a neck (see Wells, 1978), the horizontal visual plane of the eyes cannot move much relative to the base of the head and therefore the interaction of the arms with the environment also keeps, through the "physical feedback" (**Figure 3**), a stable horizontal view of the external world. If this principle is indeed implemented as the biomechanical basis of arm-propelled locomotion, it would imply that octopus locomotion is unlikely

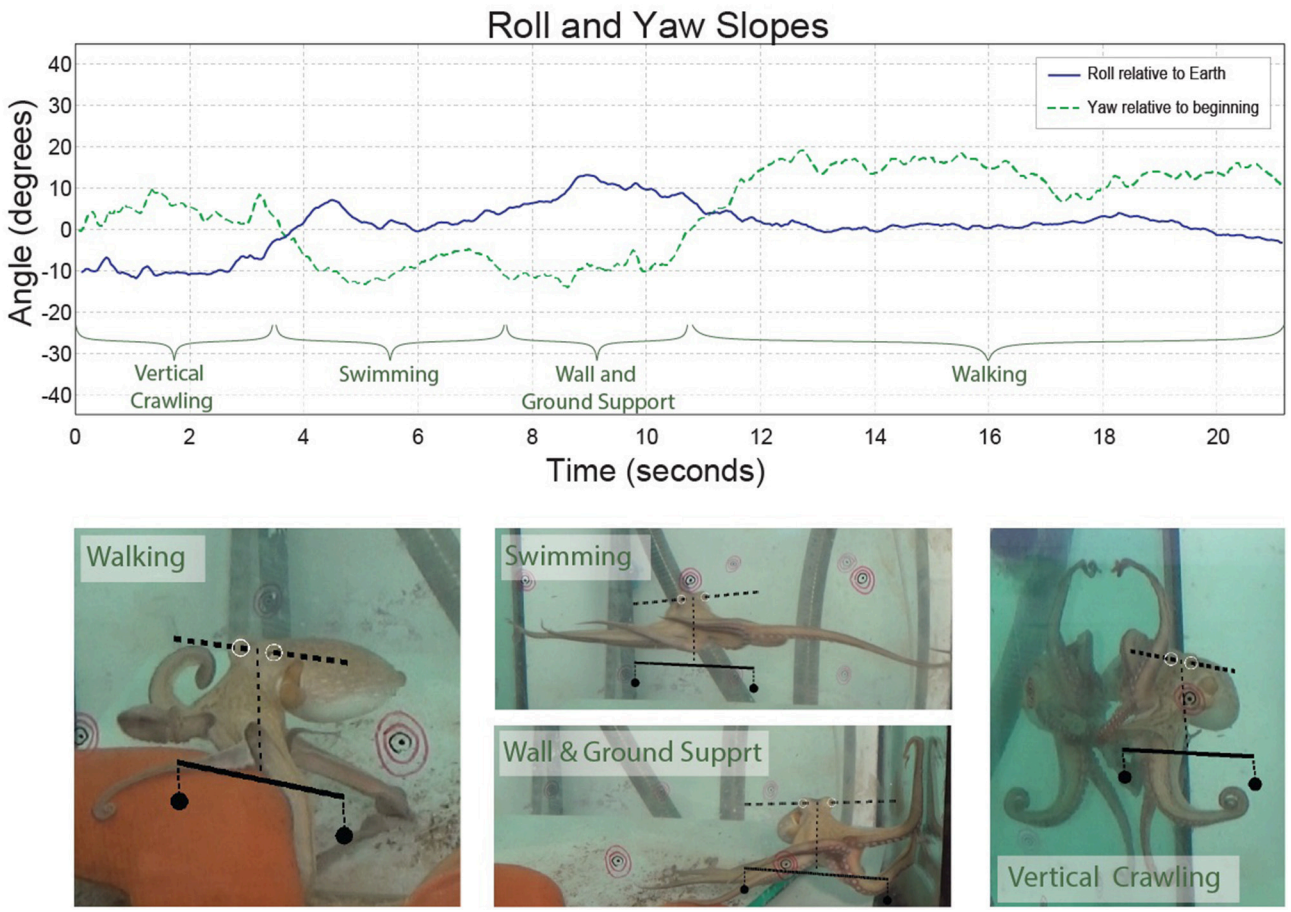

FIGURE 2 | The slope between the eyes during behavior (roll–blue). The yaw slope (green dashed line) was added for comparison. The time through which the octopus showed each type of locomotion is marked on the plot and video images. The slope of the axis that runs between the eyes (dashed line) and the scale below it (continuous line) are shown on the video and images. The scale shows what would be zero-degree orientation relative to Earth. The concentric black and red circles were physically on the aquarium and served for calibration of the three cameras to reconstruct the 3D position of the eyes.

to be based on a motor program involving a robust feedforward component, as clearly apparent in locomotion of all skeletal animals which are driven by CPGs. Indeed, our kinematic analysis of octopus crawling and walking (**Figure 1**) suggests that both these locomotion maneuvers are controlled by what we would suggest to term a "probabilistic" strategy of moment-to-moment changes in the probability of recruiting of those arms that have the better chances of moving the body in the desired direction. **Figure 1** shows that in walking (D), like crawling (C), there is no clear order in the pattern of arm recruitment. Nor does the FFT analysis of the walking velocity indicate the involvement of any CPG. Note that the lack of involvement of CPG in walking is functionally more significant than the lack of a CPG in crawling because in crawling there is no need to care for body stability as the body rests on the substrate. In walking, on the other hand, arm coordination must deal also with stability because the center of body mass is above the ground; walking control must take into consideration that at least two arms need to be in contact with external support to stabilize the body above the ground (**Figure 3B**).

The octopus' probabilistic control strategy, together with the radial organization of the arms around the body, creates yet another unique feature in the control of octopus locomotion. In contrast to all bilaterian animals (animals with bilateral body symmetry), the octopus can locomote in any direction relative to its facing direction and, as shown for crawling in Levy et al. (2015), at the same time it can independently control the orientation in which its body faces.

These findings further support the theory that *embodied organization* of behavior has led to the evolution of a unique body plan that enables the existence of efficient motor control mechanisms that overcome the huge complexity involved in the control of hyper-redundant soft bodied animal. In other words, the special morphology of the octopus enabled the selection of control strategies that require the nervous system to deal with a rather small number of controlled variables.

ETHICS STATEMENT

The research was conducted under the Directive2010/63/EU.

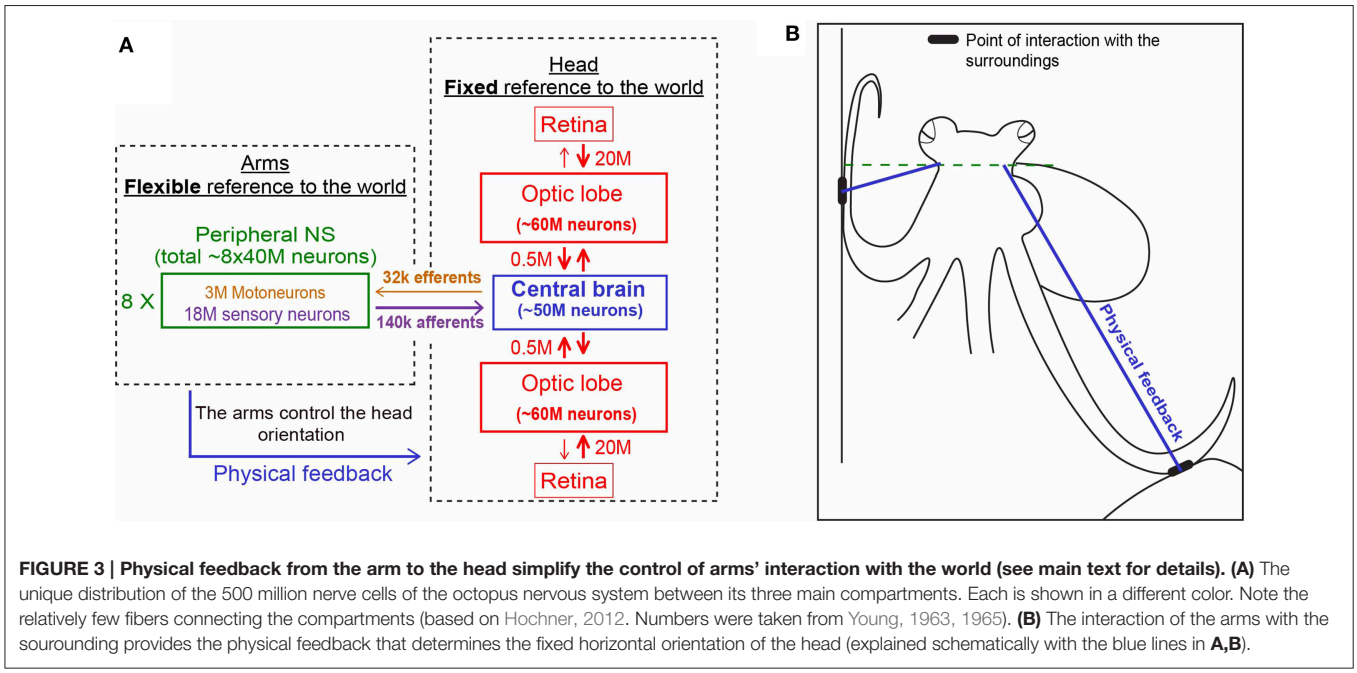

FIGURE 3 | Physical feedback from the arm to the head simplify the control of arms' interaction with the world (see main text for details). (A) The unique distribution of the 500 million nerve cells of the octopus nervous system between its three main compartments. Each is shown in a different color. Note the relatively few fibers connecting the compartments (based on Hochner, 2012. Numbers were taken from Young, 1963, 1965). **(B)** The interaction of the arms with the sourounding provides the physical feedback that determines the fixed horizontal orientation of the head (explained schematically with the blue lines in **A,B**).

AUTHOR CONTRIBUTIONS

GL did the research and the analysis. GL and BH designed the experiments, interpret the results and wrote the paper.

FUNDING

This work was supported by European Community EP7 STIFF-FLOP project number 287728.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 05 January 2017
doi: 10.3389/fphys.2016.00660

Saccadic Movement Strategy in Common Cuttlefish *(Sepia officinalis)*

Desiree Helmer[1†], Bart R. H. Geurten[2†], Guido Dehnhardt[1] and Frederike D. Hanke[1]*

[1] Sensory and Cognitive Ecology, Institute for Biosciences, University of Rostock, Rostock, Germany, [2] Department of Cellular Neurobiology, Schwann-Schleiden Research Center, Georg-August-University of Göttingen, Göttingen, Germany

Most moving animals segregate their locomotion trajectories in short burst like rotations and prolonged translations, to enhance distance information from optic flow, as only translational, but not rotational optic flow holds distance information. Underwater, optic flow is a valuable source of information as it is in the terrestrial habitat, however, so far, it has gained only little attention. To extend the knowledge on underwater optic flow perception and use, we filmed the movement pattern of six common cuttlefish *(Sepia officinalis)* with a high speed camera in this study. In the subsequent analysis, the center of mass of the cuttlefish body was manually traced to gain thrust, slip, and yaw of the cuttlefish movements over time. Cuttlefish indeed performed short rotations, saccades, with rotational velocities up to 343°/s. They clearly separated rotations from translations in line with the saccadic movement strategy documented for animals inhabiting the terrestrial habitat as well as for the semiaquatic harbor seals before. However, this separation only occurred during fin motion. In contrast, during jet propelled swimming, the separation between rotational and translational movements and thus probably distance estimation on the basis of the optic flow field is abolished in favor of high movement velocities. In conclusion, this study provides first evidence that an aquatic invertebrate, the cuttlefish, adopts a saccadic movement strategy depending on the behavioral context that could enhance the information gained from optic flow.

Keywords: cephalopods, optic flow, vision, motion vision, prototypical movements, saccades

OPEN ACCESS

Edited by:
Graziano Fiorito, Stazione Zoologica Anton Dohrn, Italy

Reviewed by:
Cinzia Chiandetti, University of Trieste, Italy
Christelle Jozet-alves, Université de Caen, Basse-Normandie (UNICAEN), France

***Correspondence:**
Frederike D. Hanke
frederike.hanke@uni-rostock.de

[†] These authors have contributed equally to this work.

Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *01 October 2016*
Accepted: *15 December 2016*
Published: *05 January 2017*

Citation:
Helmer D, Geurten BRH, Dehnhardt G and Hanke FD (2017) Saccadic Movement Strategy in Common Cuttlefish (Sepia officinalis). Front. Physiol. 7:660. doi: 10.3389/fphys.2016.00660

INTRODUCTION

It is largely unknown which cues underwater species use to navigate safely through their environment. Only recently optic flow, defined as the visual pattern elicited on the retina of a moving observer (Gibson, 1950), has reattracted notice as possible source of information in the underwater world (Gläser et al., 2014; Scholtyssek et al., 2014). Extending these studies, Geurten et al. (under revision) showed that harbor seals adopt a saccadic movement strategy comparable to terrestrial species such as insects (see e.g., Collett and Land, 1975; Zeil, 1986, 1996; Zeil et al., 1996; Van Hateren and Schilstra, 1999; Tammero and Dickinson, 2002; Ribak et al., 2009; Boeddeker et al., 2010; Geurten et al., 2010; Kress and Egelhaaf, 2012, 2014) or birds (Eckmeier et al., 2008; Kress et al., 2015; Pete et al., 2015). These animals perform short rotations of the eyes, the head, or the body depending on species. These rotations are called saccades and minimize the time during which spatial information cannot be derived from the optic flow field as all objects irrespective of their distance to the observer move with the same rotation velocities (Koenderink and van Doorn, 1987). In contrast, these animals predominantly translate through their environment as

translational movements allow the extraction of distance information from optic flow as the closer the objects, the faster they move.

To analyze if the saccadic movement strategy is as widespread underwater as it is in the aerial habitat, we studied the movement pattern of another aquatic animal, the common cuttlefish *(Sepia officinalis)*, which has a completely different movement pattern and lifestyle than seals. Furthermore, their last common ancestors are the bilaterians, which lived ≈500 Mio years ago. Cuttlefish are benthic cephalopods which have well-developed eyes and good vision (Budelmann, 1995; Hanlon and Messenger, 1996). Their eyes are very mobile and show optokinetic responses as a response to moving stimuli (Collewijn, 1970; Messenger, 1970), and eye movements seem to precede and compensate body movements during rotations (Messenger, 1968; Collewijn, 1970).

Cuttlefish actively prey upon fish or crustaceans which they capture by ejecting their extensible tentacles or by jumping on and enveloping the item with all arms, called arm attack (Sanders and Young, 1940; Wilson, 1946; Messenger, 1968; Nixon and Dilly, 1977; Duval et al., 1984). The latter occurs mainly with slow moving prey. Their attacks on prey are predominantly visually-driven with an attention, positioning, and seizure phase (Sanders and Young, 1940; Messenger, 1968, 1977; Chichery and Chichery, 1988). During an attack, cuttlefish seem to estimate the distance to the prey item as (1) they either retreat from or approach the object, (2) they modify the ocular convergence depending on the distance to the prey object (Messenger, 1968), (3) unilaterally blinded animals or animals in which the optic commissure and the basal lobes are divided are less accurate in seizing prey in comparison to normal sighted animals (Messenger, 1977), and (4) they seem to possess size constancy (Messenger, 1977). Cuttlefish might gain distance and depth information by accommodation as a change in refractive state was observed just before the cuttlefish attacked the prey item (Schaeffel et al., 1999), by the W-shaped pupil being a monocular in-or-out-of-focus detector (Schaeffel et al., 1999; Mäthger et al., 2013) or by using texture density gradients (Josef et al., 2014). Another mechanism that would allow for visual distance estimation in a feeding and non-feeding context, as outlined above, is translational optic flow. As a first approach to analyze if optic flow perception is used in cuttlefish to measure distances, we recorded the movement pattern of a small group of six cuttlefish to analyze if cuttlefish move their bodies saccadically in line with the saccadic movement strategy documented for other animals.

MATERIALS AND METHODS

Experimental Animals

The experiment was conducted with six cuttlefish *(S. officinalis)* individuals at the Marine Science Center, Rostock, Germany. The cuttlefish hatched in captivity in January 2015 at the Max-Planck-Institute for Brain Research, Frankfurt, Germany, and were thus half a year old when their movement pattern was recorded. The animals were kept in accordance with current maintenance protocols for cephalopods (Andrews et al., 2013; Smith et al., 2013; Fiorito et al., 2014, 2015) in line with the Directive 2010/63/EU. Approval (6712GH00113) was given by local authorities (Staatliches Amt für Umwelt und Natur Rostock) according to §42 of the German law on nature protection.

One up to two cuttlefish individuals shared one compartment of a 3000 l sea water aquarium system. Water quality was regularly controlled, and salinity and temperature were adjusted to 32 g/kg and 21°C, respectively. The bottom of the aquarium was covered with small pieces of corals or sand, which allowed the cuttlefish to burry themselves. The tank was artificially illuminated (daylight spectrum) with a natural day–night-cycle of 12 h/12 h. The day cycle included a phase of dawn and dusk of 1 h. To ensure a balanced diet, the animals were fed one to three times a day with *Palaemon* sp., deep frozen fish or fish pieces from *Osmerus eperlanus, Sprattus sprattus,* or *Clupea harengus* or shrimp *(Pandalus borealis)*.

Experimental Procedure

For 4 days, during which the movement pattern of cuttlefish was recorded, cuttlefish were housed together in a large compartment (150 × 51.5 × 85 cm) in a group of six individuals to maximize the time at least one individual was visible in the field of view of the camera. Within the field of view of the camera, a red PVC board (50 × 50 cm) was placed on the bottom of the compartment. The cuttlefish were lured onto the board with *Palaemon* sp. that were inserted in fasteners. The fasteners could be moved with fine thread not causing water disturbances at the water surface that would have lowered the quality of the recordings. The cuttlefish attacked the lure and removed the prey from the nut. Filming cuttlefish on the red board increased the contrast of the otherwise cryptically colored animals, which facilitated video analysis. To additionally facilitate video analysis, the luminance of the region of interest was increased with external lamps that were switched on only during filming.

The movement pattern of the cuttlefish was filmed with a black-and-white high speed camera (Photon focus DR1-D1312-200-G2, Lachen, Switzerland) with an objective with a focal length of 16–100 mm (Varifocal SC-VZ-16100M, SpaceCom, Tokyo, Japan) at 200 frames/s. The camera was installed 50 cm above and orthogonal to the water surface. We are confident that we can adequately describe the movements of the cuttlefish from video recordings from above as we moved the prey items mainly close to the bottom avoiding large vertical movements and as the movement of cuttlefish with their benthic lifestyle (Russell-Hunter, 1979) is predominantly two-dimensional. This assumption is supported by only small vertical movements amounting to 5.7 ± 4.7% quantified on the basis of the maximal difference in dorsal mantle length of the cuttlefish.

Video Analysis

The video recordings were analyzed with the help of the software ivTrace Image Analysis (https://opensource.cit-ec.de/projects/ivtools). We analyzed all video sequences obtained and only omitted those video recordings with obvious interactions between cuttlefish individuals. On the recordings, the center of mass of the cuttlefish body was tracked over time. Additionally, the orientation of the cuttlefish body and its coordinates in a two-dimensional space were determined. Using these parameters, the

movement of the cuttlefish could be described as thrust, slip, and yaw movement defined as for-/backward movement, movement to the side, and rotations around the body axis (**Figure 1** insets). Velocities of these three movement directions were calculated from the change in position and orientation between subsequent frames. Movements with velocities exceeding 3000°/s or 7000 mm/s were classified as artifacts and were consequently excluded from the analysis.

The subsequent analysis steps were conducted with the help of custom written programs in Matlab (The Mathworks, Natick, Massachusetts, USA). The velocity of yaw, thrust, and slip movements of the body were determined by calculating the angle covered or the distance moved by the body between two frames. To convert distances moved from pixel into mm, the size of the red board, which was placed on the bottom of the tank, was taken as scale.

Furthermore, a cluster analysis was conducted to describe the prototypical movement pattern of cuttlefish. Therefore, the velocity data was z-scored (normalized to a 0 mean and a standard deviation of 1) to account for numeric differences between rotational and translational speeds. For every frame on which the animal had moved a three-dimensional velocity vector consisting of thrust, slip, and yaw velocity was then fed into a hierarchical agglomerative clustering routine (MatLab Statistics Toolbox). As the whole data set was too large to be clustered at once, it was split up into 2% chunks that were clustered sequentially (Hastie et al., 2009a; Murtagh and Contreras, 2012). We used the squared Euclidean distance and "Ward criterion" to build hierarchical clusters. This first step of analysis rendered a possible number of clusters between 2 and 50. We subsequently clustered the complete data set again with the k-means algorithm (MacQueen, 1967; Milligan and Cooper, 1987; Hastie et al., 2009b). We clustered all classes between 2 and 20. For 20–50 classes, only every fifth class was analyzed because we rarely saw stable cluster combinations with these large numbers of classes (Geurten et al., 2010, 2014; Hofmann et al., 2014). To determine the number of classes that represent our data best, we used the quality and stability criteria described in Braun et al. (2010).

Statistical Analysis

We employed Fisher's permutation tests (Fisher, 1954) on the differences between the medians of different experimental groups, which were refined by various authors (see e.g., Crowley, 1992; Ernst, 2004). We corrected the p-values with the Benjamini–Hochberg false detection rate procedure (Benjamini and Hochberg, 1995; Groppe et al., 2011) using the Matlab implementation of Benjamini and Hochberg's procedure by David M. Groppe (https://de.mathworks.com/matlabcentral/fileexchange/27418-fdr-bh).

RESULTS

Altogether 202 videos including 256,830 single frames could be analyzed. **Figure 1A** illustrates a characteristic trajectory of a cuttlefish moving over a time frame of ≈10 s. The black line connecting the dots describes the movement of the center of mass of the body over time, whereas the short lines represent the yaw orientation of the body. During the first phase of the movement, the cuttlefish was moving forward positioning itself with the moving prey item. This phase ends when the cuttlefish jumped on the prey item at the upper tip of the loop. The seizure of the prey was accompanied by fast thrust and slip movements (**Figures 1C,D**). In the last phase, it retreated from the point of prey capture with a fast back- and sideward movement (**Figures 1C,D**).

It is evident from this example trajectory that the body was not necessarily aligned with the swimming direction. This phenomenon was also generally revealed by the ψ-angle analysis (**Figures 2A,B**) that describes the angle between the body long axis and the movement direction. Only during a phase at the beginning of the movement and in a short retreat phase after prey capture of the example trajectory (**Figure 1**), a clear alignment of body and the direction of movement could be observed. In general, during hunting trajectories, there was a clear bias to ψ-angle of either 0° or 180° (**Figures 2C–F**). This emerged from the cuttlefish's preference to align prey and body axis during the phases of the attack (Messenger, 1968) and moreover to use its fast siphon jet propulsion to approach prey and to leave the place where it has just caught its prey on the fastest way. Siphon propulsion was used significantly more often during attacks than during normal cruising ($p < 0.001$ Fisher's permutation test; Benjamini Hochberg false detection rate correction; **Figure 2G**). A pronounced biphasic distribution of the ψ-angle was especially prominent during failed attempts to catch a prey item. After an unsuccessful tentacle strike, the animal moved backward to aim for its target a second time (**Figure 2D**). In contrast, the 180° ψ-angle component is largely missing if the cuttlefish has unsuccessfully tried to seize the prey with an arm attack as they did not retreat in this situation but continued to follow the prey item (**Figure 2F**).

The example trajectory moreover shows that there are periods during which the body showed a constant orientation over time (**Figure 1B**). However, changes in orientation were fast and short, which is characteristic for saccadic turns (**Figures 1B,E**). During this example movement, six saccades marked by red circles in **Figure 1E** could be detected. Saccades were generally defined as short rotations reaching velocities of ≥125°/s. **Figure 3** characterizes all 136 saccades documented in the video material. During these saccades, the body reaches a mean rotation velocity of 168 ± 44.6°/s (**Figure 3A**). Generally, saccades vary in velocity between 125 and 343°/s, and the body rotates with a mean yawing angle of 20.6 ± 16.2 ms (**Figure 3B**). The angles covered by the body from frame to frame ranged between 9 and 85°. Thrust velocity is on average faster during saccades than during translational bouts (154–124 mm/s; **Figure 3C**), as are slip and yaw velocities increased (slip: 28–66 mm/s, yaw: 24–88°/s; **Figures 3D,E**). This shows that translational and rotational velocities do not coincide, but that fast rotations are segregated from other movements in line with a saccadic movement strategy. Cuttlefish saccades range in duration from 110 to 720 ms with a mean duration of 237 ± 98 ms (**Figure 3F**). In contrast, cuttlefish perform translations lasting 3.7 ± 3.5s ms on average (**Figure 3G**). Thus, translational bouts are significantly ($N = 202$,

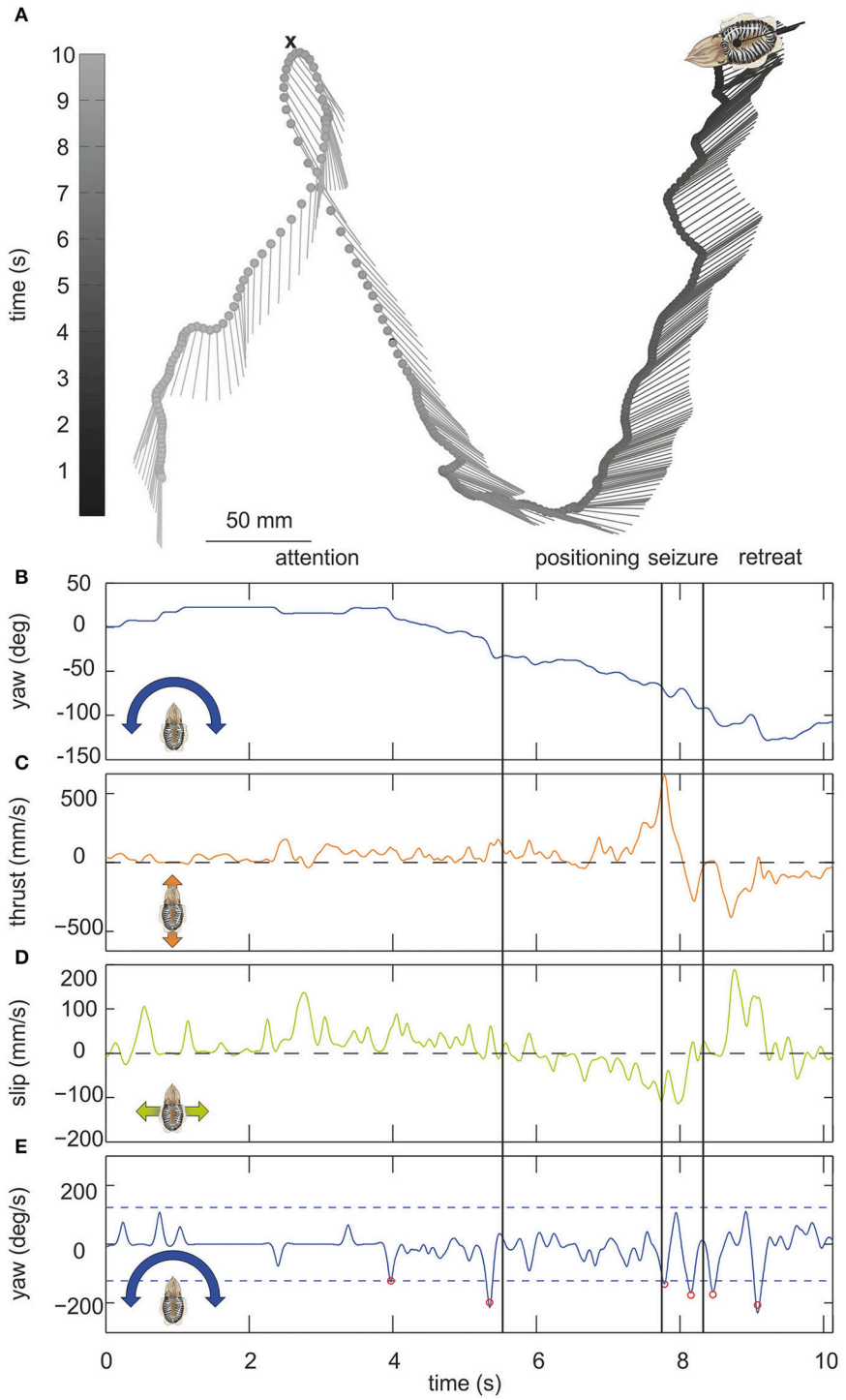

FIGURE 1 | (A) Example trajectory of a moving cuttlefish. The cuttlefish moved in forward direction from the lower left corner to the upper tip of the loop where it captured a small crab (indicated by a cross). It then moved backwards up to the upper right corner. The lines mark the long axis of the cuttlefish, the dots indicate the center of mass of the cuttlefish body over time (in s) which is depicted in gray scale from light gray representing the start of the movement to dark gray end of the movement. The position of the center of mass is plotted every 100 ms. The scale for dimensions is 50 mm. **(B–E)** Parameters of the cuttlefish's movement with **(B)** the yaw angle (in °), **(C)** the thrust and **(D)** slip velocities (mm/s), and **(E)** yaw velocity (in °/s). In **(E)** saccades, defined by velocities ≥125°/s (dashed lines), are marked by red circles. Vertical lines mark the end/start of the phases attention, positioning, seizure and retreat as indicated above the figures.

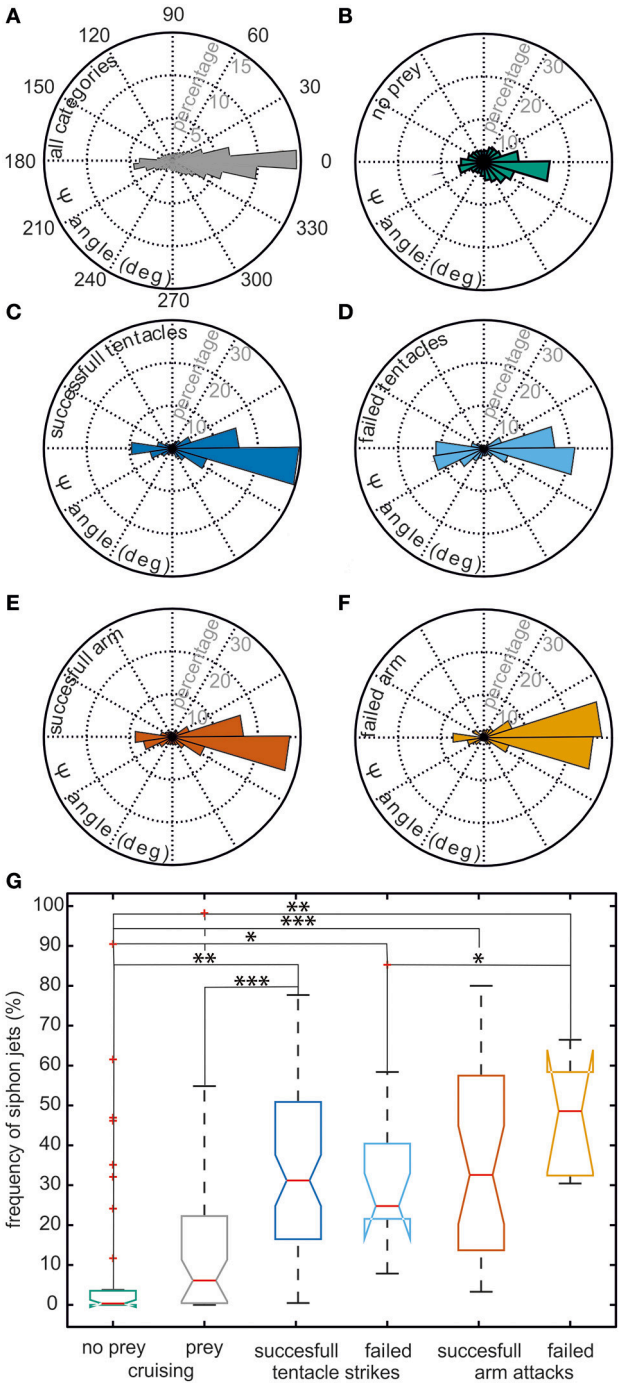

FIGURE 2 | Context dependent analysis of the ψ-angle distribution and usage of siphon jet propulsion. Cuttlefish trajectories were categorized according to the following contexts: successful tentacle strikes (n =71 trajectories), failed tentacle strikes (n = 13), successful arm attacks (n = 31), failed arm attacks (n = 7), cruising sequence with prey in the animals' vicinity (n = 43), and cruising sequences without prey (n = 37). **(A–F)** The angle between the body long axis and the movement direction (ψ-angle) is plotted as a rose plot. A ψ-angle of 0° codes for a forward movement, whereas a ψ-angle of 180° describes a backward movement. The ψ-angle distribution occurring in all trajectories is depicted in **(A)**, Panels **(B–F)** show the ψ-angle *(Continued)*

FIGURE 2 | Continued
distribution for different contexts. ψ-angle obtained from trajectories in which **(B)** no prey animals were present, **(C)** successful or **(D)** failed tentacle strikes, or **(E)** successful or **(F)** failed arm attacks were documented. In **(G)**, the frequency with which siphon jet propulsion occurred during different behavioral contexts is plotted. There is no significant difference when comparing different types of attack and their outcome. However, the frequency of jet propulsion differs significantly between cruising and attacks and between the presence of prey items or their absence. Significance was determined using Fisher's exact permutation test and corrected via Benjamini–Hochberg false detection rate procedure (see Section Statistical Analysis). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

$p < 0.001$, Fischer's exact permutations test) longer than saccades (**Figure 3H**).

The cluster analysis yielded the best stability and quality for 12 clusters. In those clusters (**Figure 4**), the two movement types of cuttlefish (Russell and Steven, 1930) are apparent: the first type of movements is elicited by a complex movement of the fins with an average movement velocity of 138 m/s, the second by the jet expelled from the siphon during which the cuttlefish reached velocities of 430 m/s, which they predominantly use during hunting (**Figure 2G**). These movement types go along with two different strategies. Whereas, during fin motion, rotations and translations are clearly separated (cluster 2, 3, 11, 12, **Figure 4**), rotations and translations are coupled during jet propulsion (cluster 4–6, **Figure 4**). Overall forward movements, however, dominate over back- and sideward movements as also revealed by the ψ-angle analysis (**Figures 2A–F**).

DISCUSSION

The results of this study revealed that cuttlefish employ a saccadic movement strategy. We analyzed body movements as a first approach as eye movements could not be resolved on our recordings. However, we assume that the eyes of cuttlefish also move saccadically in support of the saccadic body movements. Evidence supporting this hypothesis stems from previous studies (Messenger, 1968; Collewijn, 1970; Chichery and Chichery, 1987, 1988) in which it was shown that cuttlefish perform eye movements, ocular saccades in particular, in compensation of body rotations. During the saccades, only the rotation direction and velocity is perceptible from the optic flow field. This information could be useful for the animal's positioning as it is directly available in contrast to information from statocysts (Budelmann et al., 1973; Budelmann, 1979), which have a longer latency. If and how the optic flow information is integrated into the signal of the statocysts has to be analyzed in future studies.

We did not observe directed movements of the head in relation to the mantel cavity, similar to the head stabilization of birds (Pratt, 1982; Wohlschläger et al., 1993). Although, a closer investigation of the mantel orientation might reveal further stabilization strategies, the most obvious place for further gaze stabilization would be the moveable eyes of *S. officinalis*. In conclusion, by performing body saccades

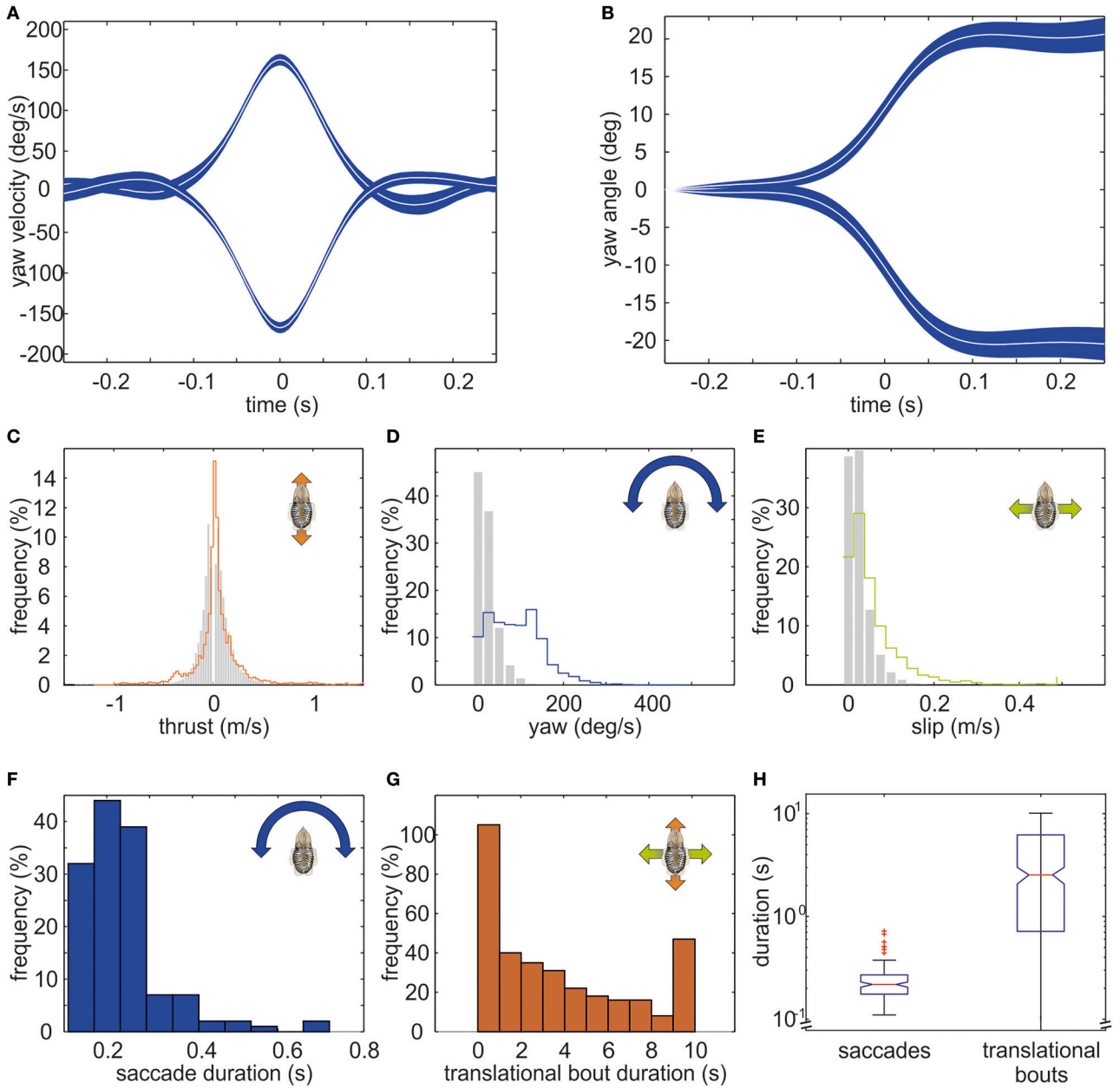

FIGURE 3 | Detailed analysis of saccades of cuttlefish. The mean course of saccades in cuttlefish ($N = 136$) is depicted as mean yaw velocity (in °/s) in **(A)** and as mean yaw angle (in °) in **(B)**. White curves indicate the median. The blue shaded area depicts the 95% confidence interval of the median. Saccades to the right (positive values) and to the left (negative values) are displayed separately. In general, saccades are short events that last for 217.5 ms on average. During a saccade, the body reached a mean rotation velocity of 152°/s (±*SD*) and covered a mean rotation angle of 18.8°. **(C–E)** Frequency (in %) of translations (gray bars) and saccades (colored lines) during **(C)** thrust, **(D)** yaw, and **(E)** slip movements. While the distribution of thrust velocities is rather similar during translations and saccades, sideways and yaw velocities are faster during saccades. **(F)** Frequency (in %) with which saccades of different durations occurred (binned in 0.1s). **(G)** Frequency (in %) with which translational bouts of a specific duration (in s) occurred. **(H)** Boxplots for the duration (in s; logarithmic scale) of saccades and translational bouts with the boxes indicating the quartiles, the red line indicating the median, and the 1.5 interquartile distance is shown by the whiskers. Outlying data points are marked with red crosses. The notches in the boxes exhibit the 95% confidence interval of the median. Saccades are significantly longer events than translations ($p < 0.01$).

most likely in combination with eye movements, cuttlefish reduce the time of rotations as rotations complicate the extraction of distance information from optic flow. Thus, this study most likely adds a mechanism to the already reported distance/depth estimation mechanisms in cuttlefish (Schaeffel et al., 1999; Mäthger et al., 2013; Josef et al., 2014). Distance estimation from optic flow offers the advantage that it provides distance information for much larger distances than

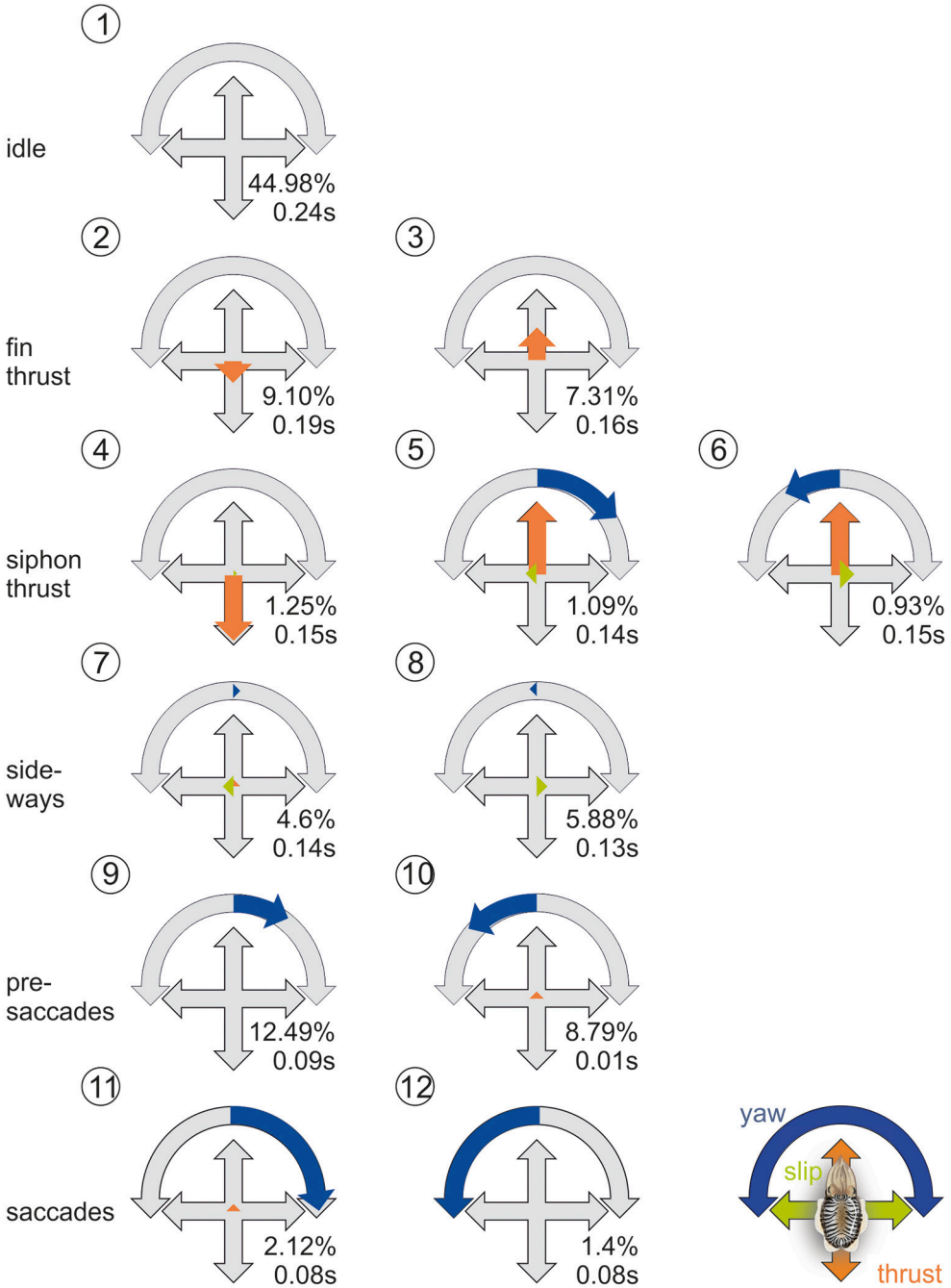

FIGURE 4 | Prototypical movements of the common cuttlefish. Normalized thrust, slip and yaw rotation velocity (thrust and slip were normalized to their maximum, yaw on its absolute maximum) for the 12 clusters as well as frequency as percentage of total events ($N = 256{,}628$) and mean duration of the behavioral element. PM2-6 are thrust dominated, PM7-8 describe slight sideways movements, and PM9-12 are characterized by an increase of rotational yaw movements. Blue arrows denote yaw rotations to the left or right, green arrows denote slip movements to the left or right, orange arrows denote thrust movements to the front or back.

the alternative mechanisms. Moreover these data add to the overall picture that all moving animals irrespective of their eye type, mode of locomotion, visual environment including the medium, in which they operate, use optic flow to guide their movements.

In contrast to terrestrial species as well as to harbor seals, cuttlefish show a context dependent strategy as revealed by the cluster analysis. During fin motion, cuttlefish move at relatively low speeds and clearly separate their body movements into saccades and translations. This behavior corresponds to the

saccade movement strategy documented in terrestrial species (see e.g., Collett and Land, 1975; Schilstra and Hateren, 1999; Blaj and van Hateren, 2004; Eckmeier et al., 2008; Ribak et al., 2009; Geurten et al., 2010, 2014; Kress and Egelhaaf, 2012) as well as in the harbor seal (Geurten et al., under revision). In contrast, the cuttlefish abolishes optic flow analysis to gain distance information when it moves its body at high velocity with the pulsed jet of its siphon. Thus, cuttlefish seem to trade their swimming velocity and the extraction of distance information form optic flow depending on the context. Siphon movements predominantly occurred shortly before and after a prey capture event. In this situation, the cuttlefish seem to primarily focus on speed to catch the prey item and to leave the location of prey capture. Especially under competition pressure, the best strategy for an animal is to escape in a straight line with high velocities. Such an escape behavior has e.g., also been shown for the African ball-rolling dung beetle that rolls its dung ball on a straight path from the dung pile at which it encounters intense competition among conspecifics (Byrne et al., 2003; Dacke et al., 2003a,b,c, 2011, 2013). A very fast escape movement in cuttlefish might have evolved because they are soft-bodied animals with many predators.

This study provided a detailed characterization of body saccades in cuttlefish. Cuttlefish saccades were defined as rotations exceeding a rotation velocity of 125°/s. This velocity threshold seems conservative when compared to the results of optokinetic studies (Collewijn, 1970; Messenger, 1970). In these studies, low gain optokinetic responses up to a rotational velocity of the optokinetic drum of only 35°/s were reported. However, the gain function published by Collewijn (1970) suggests that the cuttlefish might have also responded to higher rotational velocities if these had been tested. This claim is supported by Boulet (1960) who documented ocular reactions to a target movement of up to 51°/s and also by Cartron et al. (2013) who state that cuttlefish followed drum movements up to 100°/s but failed at a stimulus velocity of 130°/s. Cuttlefish body saccades lasted for 217.5 ms on average. It is very probable that the eyes even move faster although Collewijn (1970) reported that it took a cuttlefish eye 0.5 s to complete a saccade. The cuttlefish rotated their bodies by an angle of 9–85°. From observations and as documented by Messenger (1968), cuttlefish rotate their eyes together with the body by almost 180° in the first phase, the attention phase of their attack. The overall goal in this phase of the attack is to align the optical and the prey axis. It is very likely that we did not record such wide angles as we inserted the prey predominantly within the anterior visual field of a cuttlefish close to the platform. Thus, there was no need for the cuttlefish to turn by a large angle. The behavior we documented thus predominantely describe the movement pattern of cuttlefish in the second and third phase of the attack, positioning, and seizure (Messenger, 1968).

Cuttlefish and seal body saccades (Geurten et al., under revision), the only saccades documented for swimming animals up to now, are very similar in respect to their mean and maximum rotation velocities. These velocities are achieved by slightly smaller angles covered in a shorter period of time in cuttlefish in comparison to harbor seals that rotate in larger angles which also takes more time. These differences are most likely due to the larger body size of harbor seals as compared to cuttlefish. Body saccades of these two aquatic species are surpassed in rotation velocity by most flying species (Blaj and van Hateren, 2004; Eckmeier et al., 2008; Geurten et al., 2010), which is probably due to the higher viscosity and density of water vs. air. Whereas, harbor seals change between active swimming and gliding (Geurten et al., under revision), cuttlefish switch between two active swimming modes, fin motion, and jet propelled swimming. However, the movements made by cuttlefish are characterized by movements along as well as perpendicular to the body axis, the latter not occurring in harbor seals.

In conclusion, this study revealed that cuttlefish move their bodies saccadically thereby probably optimizing the extraction of distance information from optic flow. Future studies however need to be performed to proof the usage of optic flow in *S. officinalis*. Cuttlefish change between a saccadic moment strategy and high movement velocities, during which they abolish the separation of rotational and translational movements, a flexibility that is unique till now. Moreover the finding of a saccadic movement strategy in another aquatic species besides harbor seals suggests that this strategy might be as wide-spread underwater as it is in the terrestrial habitat.

AUTHOR CONTRIBUTIONS

All authors designed the study, DH, BG, FH collected and analyzed the data, BG, FH wrote the manuscript, all authors edited and approved the manuscript.

FUNDING

This study was supported by a grant of the VolkswagenStiftung to GD.

ACKNOWLEDGMENTS

The authors would like to thank the Max-Planck-Institute for Brain Research, Frankfurt, Germany, and Michael Kuba and Tamar Gutnick for providing the experimental animals and helpful advice on handling the cuttlefish. We acknowledge the assistance of Luisa Hofberger and Verena Klüver, University of Göttingen, during data collection and analysis, Martin Göpfert, University of Göttingen, for financial support to BG and Christian Spalthoff, University of Göttingen, for the cuttlefish drawing. Lars Miersch, Marine Science Center Rostock, is thanked for technical support with the aquarium system. We acknowledge the support of the "Cost action FA1301-CephsInAction."

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 27 March 2017
doi: 10.3389/fphys.2017.00173

Going Up or Sideways? Perception of Space and Obstacles Negotiating by Cuttlefish

Gabriella Scatà[1,2]*, *Anne-Sophie Darmaillacq*[3], *Ludovic Dickel*[3], *Steve McCusker*[1] and *Nadav Shashar*[1]

[1] Eliat Campus, Department of Life Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel, [2] Queensland Brain Institute, University of Queensland, St. Lucia, QLD, Australia, [3] EThOS Ethologie Animale et Humaine (UMR, Centre National De La Recherche Scientifique 6552), Team Cognitive Neuro Ethology of Cephalopods, Normandie Université, Caen, France

While octopuses are mostly benthic animals, and squid prefer the open waters, cuttlefish present a special intermediate stage. Although their body structure resembles that of a squid, in many cases their behavior is mostly benthic. To test cuttlefish's preference in the use of space, we trained juvenile *Sepia gibba* and *Sepia officinalis* cuttlefish to reach a shelter at the opposite side of a tank. Afterwards, rock barriers were placed between the starting point and the shelter. In one experiment, direct paths were available both through the sand and over the rocks. In a second experiment the direct path was blocked by small rocks requiring a short detour to by-pass. In the third experiment instead, the only direct path available was over the rocks; or else to reach the goal via an exclusively horizontal path a longer detour would have to be selected. We showed that cuttlefish prefer to move horizontally when a direct route or a short detour path is available close to the ground; however when faced with significant obstacles they can and would preferentially choose a more direct path requiring a vertical movement over a longer exclusively horizontal path. Therefore, cuttlefish appear to be predominantly benthic dwellers that prefer to stay near the bottom. Nonetheless, they do view and utilize the vertical space in their daily movements where it plays a role in night foraging, obstacles negotiation and movement in their home-range.

Keywords: space perception, cuttlefish, cephalopod, obstacles negotiation, three-dimensional space

OPEN ACCESS

Edited by:
Daniel Osorio,
University of Sussex, UK

Reviewed by:
Bruce R. Johnson,
Cornell University, USA
Laura Ann Miller,
University of North Carolina at Chapel Hill, USA

***Correspondence:**
Gabriella Scatà
gabry.scata@gmail.com

Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *19 October 2016*
Accepted: *07 March 2017*
Published: *27 March 2017*

Citation:
Scatà G, Darmaillacq A-S, Dickel L, McCusker S and Shashar N (2017) Going Up or Sideways? Perception of Space and Obstacles Negotiating by Cuttlefish. Front. Physiol. 8:173. doi: 10.3389/fphys.2017.00173

INTRODUCTION

Navigation has been extensively studied in two-dimensional environments, where the animal has to locate a goal by moving across a horizontal surface, neglecting the vertical dimension. However, the world is three-dimensional and since all animals have to move along the vertical plane at some point, they need to take the vertical component of space into account. The importance of vertical space has indeed recently been recognized for the conservation of several species, as well as for the welfare of animals kept in captivity (O'Neill-Wagner, 1994; Clarence et al., 2006; Tracey et al., 2014). Taking the vertical dimension into account makes navigation more complex; for example, the amount of space to be represented is larger than when encoding a planar two dimensional environment (Jeffery et al., 2013). This is especially true for animals that are able to move from and to any point in a volumetric space.

It has been suggested that the locomotor style of an animal is correlated with the accuracy with which spatial information in the horizontal and vertical planes is encoded and which of this information is prioritized (Flores-Abreu et al., 2014). Animals able to move freely in the three dimensions (e.g., fish, bats, bees, birds) encode the vertical information with either equal or higher accuracy than the horizontal information and seem to prefer vertical to horizontal information, while animals constrained to a surface (e.g., rats) do the opposite (Hurly et al., 2010; Holbrook and Burt de Perera, 2013; Davis et al., 2014; Flores-Abreu et al., 2014; but see Ulanovsky, 2011; Savelli and Knierim, 2013; Yartsev and Ulanovsky, 2013; Scatà et al., 2016). However, species of bees that differ in their use of vertical space also differ in the accuracy with which they learn height and in their ability to communicate this information (Nieh et al., 2003; Dacke and Srinivasan, 2007; Eckles et al., 2012). In addition, the performance of a number of species of birds in solving a detour task exclusively on the ground seems to be correlated with the extent to which they move vertically. Canaries for example, which are more used to move in all three-dimensions and fly over barriers, find it almost impossible to detour around a ground obstacle (Zucca et al., 2005). This suggests that the way animals negotiate obstacles reflects the degree to which they normally exploit the vertical vs. horizontal space. Thus, it could be the ecology of the species and its main plane of behavior, or as Nardi and Bingman suggest "its 3D occupancy profile" (Nardi and Bingman, 2013), that explains which component of spatial information is the most relevant to and preferred by the animal.

In previous experiments, we showed that *Sepia officinalis* cuttlefish, which are mostly benthic but can also move freely in a volumetric space, are able to learn spatial information in the vertical dimension, and prefer vertical over horizontal spatial cues when faced with conflicting situations (Scatà et al., 2016). Similar results have been reported for both benthic and pelagic fish (Holbrook and Burt de Perera, 2011; Davis et al., 2014). This dominance of vertical spatial information in fish was suggested to depend on the ability of fish to detect changes in hydrostatic pressure, a salient cue unique to vertical space (Davis et al., 2014; Holbrook and Burt de Perera, 2011). However, cuttlefish buoyancy system is mostly independent of depth (Webber et al., 2000) and pressure sensitivity in other cephalopods appears to be quite low (Rice, 1964; Jordan, 1988). Alternatively, it is possible that cuttlefish are more likely to use vertical information because their main activities - vigilance from predators, foraging, and movement—are performed along the vertical plane (Barbosa et al., 2008; Ulmer et al., 2013).

Cephalopods present a full range of use of space. While most octopus species are mostly benthic (though there are fully pelagic species), and squids are neritic to pelagic, cuttlefish present an in-between case where they spend most of their time as benthic predators, yet move up into the water column at will (Hanlon and Messenger, 1996). Indeed, although known as bottom-dwellers, cuttlefish were reported to become neutrally buoyant and move upwards in the water column at night (Denton and Gilpen-Brown, 1961; Wearmouth et al., 2013). This diel migration pattern has been observed mainly in laboratory conditions, with Aitken et al. (2005) reporting it in the field by tracking the giant Australian cuttlefish (*S. apama*), which moves deeper at night. Little is known about the navigational strategies of cuttlefish and whether or not they move equally in all three dimensions, or have a preferred dimension of locomotion. Their movement patterns have rarely been investigated, especially along the vertical plane. When presented with a vertical wall maze with only two escape holes, one lower and to the left and the other higher and to the right (10 and 60 cm from the bottom, respectively), cuttlefish escaped mostly by the lowest hole (13 out of 18 cuttlefish) (Karson et al., 2003). This setup required cuttlefish to swim upwards at least 6 body heights to escape through the top hole. Thus, it is interesting that a small percentage of the tested animals (5 out of 18) still selected the top hole and maintained such preference across trials.

In the current study, we examined the use of three-dimensional space in the cuttlefish *Sepia officinalis* and *S. gibba* in daytime spatial orientation. In particular, we investigated the use of horizontal versus vertical paths in a navigational task in which the animal has to negotiate a barrier to reach a shelter. We also examined the use of vertical space as time spent at different water depths at night by *S. gibba* cuttlefish, for which data on this is absent in the literature. *S. officinalis* is a nekton-benthic species which is mostly found on sandy or rocky bottoms from shallow coastal water (2–3 m depth) to 200 m depth (Guerra, 2006). *S. gibba* is associated with coral reefs, a more complex three-dimensional environment, which also requires more agility and maneuvering skills (Jastrebsky et al., 2016). It can be found in very shallow waters (1 m), yet not much is known of this species (Reid, 2005). A difference in the use of vertical routes between these two species could relate to the degree of vertical complexity of their natural habitat.

MATERIALS AND METHODS

Two different setups were used for testing young *Sepia officinalis* and *S. gibba* cuttlefish, each examining a different level of complexity in obstacles negotiation. *S. officinalis* were raised and tested in France while *S. gibba* were examined in Israel. Therefore, each experimental setup is presented separately. The movement and use of vertical space of *S. gibba* cuttlefish only was analyzed at night.

S. officinalis Experiments

Subjects

Sixteen young [7–8 weeks old, mantle length of 15–20 mm, about 5 mm tall (body height, bh) and 8 mm wide] *S. officinalis* cuttlefish took part in an experiment testing their path preference while bypassing barriers. Cuttlefish were hatched from eggs collected in the vicinity of Luc-sur-Mer (France). Eggs, initially laid in clusters, were separated to ensure optimal development and were put in shallow tanks at the Centre de Recherches en Environnement Côtier (CREC, Luc-sur-Mer, France). All tanks were supplied with running oxygenated sea water at 17 ± 1°C. After hatching, cuttlefish were first housed in small groups and then, 1 week before experiments began, housed in individual tanks. They were provided with enriched habitats following previous studies which showed that an enriched environment

facilitates development of learning and memory capabilities in young cuttlefish (Dickel et al., 2000; Poirier et al., 2004, 2005). These enriched habitats consisted of tanks with rocks, plastic seaweed and PVC tube as shelters. Animals were fed daily with live shrimp (*Crangon crangon*) and crabs (*Carcinus maenas*) of suitable size.

Experimental Setup—Experiment 1

Training and experiments took place in the same tank (**Figure 1**). Tank was made of opaque white plastic 20 × 10.5 × 7 cm (length × width × height). The tank was filled with seawater to its top. A 5 cm wide shelter was set at one side of the tank, with the dimensions of 5 × 10.5 cm. 3 low (no more than 1.5 cm in height) but wide stones were positioned in the tank. In the training session the stones were set along the sides of the tank, 2 in one side and one on the other. During the test, the stones were set diagonally in the tank such that they blocked any direct path, but could be negotiated and passed by going around them. Direction of the diagonal was changed randomly.

Training and testing took place in the same water table in which the animals were raised. Hence the animals experienced the same lighting and temperature conditions. Seawater was replaced between each training/experimental run to prevent possible odor cues.

Training and Testing—Experiment 1

Cuttlefish were given 3 training presentations to learn to reach the shelter at the opposite side of the experimental tank, with the training setup of the stones obstacles (**Figure 1a**). In each run, the animal was placed in the "starting area" at one side of the tank. Once the animal had settled and after at least 30 s, the wall was raised, and the animal was allowed to move to the shelter. After the animal had reached the shelter, it was rewarded with a 5-min rest in it, after which it was returned to the holding tank.

After having 3 training runs the animal was tested once with a different configuration of the obstacle stones (Experiment 1) (**Figure 1b**). This time there was no direct line to the shelter but the animal had to choose how to path them. It could stay on the bottom and go around the stones or it could go up and swim over them.

Each animal had no more than 2 sessions per day with at least 4 h between them. Training and testing sessions were videotaped from above.

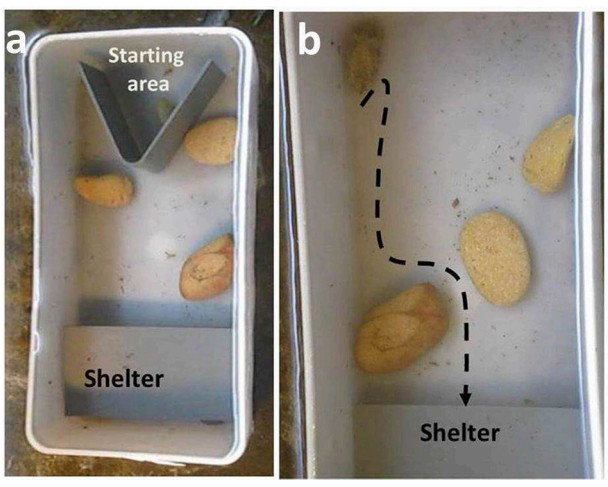

FIGURE 1 | Experiment 1- Rock obstacles: (a) training setup, **(b)** testing setup. Cuttlefish were set at the starting area and after settling on the bottom of 30 sec. were allowed to move into the shaded shelter. In training sessions the rocks were set along the sides of the tank and a direct line to the shelter was available. In the testing setup the rocks prevented such a direct line and the animals had to go around or above them. Dashed line shows the path of the cuttlefish in this image.

S. gibba Experiments

Subjects

Twelve juvenile *S. gibba* were used in two experiments (Experiment 2 and Experiment 3). Four of the *S. gibba* cuttlefish used for experiment 2 and experiment 3 were used for the night observation experiment. The animals were reared from wild-caught eggs up to 2 months old. During rearing cuttlefish were housed as a group, in an indoor holding tank (40 × 36 × 20 cm; width × length × height, 18 cm water level) with running seawater at sea temperature, at the Underwater Observatory in Eilat, Israel. At 2 months of age, when the animals were about 15 mm mantel length and about 5 mm tall (body height) and 8–9 mm wide, they were transferred into a different holding tank of running seawater in the outdoor facilities of the Inter-University-Institute of Eilat (IUI). Both tanks had a sandy bottom, shelters, and rocks to provide an environment resembling natural conditions as much as possible. This enriched environment promotes learning in cuttlefish (Dickel et al., 2000; Poirier et al., 2004, 2005). Animals were fed with shrimps (*Artemia*), which were administered such that food was constantly available in the tank (*ad libitum*).

Experimental Setup—Experiments 2 and 3

The same experimental tank was used for training and testing for the two different experiments. This was a rectangular container made of opaque white plastic 26 × 16 × 11 cm (length × width × height). The tank was arranged into two areas along its long axis: in one half of the tank a sandy bottom and a shelter were provided, the other half of the tank was empty and comprised the "starting area." Two different experimental setups were used for the two experiments (named for consistency 2 and 3). In Experiment 2, the shelter was placed centrally at the end of the sandy area (**Figures 2a,b**). A transparent plastic separator was used to constrain the animal in the "starting area," which consisted of the first portion of the empty area about 3 cm long and as wide as the tank itself (**Figures 2a,b**). During training only the sand and the shelter were present in the tank (**Figure 2a**). During the test (Experiment 2), a small "rock fence" was placed at the beginning of the sandy area, between the shelter and the "starting area." This "rock fence" consisted of 3 small rocks, two smaller ones placed laterally at each side of the tank and a wider one placed centrally. Thus, only two narrow passages over the sand were available to reach the shelter behind the rocks (**Figures 2a**, **4a**). The shelter was always visible to the animal from its starting position both in between and beyond the rocks.

The animal could thus reach the shelter by passing over one of the three rocks or around them through one of the two sandy passages in between them. Any vertical route over one of the rocks was always slightly longer (by 2–6 cm, equivalent to 1.5–4 body lengths) than a route through the sand, as the test always started when the animal had settled on the bottom in the starting area.

In Experiment 3 "rock barrier," the shelter was placed in the left corner of the sandy area (**Figures 3a,b**). Two plastic separators were used to delimit the "starting area" at the left corner of the empty area (**Figures 3a,b**). During training only the sand and the shelter were present in the tank to allow the animals to learn how to reach the latter (**Figure 3a**). During the test a single rock (13 cm wide) was placed between the start point and the shelter, blocking the left-central area of the tank (**Figure 3a**). Also in this case, the shelter was visible to the animal if peeking over the top edge of the rock (**Figure 4b**). The animal could reach the shelter either by swimming straight over the rock (20–25 cm), or by swimming around it (27, 6 cm) through a narrow passage over the sand on the right side of the rock (**Figures 3b**, **4b**). The straight path over the rock was therefore shorter than the horizontal detour around it. All the rocks used as barriers were 2–3 cm tall, which was at least 3 body heights of the animals (Figure S1). Water level was maintained at 10 cm in all experiments, therefore at least 6 cm of water column were available to the animals to swim above the barriers.

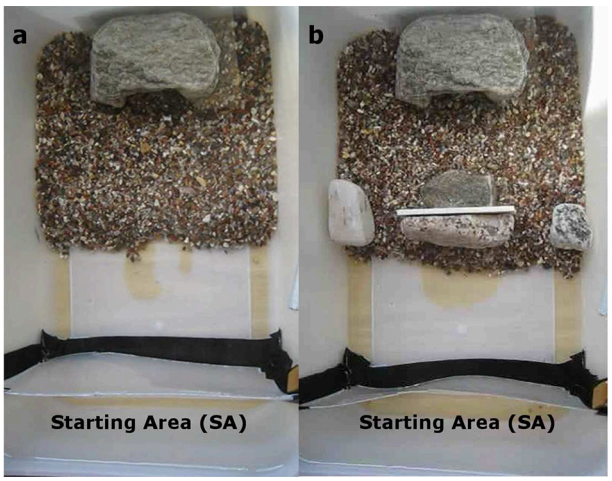

FIGURE 2 | Experiment 2 - "Rock Fence": training setup (a) and test setup **(b)**. The experimental tank is divided into 2 areas: one half has a sandy bottom and a shelter placed centrally, the other half is empty and comprises the "starting area" (SA). This consists of the first portion of the empty area. In the "Rock Fence" test setup there are two narrow passages in the fence.

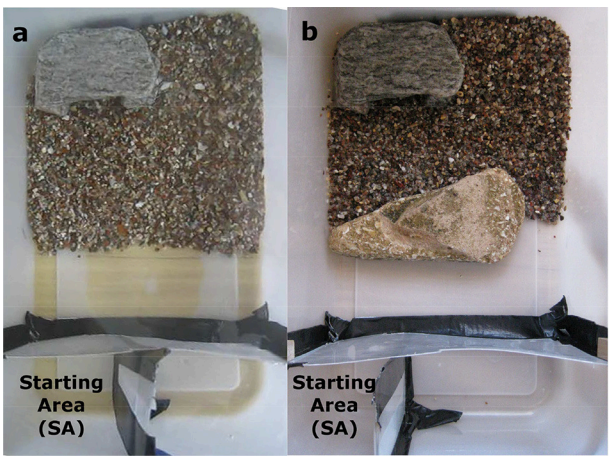

FIGURE 3 | Experiment 3—"Rock Detour": training setup (a) and test setup **(b)**. The experimental tank is divided into 2 areas: one half has a sandy bottom and a shelter placed in the left corner, the other half is empty and comprises the "starting area" (SA). This consists of the left corner of the empty area. In the "Rock Detour" test setup the cuttlefish are released at the left side of the tank and need to go above the rock or all the way around it.

Training and Testing—Experiments 2 and 3

The general training and testing procedures were the same for both experiments (Experiment 2, 3). The animals were always tested in the afternoon. Cuttlefish were given 5 training presentations in a row to learn to reach the shelter placed at the opposite side of the experimental tank. In each trial, the animal was placed in the "starting area" (SA) at one side of the tank, in the empty half of the apparatus. Once the animal had settled and after at least 30 s, the transparent wall was raised, and the animal was allowed to move to the shelter. Once the animal had reached the shelter, it was rewarded with 15 min rest in it.

After 5 training runs, a test trial was given. The test trial was performed as the training, but a rock barrier was placed in between the starting point and the shelter. This barrier consisted of a small rock fence (Experiment 2) or a wide rock (Experiment 3). The animal route to the shelter was recorded. The animal was given 15 min rest in the shelter as a reward once it had reached it.

All training and test trials were video-recorded.

Experimental Setup—Night Observation Experiment

A tank (40 × 18 × 25 cm, length × width × height) was filled with sea sand, 5 rocks and a shelter made of rocks. All rocks were tall about 4–12 body heights the cuttlefish (**Figure 5**). Water level was maintained at 20 cm. The animals were placed in the tank before sunset, and fresh live *Artemia* was added so that the animals could eat at libitum. The behavior of the animals was video-recorded overnight with an infrared camera and light both positioned in front of the tank.

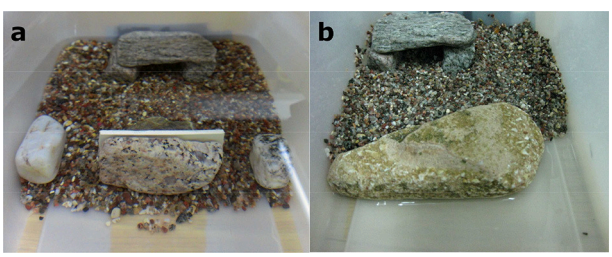

FIGURE 4 | Front views of the test setups: Experiment 2—"Rock Fence" (a), and Experiment 3—"Rock Detour" **(b)**.

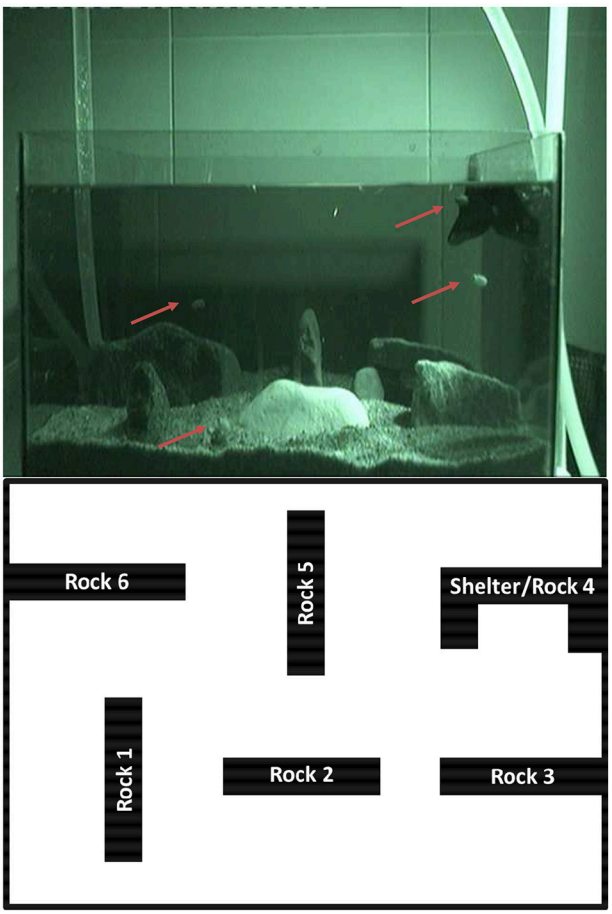

FIGURE 5 | Night observation experiment. Video image of four juvenile *Sepia gibba* cuttlefish hunting at night, video recorded with infrared lights. Cuttlefish can be seen using the entire water column during hunting (above). Scheme of the tank used for the night observation providing a top view of the 6 rocks arrangement (below).

For the analysis, we divided the video-image of the tank in 3 equal depth levels (level 1, level 2, level 3). The top border of level 1 matched the top of the tallest rocks. We recorded the percentage of time each cuttlefish spent in each of the three levels for the first 3 h 30, as nocturnal animals are most active in the first hours after sunset. In addition, we also recorded for each cuttlefish the time spent settled on the bottom, settled on one of the rocks, at the surface and the time in which the animal was not visible because it was behind one of the rocks.

Statistical Analysis

A non–parametric Binomial test with even (0.5) expectancy was used to assess whether the animals' choices during the tests deviated from chance. A *t*-test was used to assess whether there was a difference between the mean time to reach the shelter during the last training trial and the test in Experiment 1 and 2. In Experiment 3, as data were not normal, a Wilcoxon signed-rank test was used to assess whether the mean time to reach the shelter during the last training trial differed significantly from the time needed during the test. As for the night observation experiment, for the values related to the last 2 h of the analyzed video, when animals were clearly distinguishable from each other, a one-way repeated measures Anova was used to compare the percentage time spent at each level. A paired *t*-test was used to compare only the time spent at the first level with the time spent at the second and third levels pooled together. Statistical analyses were performed using R. version 0.98.501 (RStudio, Boston, MA, USA).

RESULTS

S. officinalis Experiments

Experiment 1—"Rock Obstacles"

All 16 young *S. officinalis* cuttlefish moved to the shelter during training and testing. However, while in the first training session it took them on average 9.33 ± 4.5 min (mean ± SD), on the last one they did it in under 5 min (4.67 ± 2.4 min; *t*-test $p < 0.01$). In test runs it took them a little longer but still similar to the last training session (5.25 ± 3.58 min; *t*-test $p > 0.5$). All but one cuttlefish chose the longer yet closer to the ground path (ex. **Figure 1b**), with a single animal going over the top of a rock and swimming rapidly into the shelter.

S. gibba Experiments

Experiment 2—"Rock Fence"

During the test runs, 11 out of 12 animals swam around the rocks, although 3 animals swam at the same height as the top edge of the rocks or immediately below it (**Figure 6A**) (Binomial test: $p < 0.001$). Only one of the 12 animals swam to the shelter by hovering over the left edge of the rock on the right. One animal, despite choosing to swim over the sandy passage on the right, reached it by swimming at the edge of the rock barrier; therefore it definitely swam higher than the rocks. Most animals selected the sandy passage most close to their starting position, where they had settled before. Only one animal started from the left corner of the starting area and swam to the shelter via the sandy passage on the right instead of using the closest one on the left. Animals took a mean of 10.9 ± 6.7 s (mean ± SD) to reach the shelter in the last training trial, and 9.27 (± 3.4 SD) seconds in the test trial. There was no significant difference between the time needed to reach the shelter in the last training trial and in the test (*t*-test $p = 0.4$). We excluded from the calculation of these mean values the latency to reach the shelter needed by an individual which stopped at the rock for 43 s before moving to the shelter during the test run. This animal took 58 s in total to reach the shelter.

Experiment 3—"Rock Detour"

During the test runs, nine out of 12 animals swam over the rock while 3 animals swam around the rock via the sandy passage on the right side (**Figure 6B**, Binomial test: $p = 0.073$). Since the animal started always from the left corner of the empty half of the tank, swimming over the left-central part of the rock represented the shortest route to the shelter. Of the 9 animals that swam over the rock, 5 swam over its central part—which was the shortest way, 2 over its left edge and 2 over its right edge.

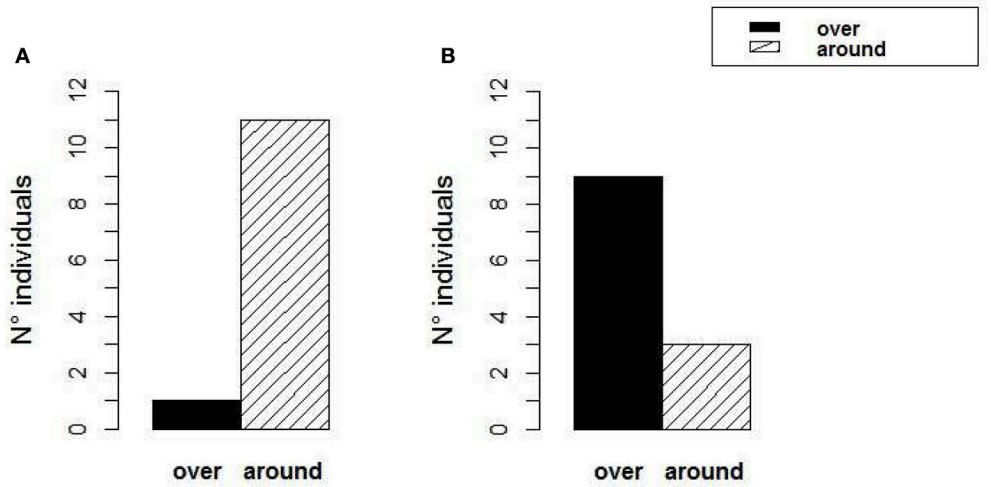

FIGURE 6 | *Sepia gibba* choices. (A) Experiment 2—"Rock Fence": 11 out of 12 animals chose to reach the shelter through one of the two sandy passages around the rocks, while one animal swam over one of the rocks. **(B)** Experiment 3—"Rock Detour": 9 out of 12 animals chose to reach the shelter by swimming over the rock, and only 3 animals made a detour around the right edge of the rock and reached the shelter via the sandy passage on the right.

Only 5 out of the 9 animals who swam over the rock went on a straight path directly from their starting position to the shelter over the left-central part of the rock. However, it seems that the remaining 4 animals selected the shortest route possible from the point from which they started heading toward the shelter crossing the rock barrier. Animals took a mean of 10.45 (± 5.4 SD) seconds to reach the shelter in the last training trial, and 26.18 (± 16 SD) seconds in the test trial (Wilcoxon signed-ranks test: $p < 0.05$). We excluded from the calculation of these mean values, one animal which took 6 min and 17 s to reach the shelter in the test, as it settled at the rock barrier for a long time.

Night Observation Experiment

During the first hour and a half, at least two animals were not visible and behind the same rock at the same time; hence for this period we pooled the data of the 4 animals together and calculated a total percentage of time for each parameter. For the remaining 2 h, the 4 animals were always discriminable and time periods were calculated for each of them separately.

Animals spent most of the time in the lowest water level. In the first hour and a half animals spent a total of 83.2% of time in level one (the lowest level), 7.4% of time in the middle level (level two) and 9.2% of time in the highest level (level three) (**Table 1**). In the following 2 h, animals spent a mean time of 64.8 ± 21.5 % in level 1, 18 ± 10.9 %in level 2 and 17 ± 17.78% in level 3. There was a significant difference among levels (One-way repeated measures ANOVA: F0.05(1),2,3 = 6.62, $p < 0.05$). However, there was no difference between time spent in the first level and time spent in the second and third levels when these last two levels were considered together [paired t-test: $t_{(3)} = 1.3$, $p = 0.26$].

During the first hour and a half, animals spent 7% of time settled on the bottom. However, since animals were invisible to us behind one of the rocks for 31% of the time, we could not know whether in this period they were settled on the bottom or moving around near the bottom (always within the first level). During the following 2 h, animals spent a mean of 23 ± 25% of time settled on the bottom, but were invisible to us behind one of the rocks for 18 ± 21% of the time. Animals spent also 4.8 ± 7% of time at the surface and 15.9 ± 8% of time settled on top of one of the rocks, and one animal even spent 6.6% of time settled on the net wrapping the outflow at the top right side of the tank, a couple of body heights from the water surface. Animals mostly moved to hunt both near the bottom and in mid water or close to the water surface (**Figure 5**); and curiously, if catching a shrimp in mid water they kept eating the prey while maintaining the very same position in the water column (without moving lower or to the bottom).

DISCUSSION

In this study we investigated the use of three-dimensional space by cuttlefish. In particular, we examined the relative use of vertical vs. horizontal space in a navigational task in which the animal had to negotiate an obstacle in order to reach a shelter. We performed separate experiments which differed not only in the species examined but also in the availability of a direct horizontal (along the ground) path that led to the shelter. This direct horizontal path was present in the second experimental setup ("Rock Fence"), along with slightly longer routes over the rocks; whereas, in the first and third setups ("Rock Obstacle" and "Rock Detour") only a longer horizontal detour was available, while the shortest path included a vertical displacement over the rock. We also examined the use of different water depths at night by cuttlefish *S. gibba*.

In the second experiment ("Rock Fence"), 11 of 12 animals reached the shelter by swimming through one of the two sandy passages in between the rocks, and ten out of the them took the

TABLE 1 | Percentage of time spent by *S. gibba* cuttlefish at each water level (Level 1, 2, 3) and settled on bottom or on rocks, at the water surface or behind rocks during the night observation experiment.

Time	Level 1	Level 2	Level 3	Settled on bottom	At water surface	Settled on rocks	Behind rocks
0:00 -1:30	83.23	7.48	9.2	7	0.7	4.5	31
1:30 -3:30	64.8 ± 21.5	18 ±10.9	17 ±17.78	23 ± 25	4.8 ± 7	15.9 ± 8	18 ± 21

Data from the first hour and a half were pooled for all 4 cuttlefish.

passage that provided them with the shortest route to the shelter (the closest passage). In the first ("Rock Obstacle") experiment 15 out of 16 animals chose the slightly longer path while staying near the bottom. However, in the third experiment ("Rock Detour"), when the single narrow passage over the sand was further away from the animals' starting point, the cuttlefish more frequently swam over the rocks than along the longer horizontal detour. Therefore we conclude that, at least during day time, cuttlefish are more likely to move along the bottom, but will take a vertical path if it significantly shortens their way or if the horizontal path may be perceived as blocked. In the night observation experiment, the animals spent most of the time in the lowest water level (level 1), namely within 12 body heights. However, time spent at the first level was not significantly different from time spent above 12 body heights, in both levels 2 and 3. This suggests that at night, *S. gibba* cuttlefish as *S. officinalis* spend a considerable amount of time also far above the bottom mostly hunting and eating in the water column, but also settled camouflaged on tall structures.

The significantly longer time to reach the shelter required in the Detour but not in the Rock Fence test compared with the last training trial might be due to the reduced visibility of the shelter in the Detour test. While in the Rock Fence test cuttlefish could see the shelter from its bottom to its top, in the Detour setup they could only see its top portion behind the rock and might have needed more time to recognize it. In addition, in this case the direct path to the shelter comprised a vertical component thus it was longer than the direct path on the ground of the training run. Alternatively, cuttlefish might have moved slower during the Detour test compared to the training. Unfortunately, we could not assess the animal speed as the cuttlefish movement was recorded only from above and the animal moved both horizontally and vertically. In detour experiments with a vertical component, rats showed a preference for the horizontal-first path over the vertical-first path when both paths were equal in length. However, when the length of the previously preferred horizontal-first route was increased, rats climbing upwards to the goal chose each path equally often (Jovalekic et al., 2011). Other surface-bound species, such as ants and humans, also select routes that include a vertical displacement only when their energetic cost is less than that of an alternative horizontal route (Denny et al., 2001; McNeill Alexander, 2002; Wall et al., 2006; Layton et al., 2009; Holt and Askew, 2012). The energetic cost for vertical and horizontal locomotion in cuttlefish has not yet been thoroughly investigated. However, since cuttlefish and Nautilus have a similar buoyancy system, we can assume that, as in Nautilus (Webber et al., 2000), the cost of vertical movement is similar to that of horizontal swimming (Webber et al., 2000; Aitken and O'Dor, 2006). Hence, since in the Rock Detour the vertical route over the rock was shorter than the horizontal detour, it might have been the less energetically costly. However, in the "Rock obstacles" experiment cuttlefish chose the horizontal route even if this was slightly longer and thus more "expensive" than the vertical. Optimal path choice also depends on factors other than distance and energetic cost, such as predation risk and resource distribution (Makin et al., 2012; Shepard et al., 2013; Sparks et al., 2013). For example, wood ants *Formica rufa* prefer vertical to horizontal detours when these are equal in length (Denny et al., 2001), and this might be associated with the fact that their aphids preys galleries are spread vertically within the canopy (Skinner, 1998). In our case, the cost of using a vertical path should be regarded mainly in terms of safety: exposure to predators by moving up vs. longer exposure by being out of a shelter. The cost of a slightly longer horizontal detour was still lower than the predation cost associated with a vertical route in the Rock obstacles test, but not in the Detour test. Cuttlefish use crypsis as main anti-predatory tactic and as long as they stay camouflaged on the bottom they are hard to detect by predators even when these pass just over them (Hanlon and Messenger, 1988; Staudinger et al., 2013). Direct observations of cuttlefish antipredator behavior in the wild are rare, thus it is hard to assess whether the short vertical distances cuttlefish moved in this study are relevant to predation. However, cuttlefish have demersal and benthic fish threatening them even within the first meter above the seabed (Hanlon and Messenger, 1988; Guerra, 2006), and high predation risk is perceived when a predator swims 6 body heights above the animal (about the range of the vertical movement in this study) (Okamoto et al., 2015). Therefore, even small displacements upwards in the water column can enhance predation risk. In these experiments we used two different cuttlefish species. *Sepia officinalis* inhabits sandy, or rocky substrates and seagrass areas (Guerra, 2006; Guerra et al., 2016), whereas *Sepia gibba* is found in coral reefs, which are among the most complex marine habitats not only along the horizontal but also in the vertical plane (Luckhurst and Luckhurst, 1978; Reid, 2005; Tokeshi and Arakaki, 2012). The availability of preys and shelters in the vertical plane is likely higher in coral reefs than in the habitat of *S. officinalis*. Therefore, we expected *S. gibba* to be more prone to use the vertical space than S. officinalis. However, each species was given its own set of experimental tasks. Under such diverging conditions it is hard to come to clear cut conclusions on habitat-driven behavioral differences or to expand conclusions to an entire genus. In our experiments, both species demonstrated a

preference to swimming close to the bottom and while capable of swimming vertically, they did it only adjacent to the surface of the rock. Nonetheless, we showed here that although cuttlefish of both species are basically bottom dwellers, they do use the vertical dimension even by day and move up into the water column when needing to reach a desired location.

Cuttlefish selected paths containing a vertical component more often than the horizontal-only path not only in the Rock Detour when this was much shorter, but also in the "Rock Fence" 3 of the 12 animals moved through the sandy passage by swimming at the same height as the top edge of the surrounding rocks and one of the animals even chose a route over one of the rocks. Also in the "Rock obstacle" experiment one cuttlefish preferred to swim over the rock. Similarly, in the study of Karson et al. (2003), when cuttlefish had to move much higher than in our setup, a few of them still preferred the upper escape hole. Therefore, we believe that these findings (Karson et al., 2003) support our conclusion that, despite being predominantly benthic and preferring to move close to the ground, cuttlefish do also select routes away from the bottom and move in the vertical plane as well. However, as moving vertically away from the bottom is more risky, cuttlefish might need a greater accuracy in the evaluation of positions in this plane. This could explain the preference for vertical information showed in our previous study, where cuttlefish preferentially relied on the correct vertical coordinate rather than on the correct horizontal coordinate of a learned 3D location when these were in conflict (Scatà et al., 2016). The fact that cuttlefish do use the vertical space not only at nightime, but also when needed during the day, suggests that vertical space may be quite important for these animals. High contrast visual cues in the vertical plane seem to be more relevant to a camouflaging cuttlefish than horizontal ones (Mäthger et al., 2006; Ulmer et al., 2013), possibly because masquerading as a nearby object is more effective than blending to the substrate (Buresch et al., 2011). Thus, cuttlefish may as well remember the position of vertical structures in their environment to return to specific locations for effective camouflage or shelter. For example, a single cuttlefish was followed in the field swimming up 3 m to overcome a vertical wall and reach a crevice behind it (Jozet-Alves et al., 2014). Our results are corroborated by a recent preliminary study, when laboratory-reared *Sepia officinalis* cuttlefish were observed using shelters at different heights along the water column (up to 7 body heights higher) and moving to such shelters even during the day (G. Scatà, N. Shashar, and C. Jozet-Alves unpublished results, obtained during a COST Action FA1301 - STSM project). This also suggests that even cuttlefish species living in less complex environments could quickly adapt to vertical structures when available. Cephalopods have highly flexible behavior and they do not seem to need complex habitats for example to express sophisticated camouflage (Shohet et al., 2007; Bush et al., 2017).

S. officinalis cuttlefish are mostly active at night, when they move upwards, most likely to forage (Denton and Gilpen-Brown, 1961; Castro and Guerra, 1989; Guerra, 2006; Wearmouth et al., 2013). We observed such behavior also in lab-reared juvenile *Sepia gibba* cuttlefish, which at night moved often upwards to hunt, even all the way to the surface, and settled from time to time on top of the tall rocks (4–12 body heights). Our experiments were conducted during daytime and under constant artificial light conditions and thus may have inhibited the upward movement displayed naturally at night by cuttlefish (Denton and Gilpen-Brown, 1961; Wearmouth et al., 2013). For example, cockroaches that are nocturnal like cuttlefish, tunnel underneath an obstacle in light conditions but climb over it in the dark (Harley et al., 2009). However, at least in captivity, *S. officinalis* spend most of the night close to the water surface with little or no return to the bottom, while during the day remain on the bottom with occasional upwards trips (Wearmouth et al., 2013). In our experiment *S. gibba* cuttlefish did not spend most of the analyzed night time close to the water surface as described for *S. officinalis*. This could be due to different experimental conditions: in our study the animals were provided with *ad libitum* food throughout the night and day, therefore the need to hunt might have been reduced in our animals compared to the *S. officinalis* cuttlefish used in previous studies (Denton and Gilpen-Brown, 1961; Wearmouth et al., 2013); in addition we also used juvenile instead of adult cuttlefish which might as well show a different behavior. However, they did spend similar amount of time close to the bottom as in the upper water levels, and relatively little time settled on the bottom. Therefore, it seems more appropriate to study shelter seeking during daytime when this behavior is more natural.

Nonetheless, cuttlefish seem to change their behavioral patterns between night and day, and vertical movement may be more important at night.

We believe that the relatively low water level (20 times the animals bh), and the space above the rocks (about 12 bh) was enough to allow natural swimming behavior over the rocks. Indeed, some cuttlefish in our study did move even all the way to the surface in the experimental tank before heading to the shelter. Thus, the animals were not inhibited to move upwards by the shallow water. However, it is possible that in deeper waters cuttlefish swim more frequently over vertical obstacles. Further studies are needed to investigate whether this is the case and to explore vertical space use during other behaviors and day/night conditions.

ETHICS STATEMENT

The experiments were carried out in accordance with the recommendations of the French and Israeli National Legislation for animal experiments and with the recommendations of the EU directive 2010/63 on the protection of animals used for scientific purposes (Fiorito et al., 2014; Smith et al., 2013). The study was approved by the Committee for experimental with animals, Ben Gurion University, Israel.

AUTHOR CONTRIBUTIONS

Conceptualization: NS, GS, LD, AD; Methodology: NS, GS, AD; Investigation: NS, GS, SM; Formal analysis: GS and NS; Writing:

GS, NS, LD, AD; Funding acquisition: NS, LD, AD; Resources: NS, LD, AD; Supervision: NS and LD

FUNDINGS

This research did not receive a specific grant from any funding agency in the public, commercial or not-for-profit sectors. NS was an invited scholar at the Univ. of Caen, Normandy, France, during part of the study. GS was supported by a Master's degree scholarship from Ben Gurion University of the Negev, Eilat, Israel.

ACKNOWLEDGMENTS

We thank the staff of the Centre de Recherches en Environnement Côtier (CREC, Luc-sur-Mer, France), and in particular Jean-Paul Lehodey, Olivier Desmurs and Frédéric Guyon for their continual technical and logistical assistance. GS and NS are grateful to the CREC staff for making our stay a most pleasant experience. We thank the hospitality and technical support of the staff of the H. Steinitz Marine Biology Laboratory, the Interuniversity Institute for Marine Sciences in Eilat. We also thank the Underwater Observatory, in particular Shiri Ayal, for the supply of *S. gibba* cuttlefish and for assistance in the care of the animals. In addition, we thank our research group, in particular Aiste Klimasauskaite and Noam Josef, for their assistance, input and suggestions, and Lilac Shashar for assistance in experiments in France. Special thanks to Wen-Sung Chung for assistance in species recognition and to Meghan Rousseau for her valuable proofreading of the manuscript. The experiments performed in this study complied with the French and Israeli National Legislation for animal experiments and with EU directive 2010/63 on the protection of animals used for scientific purposes (Fiorito et al., 2014; Smith et al., 2013).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00173/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 17 January 2017
doi: 10.3389/fphys.2016.00671

Size Matters: Observed and Modeled Camouflage Response of European Cuttlefish (*Sepia officinalis*) to Different Substrate Patch Sizes during Movement

Noam Josef[1,2]*, *Igal Berenshtein*[3,4], *Meghan Rousseau*[3,4], *Gabriella Scata*[3], *Graziano Fiorito*[5] and *Nadav Shashar*[3]

[1] Mote Marine Laboratory, Directorate of Marine Biology and Conservation, Sarasota, FL, USA, [2] Institute for Human and Machine Cognition, Pensacola, FL, USA, [3] Eilat Campus, Department of Life Sciences, Ben-Gurion University of the Negev, Beer Sheva, Israel, [4] H. Steinitz Marine Biology Laboratory, Interuniversity Institute for Marine Sciences, Eilat, Israel, [5] Stazione Zoologica Anton Dohrn, Napoli, Italy

Camouflage is common throughout the phylogenetic tree and is largely used to minimize detection by predator or prey. Cephalopods, and in particular *Sepia officinalis* cuttlefish, are common models for camouflage studies. Predator avoidance behavior is particularly important in this group of soft-bodied animals that lack significant physical defenses. While previous studies have suggested that immobile cephalopods selectively camouflage to objects in their immediate surroundings, the camouflage characteristics of cuttlefish during movement are largely unknown. In a heterogenic environment, the visual background and substrate feature changes quickly as the animal swim across it, wherein substrate patch is a distinctive and high contrast patch of substrate in the animal's trajectory. In the current study, we examine the effect of substrate patch size on cuttlefish camouflage, and specifically the minimal size of an object for eliciting intensity matching response while moving. Our results indicated that substrate patch size has a positive effect on animal's reflectance change, and that the threshold patch size resulting in camouflage response falls between 10 and 19 cm (width). These observations suggest that the animal's length (7.2–12.3 cm mantle length in our case) serves as a possible threshold filter below which objects are considered irrelevant for camouflage, reducing the frequency of reflectance changes—which may lead to detection. Accordingly, we have constructed a computational model capturing the main features of the observed camouflaging behavior, provided for cephalopod camouflage during movement.

Keywords: crypsis, cephalopods, vision, object size recognition, camouflage modeling, behavior, background matching, cognition

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Edited by:
Daniel Osorio, University of Sussex, UK

Reviewed by:
Erik Caroselli, University of Bologna, Italy
Eric Warrant, Lund University, Sweden

***Correspondence:**
Noam Josef
njosef@mote.org

Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *16 October 2016*
Accepted: *19 December 2016*
Published: *17 January 2017*

Citation:
Josef N, Berenshtein I, Rousseau M, Scata G, Fiorito G and Shashar N (2017) Size Matters: Observed and Modeled Camouflage Response of European Cuttlefish (Sepia officinalis) to Different Substrate Patch Sizes during Movement. Front. Physiol. 7:671. doi: 10.3389/fphys.2016.00671

INTRODUCTION

Animals often use camouflage to avoid detection by either predators or prey (Skelhorn and Rowe, 2016). Camouflage can take several forms: crypsis (avoiding detection) (Stevens and Merilaita, 2009), mimicry (resembling a defended organism) (Speed, 1993) and masquerading (resembling an inedible object) (Skelhorn et al., 2010). Crypsis in general, and background matching in particular,

are examples of adaptation, where mismatch results in high susceptibility to detection (Ruxton et al., 2004; Caro, 2005a,b).

Coleoid cephalopods (octopuses, cuttlefish, and squid) are often preyed upon by marine mammals, eels, sharks and many other fishes (Aronson, 1991). Such selective forces drove this group of animals to develop various coloration capabilities and behaviors, including adaptive camouflage (Cott, 1940; Hanlon and Messenger, 1998; Barbosa et al., 2007). Adaptive camouflage is the capacity of animals to modify their appearance according to their habitat, to resemble specific background features in their immediate surroundings, or to perform background matching and context-dependent body patterning while moving (Keeble and Gamble, 1899; Gamble and Keeble, 1900; Josef et al., 2012, 2015; Jensen and Egnotovich, 2015). Cuttlefish can dynamically and rapidly camouflage themselves against a variety of natural backgrounds (Thomson, 1920; Hanlon and Messenger, 1998) using specialized tissues: the chromatophores, iridophores, leucophores, and papillae. These marine molluscs possess a keen visual system which can rapidly assess complex visual scenes and reflect them as camouflage body patterns, reviewed in Chiao et al. (2007). Body patterning, texture and body posture is adjusted to their intended audience (Boal et al., 2004) and is effected by background intensity (Chiao et al., 2007), spectrum (Akkaynak et al., 2013), contrast (Chiao et al., 2007, 2010), 3D environment structure (Buresch et al., 2011), background orientation (Barbosa et al., 2012) and object edges (Chiao et al., 2013). As in most evolutionary arms-races, the capacity to quickly alter one's body patterns and camouflage against visual backgrounds may have facilitated the development of visual mechanisms that enhance cephalopods' predators and prey ability to identify objects of interest; examples of such mechanisms include figure/ground discrimination by relative motion and edge detection (Land and Nilsson, 2012; Cronin et al., 2014). Previous studies have categorized cuttlefish's pattern repertoire to: uniform, mottled and disruptive (Hanlon and Messenger, 1998; Hanlon et al., 2007); this was recently implemented in an automated quantitative algorithm successfully classifying images of cuttlefish into these three categories (Orenstein et al., 2016). Coleoid camouflage capabilities have been intensively studied, yet little is known about how changes in appearance operate over variable timescales, or the mechanisms involved, ranging from short term reflectance change to longer phenotypic plasticity (Nettle and Bateson, 2015).

The benthic marine environments of coral reefs, sea grass or sandy seabed are constructed of many microhabitats largely characterized by a wide range of textures, brightness levels and contrast. Furthermore, flicker, or wave induced moving light patterns, also temporarily change the appearance of these backgrounds (Mcfarland and Loew, 1983). For a given cuttlefish swimming in such an environment, responding to small and possibly transient visual stimuli in its surroundings may subject the animal to dangerous mismatching, and the allocation of unnecessary processing effort. Hence, it is likely that the moving animal will react to patterns large enough to allow matching. Therefore, we hypothesize that a minimal threshold may exist for any Camouflage Eliciting Patch Size (CEPS). We conceive that this threshold represents the smallest background patch eliciting a quick dynamic camouflaging reaction. Note that "small" could be in terms of relative size, angular size, duration of encounter, or other terms relevant to the animal. Moreover, we hypothesize the existence of a positive correlation between patch-size and change in mantle reflectance.

In this study we tested the occurrence and intensity of the moving animal's reaction to visual patchs of various sizes. We identified the minimal CEPS threshold, and provide a possible camouflage model for a swimming *S. officinalis* cuttlefish.

MATERIALS AND METHODS

In this set of experiments we were using the same experimental design and methodologies extensively detailed and described in Josef et al. (2015), modified mainly for the artificial backgrounds (see experimental design and testing procedure sections Experimental Design, Testing Procedure).

(a) Animals: Eight naive common European cuttlefish (*Sepia officinalis),* mantle length of 7.2–12.3 cm (10.2 ± 1.2 cm: mean ± SD) were collected from the Gulf of Naples, Italy and were held in separate tanks with running seawater, at the Stazione Zoologica Anton Dohrn in Italy, for 2 days of acclimatization. The cuttlefish were fed with live crabs, and maintained under a 12:12 (D: L) light regime. When experiments ended, all animals were returned to the Gulf of Naples. The experiments carried out in this study complied with the Italian National Legislation for animal experiments and with EU directive 2010/63 on the protection of animals used for scientific purposes (Smith et al., 2013; Fiorito et al., 2014).

(b) Experimental design: All visual cues and external stressors were minimized by performing the experiments in a secluded room with a curtain surrounding the set-up. An elongated tank (200 × 40 cm, water level 45 cm) was colored in a uniform 18% reflectance gray (**Figure 1A**); the reflectance throughout this study was based on a standard 18% gray card, photographed inside the elongated sea water tank, where 0 to 100% represents black and white respectively. All eight cuttlefish were placed in the elongated tank with either a control pattern (complete 18% reflectance gray; **Figure 1A**), or a dichromic pattern composed of three areas: 18% gray, 3% black, and 18% gray again (**Figure 1B** Each animal swam, one at a time, across the gray tank as a control, and over a set of six black patches of different sizes. The dark sections varied in length (3, 7, 10, 19, 29, 60 cm, all 40 cm in width spanning the width of the tank); these sections were added at the bottom center of the tank along the animals' swimming course (**Figure 1C**). Since cuttlefish preferentially respond to bottom rather than side stimuli (Taniguchi et al., 2015), the black patch covered the entire width of the tank but not the sides. The swimming cuttlefish were tracked and their mantle reflectance was continuously monitored. Since tactile information is a potential signal for camouflage, all textures were equally and completely smooth.

(c) Illumination across the tank was fairly homogeneous (350 ± 5 lux—measured with a PeakTech 5025 light meter) to

avoid shaded areas or light reflections. The water in the experimental tank was replaced prior to each trial.

(d) Testing procedure: Animals were tested separately during the daytime (9:00–17:00). After being placed at one end of the experimental tank, each animal was left to settle for at least 5 min. We then waited until two conditions were met: (1) the animal remained motionless on one side of the tank; (2) the body color became uniform and generally matched the gray background, and remained stable for at least 2 min. The animals were then observed and video recorded as they moved in the tank, mostly crossing it along its length. If the animals did not move within 15 min of observation, they were motivated to cross the tank either by simply standing at one end of the tank, or by providing a shelter at the opposite side of the tank. Under no circumstances were the animals scared or strongly motivated, to minimize stress. In both control and dichromic conditions, animals were recorded crossing the tank, mantle first, from one side to the other (hereafter: "Full-cross"). Cuttlefish possess both anterior and posterior binocular visual fields which allow them to clearly see and plan their route while swimming forward or backwards (Watanuki et al., 2000). Thus, confining data acquisition to episodes of swimming mantle-first should not bias the results. In the control background, a full crossing of the tank provided information on the animals' changes in body color during motion over a constant background. Introducing the experimental dichromic backgrounds with the variably-sized black patchs allowed assessment of color changes as the animals swam over a gray-to-black and then a black-to-gray background transition. Patch widths were randomized and a single "full-crossing" over the control background and each of the six black patches were recorded for each of the eight animals. This protocol resulted in recordings of 48 experimental full crossings with 96 background transitions: 48 gray-to-black and 48 black-to-gray.

(e) Data acquisition: The animals movements were recorded using a SONY HDR-CX110 digital video camera mounted vertically above the tank providing a top-down view. To achieve high-resolution frames for analysis, the camera was set so its field of view covered the entire width and 70% of the tank's length- filming 1440 × 1080 pixels video files of 140 cm out of the 200 cm tank's length; the final 30 cm at each end of the elongated tank was not recorded.

(f) Data analysis: Cuttlefish possess a single, mid-wavelength visual pigment making them essentially color-blind (Marshall, 1996; Hanlon and Messenger, 1998; Mäthger et al., 2006). Moreover, most of the changes in the background and the cuttlefish display are monochromatic in nature, so we chose to look only at changes in reflectance and not in color. Therefore, only the green channel from all videos were gray-scale transformed, using the green channel alone. Videos were analyzed using a designated MATLAB™ code (Matlab version R2016a, MathWorks Inc., Natick, MA, USA). The code was utilized as follows: loading a video file, transforming each frame into a gray-scale intensity image, balancing each frame according to the 18% gray standard, manually tracking the animal in $\frac{1}{10}$ of a second intervals, and measuring the animal's mantle reflectance consisted of the average value of 1000 (40X25) pixels surrounding the center of the mantel (**Figure 1D**), velocity and relative position in relation to the next background. Although cuttlefish can present three types of body patterns—uniform (little or no variation in body pattern contrast), mottled (small or large-scale light or dark patches), or disruptive (non-repetitive high-contrast patches) (Cott, 1940; Hanlon and Messenger, 1998; Chiao et al., 2007), due to the uniform background in our setup, the animals always elicited a uniform body pattern in all cases. Therefore, we used the value of the mantle sample for data analysis. To characterize trends, we extracted and analyzed each section separately, paying special attention to the start and end points of each transition in body reflectance (Detailed methodology can be found in Josef et al., 2015). Then, the chromatic transitions start and end points were determined by manually selecting points that marked the beginning or end of change in reflectance. A start or end point was only chosen if the trend was maintained for at least three consecutive measurements. Once we set the beginning and end points of all transitions, we calculated the

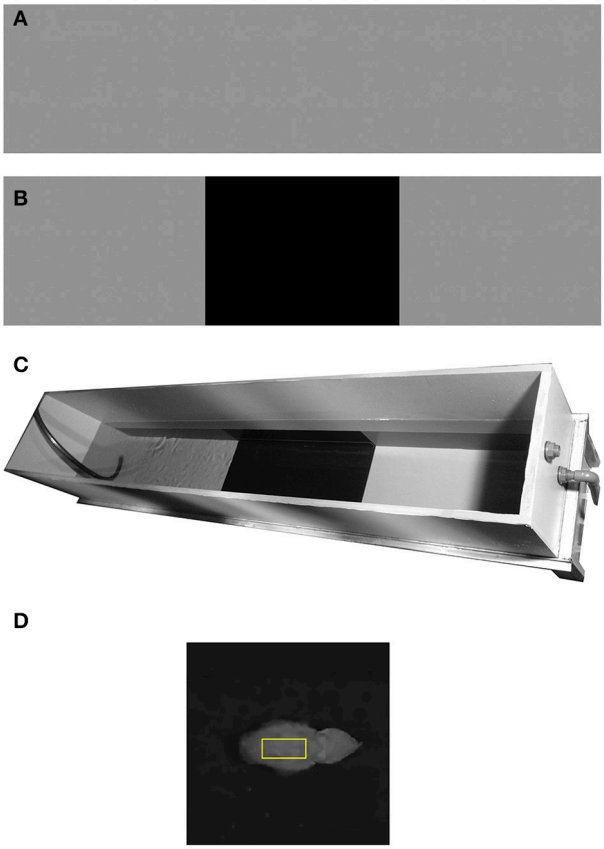

FIGURE 1 | Experimental set up. (A) Control background –uniform 18% gray. **(B)** Dichromic background—providing a change in background reflectance. **(C)** Experimental tank with the dichromic pattern. **(D)** An example of a cuttlefish mantle with a 1000 pixel rectangular sample (yellow).

average slope, which represents the rate at which the animals match their backgrounds. Reflectance change variance was measured for each patch-size, quantifying the variance reaction between animals with patch size of increasing size. The Kruskal–Wallis one-way analysis of variance was used to compare the reflectance change percentage between the different patch sizes. This test allowed us to compare animal reaction to each pattern and to determine in which patch size the control and the test become significantly different.

Camouflage Sampling Area (CSA) is an important region we have defined as a partially occluded oval sub-sample of the environment relevant for the animal's camouflage and visually sampled for dynamically matching it. In our purposed model, the cuttlefish modifies its mantle reflectance according to the mean reflectance captured by its oval field of view.

(g) Modeling the Field Of View (FOV) and the animal's behavior**:** We used MATLAB™ (Matlab version R2016a, MathWorks Inc., Natick, MA, USA) image analysis tool to simulate a dynamic environment where we sampled the changing background (emulating a swimming cuttlefish) (**Figure 2**). Using a designated MATLAB code, we performed the following:

(1) We created a virtual black and gray arena simulating the experimental tank, with increasing in size black patches.
(2) We then created an oval sampling area (4530 pixels), which is analogous to the cuttlefish' oval field of view. The algorithm then computes the average reflectance across all pixels within that oval sample, and records it. Sampling began at the bright area (18% reflectance gray), moving one pixel at a time across the virtual arena toward the black patch (3% reflectance black), and out to the bright area. The model graphs were then superimposed over the actual results graphs (**Figure 3**).
(3) Virtual mantle: We created a virtual mantel (rectangle 40X25 pixels) with a dynamic reflectance, which is modified (under certain circumstances) according to the mean reflectance, captured in the oval Camouflaging Sampling Area (CSA). Moving one pixel at a time, the sampled averages were combined with a stochastic behavior factor (±3%), reflecting behavior variance between individual animals. The full conditioning of the model is described in the discussion part and **Figure 5**.

RESULTS

As expected, while swimming in the uniform gray control tank, all eight animals maintained their overall light and uniform body coloration, matching the background throughout their movement (**Figure 3A**). As patch size increased we observed a gradual increase in number of animals that elicited a camouflage response with increasing patch size and a notable increase in intensity change (darkness level when over the black patch) was recorded for background patches ≥10 cm in length (**Figure 3**).

Comparing animals' reflectance in crossings from a gray to a black background vs. crossings from a black to gray background did not show any significant differences. For example, the trend lines' slopes of animals going onto the patch and going out toward the gray background shows high symmetry around the x axis and both follow a logarithmic trend (**Figure 4A**; $R^2 = 0.96$, $R^2 = 0.95$, respectively). Therefore, from here on, we will only address reflectance change behavior without distinguishing between whether it was from gray to black or from black to gray.

The entire cuttlefish's mantle changed simultaneously, without notable differences between posterior and anterior similar to the findings of a previous study (Josef et al., 2015).

In the control group, without visual background change, animals presented an average change in reflectance of 1.69 ± 0.40 %, while 4.02 ± 2.9% in the treatment group. The reflectance change had a positive linear correlation to patch-size ($r = 0.93$, $p < 0.01$), demonstrating that swimming over increasingly large black patches caused the animals to change their appearance faster (**Figure 4A**), and the overall reflectance-change increased with high correlation to patch size (**Figure 4B**). The Kruskal–Wallis test conducted on the seven different conditions (control and six patch sizes) validated a significant difference ($\chi^2 = 42$, df = 6, $n = 8$, $p < 0.001$) between the control and the 19, 29, and 60 size patches. As patch size increased, the first patch evoking a significantly different reaction than the control was the 19 cm patch.

When looking at the change rate (measured as the sigmoidal reflectance slopes), most animals behaved symmetrically while swimming into the patch and out of it. For both cases, logarithmic curve fit best describes the relationship between the average curve slope and the patch-size ($R^2 = 0.98$ and $R^2 = 0.989$ respectively). Symmetric behavior also expressed in a similar interception point with the x axis (2.25 and 2.897 respectively). The sigmoidal trend line ($R^2 = 0.88$) shows a lag in reflectance-change variance (**Figure 4C**). Note is that **Figure 4A** represents the cuttlefish's reflectance change-rate without addressing the magnitude of the change.

Weak but significant correlation was found between the extent of reflectance change and the animal's average swimming velocity ($r = 0.26$, $p < 0.05$) throughout the different patch sizes (See **Supplementary Figure 1**).

DISCUSSION

To remain cryptic, it is essential for a moving cuttlefish to continuously adjust its appearance according to its changing background. Josef et al. (2015) showed that these animals can also anticipate and match upcoming backgrounds resulting in a gradual, sigmoidal-like function of background matching while moving. Previous studies identified and categorized specific background features that could elicit different skin patterns (Hanlon and Messenger, 1998; Chiao et al., 2007, 2010; Barbosa et al., 2008).

A welter of shapes, brightness levels, and textures, constantly stimulates the visual system of these animals, creating an enormous amount of information. At any given moment, this information must be reduced and prioritized, to obtain a comprehensive image with minimal use of data processing and memory. In primates, for example, visual recognition of objects

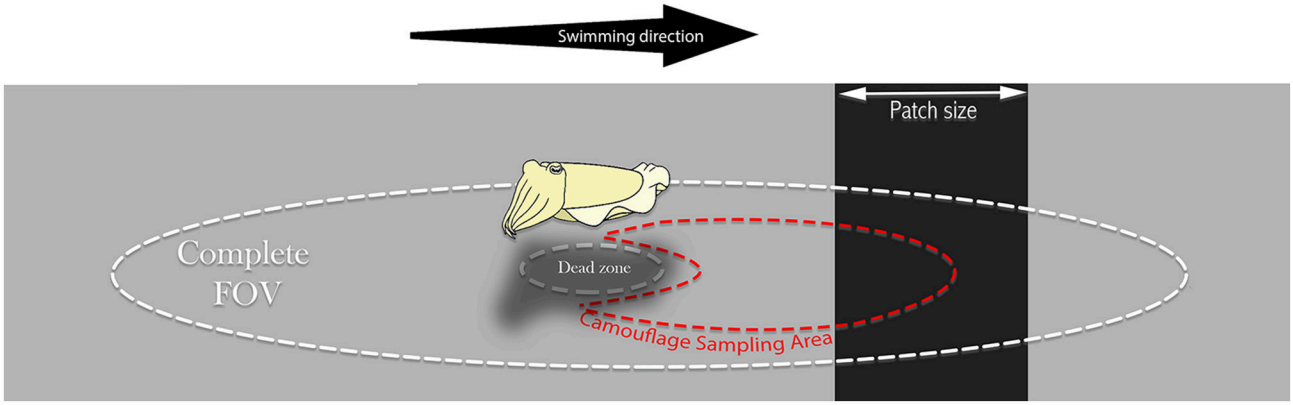

FIGURE 2 | Relevant Cuttlefish's fields of view. The oval cuttlefish Complete Field of View (FOV) is restricted only by its optic and physiological features. We suggest a Camouflaging Sampling Area (CSA) as the relevant sampling area, sized and averaged by the observing animal to match its mantle reflectance under certain conditions for background matching purposes. The CSA is likely to be skewed forward to allow anticipation. Both fields are affected by the visual dead-zone under the animal's body.

depends on the transmission of information from the striate cortex through pre-striate areas into the inferior temporal cortex (Ozaki et al., 1983). The ability to filter out irrelevant visual information is required in developing attention and addressing the most relevant cues in any given visual scene. Moreover, the filtering of irrelevant information from the receptive fields underlies the ability to identify and remember the properties of a particular object out of the many that may be represented (Moran and Desimone, 1985). In the context of camouflage, such mechanisms would be beneficial for a static animal selecting a relevant object/background to match, as well as for a moving animal screening irrelevant cues as they appear.

There are two basic aspects that constrain visual attention. The first is the limited capacity for processing information. At any given time, only a small amount of the available information can be processed and used in the control of behavior. The second is selectivity—much of the information available is not relevant to the animal's tasks and hence animals need to filter out redundant or irrelevant information.

The artificial uniform backgrounds provided a simplified visual environment in which the camouflage of a swimming cuttlefish could be examined and modeled with regard to the patch-size encountered. Here, we would like to stress that in the wild, a clear step like transition between two uniform backgrounds is rare, as most natural scenes include a blending phase comprising complex backgrounds affecting each other. Although very interesting and highly important for further understanding of this process, in the current study we tried to model one of the simplest camouflaging feature and did not study transitions between mottled or disruptive patterns. Nonetheless, this apparatus simplified the visual field and minimized the behavioral response to a single type of reflectance change without addressing complicating factors such as patterns, textures and others.

Animals responded to the size of the patches in the background, yielding stronger changes of reflectance as the patch size increased; camouflage responses occurred more rapidly and were seen in more animals as they swam over larger patches (**Figure 3**), while both intensity and rate of reflectance change increased accordingly (**Figure 4**). Although it is hard to decipher what underlies the variation between the control and the experimental patches for patches up to 10 cm in width, a noticeable difference in rates and in reflectance magnitude was found between the 10 cm patch and the following 19 cm patch size. This difference means that the animals reacted significantly more strongly than to a 19 cm (and wider) patches than to the first three (0–10 cm) patches. This is the first evidence for the existence of CEPS in moving cephalopods suggesting that a possible CEPS threshold laying somewhere between these values. It is worth noting that Chiao (Chiao and Hanlon, 2001) and Hanlon and Zylinski et al. (Zylinski et al., 2011) alluded to a CEPS in stationary cuttlefish.

The first three Patches (3, 7, and 10 cm) elicited a mild change in mantle reflectance. The larger patch sizes (19, 29, and 60 cm) elicited a much more noticeable change in reflectance (both visually and numerically) making a faster transition with a positive linear correlation to patch size. The results support the existence of a CEPS threshold somewhere between the 10 and the 19 cm patch width, for average animal mantle lengths of 10.2 ± 1.2. Hence, the suggested CEPS threshold is slightly larger than the animal's mantle length.

In addition to the basal variation seen in the control group, we found a positive correlation between patch size and variance in the animals' reflectance change; this change is represented by a sigmoidal function. This sigmoidal fit (**Figure 4C**) also demonstrates that the greatest increase in reflectance variance took place for the 10–19 cm patch size range. The limited variances within the smaller-sized patches combined with the moderate reflectance change suggests a subtle behavioral reaction to smaller patch sizes; while the larger patches induced a stronger behavioral response, followed by larger reflectance variance. This

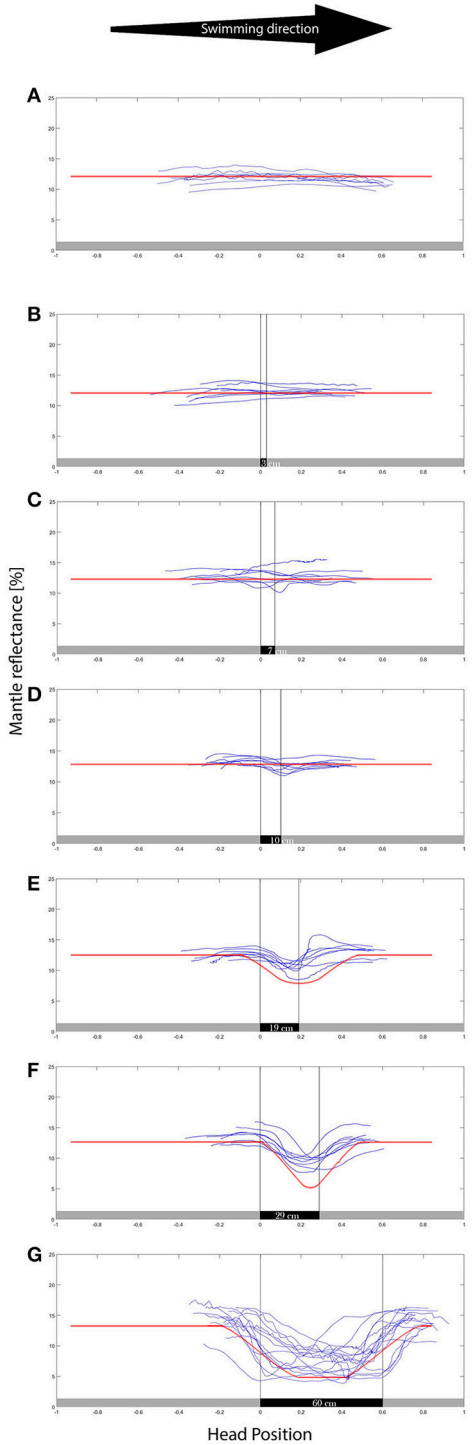

FIGURE 3 | Mantle reflectance values while swimming in the direction indicated over the different backgrounds (**A–G** representing 0–60 cm patches respectively). The X axis is the position of the head/eyes of the cuttlefish, where the 0 represents the transition between gray and the black patch. The animals showed little response to the smaller (0–10 cm) group of patches, exhibiting a growing reflectance-matching behavior to the wider (19–60 cm) background patches. $N = 8$. The red lines are superimposed over the data, representing the computer model results. The model well represents the animal camouflaging behavior.

serves as additional supportive evidence for a greater reaction to the larger patch sizes and a CEPS in the range of the animal's body size (in our case patch sizes somewhere between 10 and 19 cm).

It is conceivable that optic flow might have influenced visual perception and decision-making as our test animals swam between backgrounds. In our experimental trials, the animals swam rather gently without jetting; optic flow likely did not change during these relatively slow swim rates. However, when observed velocities did change, we saw no obvious relationship between the cuttlefishes' velocities and reflectance change. These observations support our conclusion that the visual cue of dimension/magnitude is a primary driver of the animals' reflectance change.

Matching very small background patches and paying attention to many details in it requires a large amount of attention and processing effort. Such a delicate process, is highly sensitive to errors and inconsistencies—inevitably causing the cuttlefish to be conspicuous. The lack of response to small patches might act in according to fitness considerations such that, individuals that changed their reflectance to very small landscape features may have an excess energy investment in camouflaging, or possibly that erratic change in coloration ultimately made them more conspicuous.

However, from the results, it seems there is a very weak reaction to the presence of these objects or patches. We therefore suggest that the cuttlefish preforms patch size estimation with a body-size CEPS threshold. If the threshold is not met, the cuttlefish expresses no change in mantle reflectance, whereas above this threshold they average a partially-occluded oval shaped CSA (Watanuki et al., 2000), resulting in a sigmoidal change in the sample model. This matches all observations in our previous work (Josef et al., 2015). Moreover, according to Josef et al. (2015), cuttlefish show an anticipation behavior, which might be explained if the CSA is in the direction of the animal's movement (**Figure 2**). Considering what is already known regarding cuttlefish background intensity matching (Chiao et al., 2007; Buresch et al., 2015), and the computer model results—we propose that these animals average an approximate oval-shaped subsample of the substrate in the direction of their movement. Such an averaging may also explain the moderate change in some animals in the presence of the small patches and increased reaction when larger patches are introduced. In the 3, 7, and 10 cm patches, some animals responded with a moderate reflectance change while others did not respond. If the animals had been continuously averaging a CSA and changing their mantle appearance accordingly, we would expect to see an increasing reaction throughout—even in the small patches. Since this did not happen, we suggest that a visual evaluation process is involved before the animals cross to the next background. This also corresponds with the findings of Josef et al. (2012) who showed a selective process in octopuses camouflage responses. On the other hand, the sigmoidal reaction signifies that the animals do not use an average reflectance value as the only threshold—which would result in a step function (see **Supplementary Figure 2**). Furthermore, the fact that in the 3,10, and 19 cm patches, 0, 50, and 100% of the animals respectively

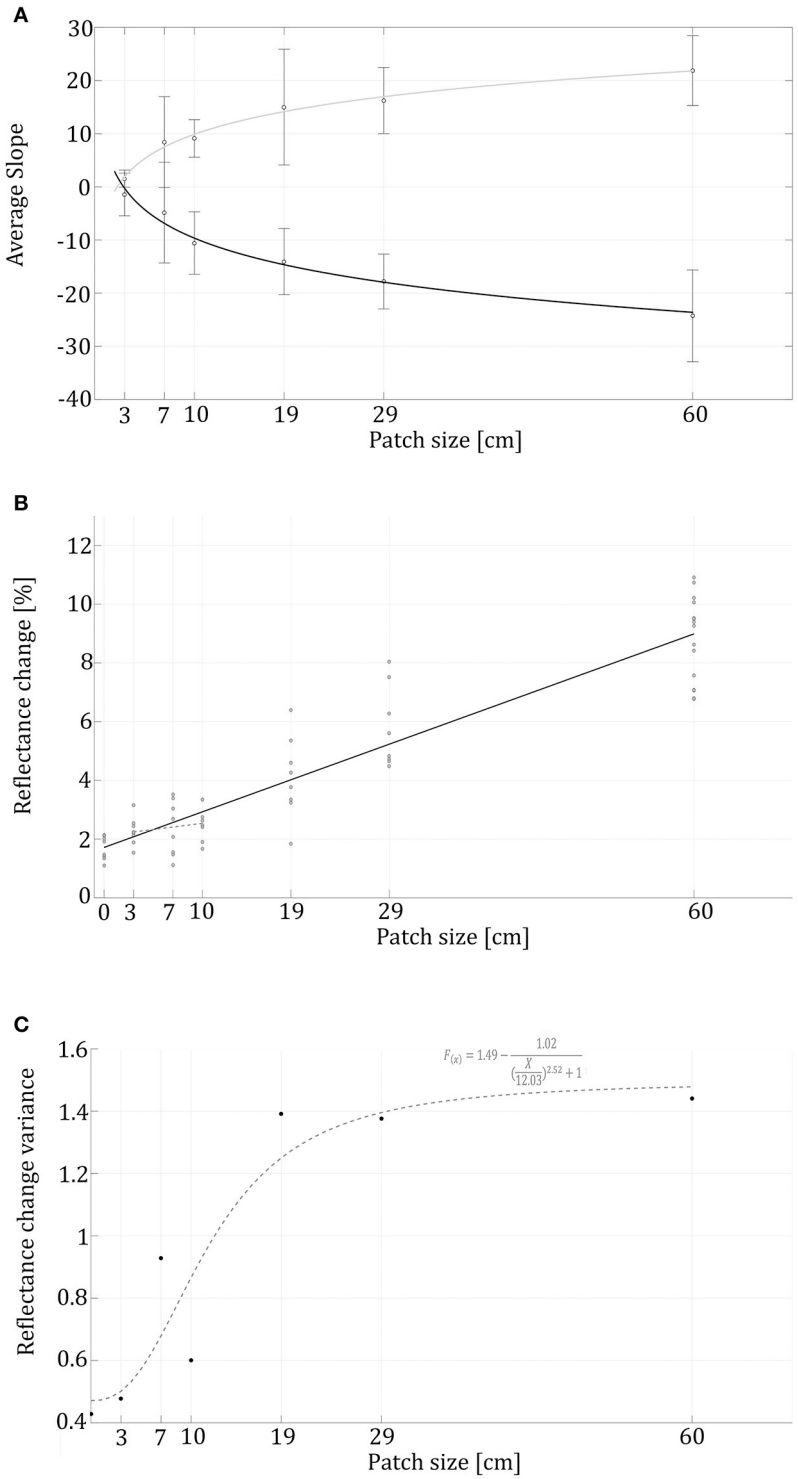

FIGURE 4 | Reflectance change rates and variance in correlation to patch sizes. (A) Logarithmic black trendline represents swimming into a patch ($R^2 = 0.96$), while the gray trendline relates to the animals swimming out of the patch onto the gray background ($R^2 = 0.95$). Reflectance change rate (slopes) validates a symmetrical behavior while swimming into the patch and out of it. **(B)** The black linear trendline ($f_{(x)} = 0.12X + 1.72$, $R^2 = 0.87$) represents the reflectance's correspondence to the patch sizes ($R^2 = 0.87$). **(C)** The variation in reflectance change within each treatment with a sigmoidal dashed trend line ($R^2 = 0.88$).

elicited a camouflage response indicate individual behavioral variation.

In conclusion, based on previous as well as our current studies, we propose a new camouflage behavioral model for a swimming cuttlefish (**Figure 5**). We believe that two relevant oval visual fields: (1) the Entire Field Of View (the complete field of view of the animal, restricted only by its optic limits), and (2) the CSA (an oval subsample, skewed to the direction of animal movement: **Figure 2**) are primarily operative in cuttlefish camouflage responses to visual stimuli. We believe that the model might operate as follows.

The cuttlefish preforms Patch Size Estimation (PSE), possibly using depth perception visual cues (Josef et al., 2014), optic flow (Sun et al., 2014) or possibly by combining multiple visual cues. Specifically, it constantly scans for patches smaller than the CEPS threshold (segment 1, **Figure 5**). As long as a patch is not identified, the cuttlefish gauges whether its Self Reflectance (SR) is significantly different from the background. The latter distinction requires the cuttlefish to assess its own Self-Reflectance (SR) and averaging an approximate oval Camouflaging Sampling Area (CSA). Then it calculates the delta between its immediate background to its own reflectance |SR-CSA|—evaluating its current cryptic status (segment 2, **Figure 5**). If the reflectance difference is larger than a threshold, the cuttlefish modifies its reflectance according to the CSA (segment 3 in **Figure 5**). If the threshold is not surpassed, no change in reflectance will be elicited (segment 4 in **Figure 5**). In cases where the PSE is smaller than the CEPS threshold (body-size in our case), no change in reflectance will be elicited (segment 5, **Figure 5**).

Cuttlefish are well known for their dynamic, responsive and rapidly adjusting camouflage patterns and background matching. In the current study we did not experimentally confirm the existence of a reflectance delta threshold, yet a self-reflectance awareness and crypsis assessment are clearly required for such responses (**Figure 5**, segment 2).

Although our suggested model captures the main features of the cuttlefish camouflage, it is likely that the mechanism is more complex. For example, the averaging of a CSA might be a weighted average, with weight higher at the center of the shape; or a missing-oval shape of the CSA might be non-symmetric and skewed forward. More studies—especially using various artificial and natural patterns—will be required to further compare cuttlefish camouflage with our conceptual model performance and to refine the model accordingly. Finally, this model provides a first step in applying cephalopod camouflage in the growing field of biomimicry, allowing the implementation of the observed camouflage behavior in machine learning protocols, dynamically camouflaging protocols for both recreational and defense purposes.

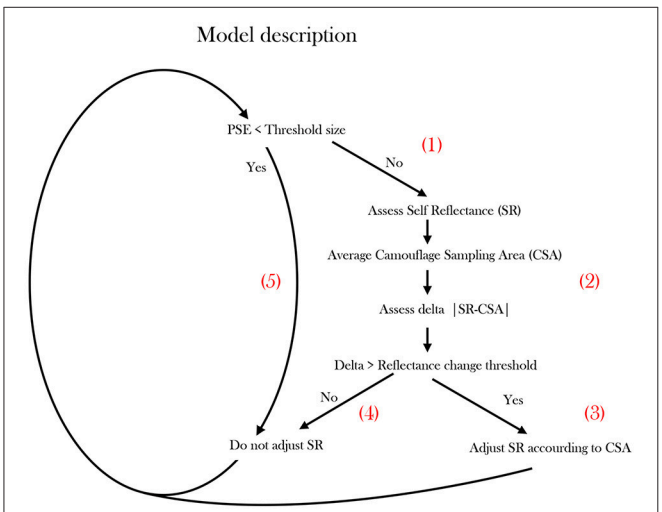

FIGURE 5 | Proposed operational model describing cuttlefish Camouflage behavior. The model scheme simplifies the camouflaging process undertaken by a swimming cuttlefish when encountering different background cues. Patch Size Estimation (PSE).

AUTHOR CONTRIBUTIONS

Conceptual contribution and study design: NJ, GS, and NS. Acquisition of data: NJ, NS, and GF. Analysis and interpretation of data: NJ, NS, IB, and MR. Drafting of manuscript: NJ, IB, GF, and NS. Critical revision: NJ, IB, MR, GS, GF, and NS.

FUNDING

NJ and NS received financial support from the European Community for the access provided to Stazione Zoologica in Naples, through the ASSEMBLE program [EC/FP7 Grant Agreement No. 227799]. The funders played no part in the design of the study, data collection and analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGMENTS

We would like to thank Tal Aruety for his technical support; Marinella Salemme and Giovanni De Martino for their assistance in preliminary experiments and technical aid; and Dr. Anna Maria Grimaldi, Ofri Mann, and Noa Moshkovitz for enriching discussions. We also would like to specially thank Dr. Aaron S. Fink for his assistance in manuscript preparation. We are thankful on the financial support of "COST action FA1301-ChephInAction." NJ and NS received financial support from the European Community for the access provided to Stazione Zoologica in Naples, through the ASSEMBLE program [EC/FP7 Grant Agreement No. 227799]. The funders played no part in the design of the study, data collection and analysis, decision to publish, or preparation of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2016.00671/full#supplementary-material

Supplementary Figure 1 | Animal's velocity had no effect on the reflectance change values. We found no correlation between the two variables (correlation coefficient is 0.26).

Supplementary Figure 2 | From the model we learn that if the animals were continually and indiscriminately averaging a sampling area (CSA) while only responding to a reflectance threshold, a step-function in reflectance would emerge. Such a sudden change in reflectance would create a drastic change in the animal appearance, in striking contrast to the results of the current and previous studies. Therefore, we conclude that the animals do not average the CSA indiscriminately and continuously, but they instead decide whether to camouflage (or not) in response to the upcoming patch on approach and not upon arrival. In this manuscript we show that the CEPS could well support this response by offering a selective threshold.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 19 June 2017
doi: 10.3389/fphys.2017.00393

Dynamic Skin Patterns in Cephalopods

Martin J. How[1]*, *Mark D. Norman*[2†], *Julian Finn*[2†], *Wen-Sung Chung*[3] and *N. Justin Marshall*[3]

[1] Ecology of Vision Group, School of Biological Sciences, University of Bristol, Bristol, United Kingdom, [2] Marine Sciences, Museum Victoria, Melbourne, VIC, Australia, [3] Sensory Neurobiology Group, Queensland Brain Institute, University of Queensland, Brisbane, QLD, Australia

Cephalopods are unrivaled in the natural world in their ability to alter their visual appearance. These mollusks have evolved a complex system of dermal units under neural, hormonal, and muscular control to produce an astonishing variety of body patterns. With parallels to the pixels on a television screen, cephalopod chromatophores can be coordinated to produce dramatic, dynamic, and rhythmic displays, defined collectively here as "dynamic patterns." This study examines the nature, context, and potential functions of dynamic patterns across diverse cephalopod taxa. Examples are presented for 21 species, including 11 previously unreported in the scientific literature. These range from simple flashing or flickering patterns, to highly complex passing wave patterns involving multiple skin fields.

Keywords: dynamic patterns, cephalopod, communication, camouflage, motion, chromatophore, skin, passing wave

OPEN ACCESS

Edited by:
Daniel Osorio,
University of Sussex, United Kingdom

Reviewed by:
Jérôme Casas,
François Rabelais University, France
Danbee Kim,
Sainsbury-Wellcome Center,
United Kingdom

***Correspondence:**
Martin J. How
m.how@bristol.ac.uk

†Present Address:
Mark D. Norman,
Parks Victoria, Melbourne,
VIC, Australia
Julian Finn,
Sciences, Museums Victoria,
Melbourne, VIC, Australia

Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *14 September 2016*
Accepted: *26 May 2017*
Published: *19 June 2017*

Citation:
How MJ, Norman MD, Finn J, Chung W-S and Marshall NJ (2017) Dynamic Skin Patterns in Cephalopods. Front. Physiol. 8:393. doi: 10.3389/fphys.2017.00393

INTRODUCTION

Cephalopods are well-known masters of camouflage, but are also unsurpassed in their ability to alter their visual appearance for communication. The most complex of the Mollusca, they have evolved a sophisticated system of neurally- and hormonally-driven active dermal units that produce variable body patterns using three distinct visual components: (1) a chromatic component provided by elastic pigment-filled structures, the *chromatophores*, (2) a color-reflective component effected by wavelength interference platelet structures, the *iridophores*, and (3) a passive reflection component produced by the *leucophores* (Messenger, 2001). Skin patterns in many cephalopods are further enhanced by a textural component, where muscular and hydrostatic forces within the architecture of the skin enable simple to complex changes in skin topography. Amongst benthic octopuses (family Octopodidae) and cuttlefishes (family Sepiidae), this variable sculpture can include flaps, ridges, and/or simple to multiple branching papillae (e.g., **Figure 1a**).

This unique dermal architecture enables many cephalopods to switch easily between matching the tone and texture of various backgrounds (**Figure 1a**), through to performing conspicuous signaling displays for intra- and inter-specific communication (**Figure 1b**). This is particularly impressive considering that the vast majority of cephalopod species are color blind, possessing only a single visual pigment and failing to demonstrate color vision in behavioral tests (Hanlon and Messenger, 1996; Marshall and Messenger, 1996). The exceptions are a deep-sea family including the firefly squid *Watascinia scintillans,* which have three spectral sensitivities, almost certainly co-evolved with their multicolored bioluminescent displays (Michinomae et al., 1994). Recent hypotheses suggesting that the optics of the cephalopod eye may provide chromatic discrimination

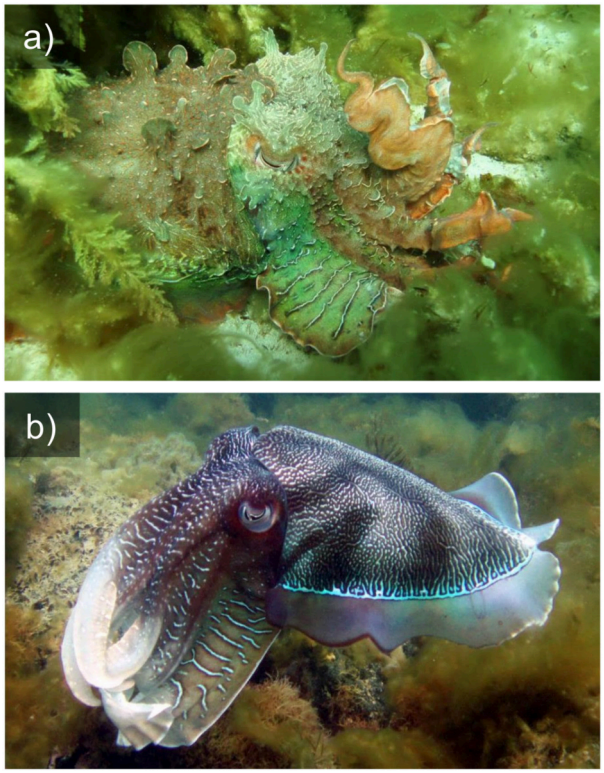

FIGURE 1 | Camouflage **(a)** and signaling **(b)** in the Giant cuttlefish *S. apama*.

in some circumstances (Stubbs and Stubbs, 2016a) seem unlikely or of limited use in their normal habitat (Gagnon et al., 2016; Stubbs and Stubbs, 2016b).

Cephalopods are not the only animals capable of color change. Many lizards, particularly the chameleons, as well as many fishes can change both color and pattern (Ramachandran et al., 1996; Stuart-Fox et al., 2006). However, it is the speed with which the skin's appearance can be controlled in cephalopods that is unique. Skin-change capability is deployed by cephalopods in diverse behaviors (Messenger, 2001) and the rapidity of pattern change puts many of these visual displays in a class of their own. With parallels to the pixels on a television screen, cephalopod chromatophores can be coordinated to produce dramatic, dynamic, and rhythmic signals in the form of "flashing" or "strobing," where fields of chromatophores are opened and closed in synchrony or as moving bands, produced by waves of transiently expanded and contracted chromatophores flowing over the body in a coordinated manner (Packard and Sanders, 1969). We define these display types here collectively as "dynamic patterns."

The best-known example of a dynamic pattern amongst cephalopods involves a moving pulse or dark band running over the body and arms. This display has been treated by previous authors under the names "passing cloud" or "wandering cloud" (Packard and Sanders, 1969; Hanlon and Messenger, 1988; Mather and Mather, 2004; Adamo et al., 2006; Huffard, 2007). In this manuscript, we avoid using the term "cloud," as it can imply that this display mimics cloud shadows or dappled light from surface waters playing over the animal, thus presupposing a hitherto unknown function. Our use of the term "dynamic patterns" is distinct from "dynamic camouflage," a term previously used to describe the general ability of cephalopods to switch between different static color patterns (Hanlon, 2007). It is also distinct from "dynamic mimicry," a term coined for the mimic octopus, *Thaumoctopus mimicus*, where an individual can fluidly morph between multiple aposematic models (Norman et al., 2001).

Due to their transient nature, the dynamic patterns of cephalopods are seldom observed and rarely recorded in the wild. As a result, very few studies have focused on the form and function of these patterns, other than mentioning them as brief anecdotes. This study attempts to collate evidence of dynamic patterns from a wide variety of sources, including public and private video recordings (presented in Supplementary Information), as well as existing scholarly descriptions, in order to examine the nature, context, and potential functions of dynamic skin patterns across diverse cephalopod taxa.

MATERIALS AND METHODS

The dynamic displays of 21 species of cephalopod were categorized and described from widely sourced digital video sequences. Ten species were filmed by the authors or colleagues, and the remaining 11 were sourced from the literature or from videos posted by the public online. The diverse range of material made it difficult to obtain standardized quantitative data, and so the study focuses on a qualitative description of the dynamic patterns in question. Where possible, example video has been included in Supplementary Information. Analysis was performed using open-source video playback software (VideoLAN, 2016) and Matlab scripts (Mathworks, 2014). Detailed analysis involved digitizing points on the body of cephalopods or their backgrounds over sequences of video frames using the Matlab analysis script "DigiLite" (Jan Hemmi, University of Western Australia) and plotted graphically using custom scripts (available in Supplementary Information). Digilite is available on request from Jan Hemmi. Alternatively, slimmed down versions of this digitisation script are included as Supplementary Information (dgigas_digitisepoints.m, olaqueus_digitisepoints.m, and latimanus_digitisepoints.m). Measures used to describe each display included temporal frequency or movement speed of the pattern, and the fine-scale behavioral context of the displays.

RESULTS

Through direct observation, video documentation and externally sourced footage of 21 cephalopod species, we recognize five categories of dynamic skin patterns, with certain species being capable of displaying more than one category: (1) *flashing* (or strobing); (2) *flickering*; (3) *chromatic pulses*; (4) *rhythmic passing waves*; and (5) *multi-directional passing waves*. Cephalopod species known to produce these categories of pattern are treated individually below.

Flashing Patterns

Flashing, or strobing, is the simplest category of dynamic skin pattern. It involves the synchronous activation of skin color or light-emitting components, often across the whole body, resulting in a repeated transition from one skin pattern to another (Hanlon and Messenger, 1996). "Flash behavior" is reported elsewhere in nature, where appendages or plumage are used to rapidly display color, contrast or specific patterns (Cott, 1940; Edmunds, 1974; e.g., deimatic displays: Umbers et al., 2015). In this paper, we deal only with patterns in which the repeated flashing is a clear component of the display. We therefore exclude the myriad examples of cephalopods producing single rapid color changes (sometimes referred to as a flash), often produced as part of anti-predation behavior (e.g., Hanlon and Messenger, 1996; Langridge et al., 2007).

Flashing patterns have been most reliably documented in the Humbolt squid, *Dosidicus gigas*. Sometimes known as jumbo squid or jumbo flying squid, *D. gigas* is one of the largest and most abundant nektonic species of cephalopod (Nigmatullin et al., 2001). The species inhabits deep ocean areas from the eastern Pacific to the Chilean coast and the Sea of Cortez, where it performs vertical feeding migrations at dusk from the deep to shallower water (Markaida et al., 2005; Gilly et al., 2006; Zeidberg and Robison, 2007; Trueblood et al., 2015). The flashing patterns of *D. gigas* have been recorded and described on numerous occasions, including a recent deployment of the National Geographic "CritterCam" (Marshall et al., 2007; Rosen et al., 2015). Briefly, flashing patterns in this species involve the rapid opening and closing of chromatophores over the whole body in tight synchrony at a frequency of around 2–4 Hz (**Figure 2a**; Supplementary Video 3.1.1). The pattern tends to occur when other *D. gigas* are nearby, suggesting an intraspecific communication function. The display probably plays a role in courtship as well as during agonistic interactions (Rosen et al., 2015), particularly given the high risk of cannibalization within the species (Markaida and Sosa-Nishizaki, 2003). Rosen et al. (2015) also report a similar dynamic pattern in another large pelagic squid, *Sthenoteuthis oualaniensis*, but data supporting this is as yet unpublished.

Several cephalopod species are known to produce flashing patterns of bioluminescence. The deep sea Dana squid, *Taningia danae*, possesses large occludable photophores on the tips of the dorsolateral pair of arms (**Figure 2b**; Roper and Vecchione, 1993). These organs have been observed producing brief, synchronous flashes of blue-green light in captured and free-ranging individuals, a display that seems to be associated with attack or escape behavior (Roper and Vecchione, 1993; Kubodera et al., 2007). Similarly, vampire squid, *Vampyroteuthis infernalis*, can dynamically occlude large photophores on the mantle, apparently as an anti-predation strategy (Robison et al., 2003) and the deep sea squid *Octopoteuthis deletron* can flash it's arm-tip photophores in various behavioral contexts (Bush et al., 2009). However, very little is known about the natural ecology of these deep-water species and so further work is needed.

Flicker Patterns

Flicker (or shimmer) patterns involve the non-synchronous activation of skin pattern elements to produce seemingly random shimmering or flickering waves, often across the whole body. Many species show low-level flickering of their skin pattern, possibly due to signal noise in the neuro-muscular control system (e.g., *Idiosepius notoides* example, Supplementary Video 3.2.1; Holmes, 1940; Suzuki et al., 2011). However, several flicker displays with an apparent function have been described in the literature.

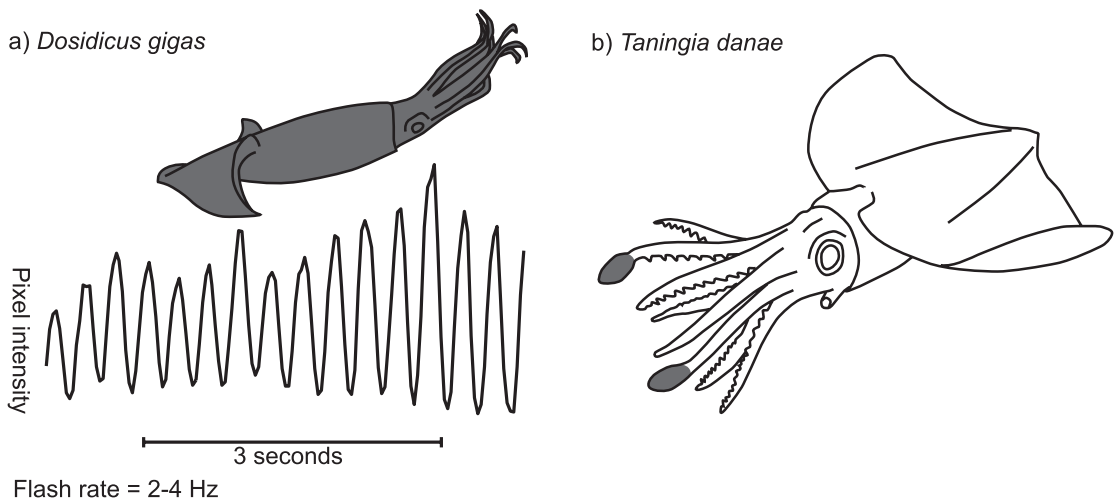

FIGURE 2 | Flashing patterns in two squid species. **(a)** Temporal characteristics of *D. gigas* flashing display. Graph shows, for a single example sequence, pixel intensity for a point on the body on the y-axis (scale not shown) and time along the x-axis. Inset diagram shows in gray the region of the body over which the signal occurs (in this case the whole animal). **(b)** Photophore flashing in *Taningia danae*. Two large bioluminescent organs are located at the distal tips of the dorsolateral arms (gray shaded areas), each of which can be flashed on and off by moving a pigmented cover (redrawn from Richard Ellis).

The "CritterCam" study of Rosen et al. (2015) recorded multiple instances of flicker patterns in the Humbolt squid, *D. gigas*. They describe the pattern as having a "*noisy wave-like appearance*" across the whole body and observed that it occurs as a "*basal level of chromatophore activity in the absence of flashing*" (Supplementary Information 3.2). The authors go on to suggest that the pattern may act as a form of dynamic crypsis, mimicking the pattern of down-welling light in shallow waters. How the addition of flicker dynamic skin patterns to naturally occurring caustic flicker could have the effect of reducing animal conspicuousness remains to be demonstrated.

A second example of a flicker display can be found in the deep-sea finned octopod *Stauroteuthis syrtensis* and is described from captured and free-ranging specimens by Johnsen et al. (1999). This species possess many small bioluminescent photophores in place of its suckers on the underside of each of its eight arms. These organs can be induced to flash when disturbed, similar to other species. But interestingly, individual photophores were observed blinking on and off asynchronously at about 0.5 to 1 Hz, producing a "*twinkling*" effect. *In situ*, one individual was seen "*spread in the horizontal plane with the mouth upwards,*" leading the authors to suggest that the twinkling photophores may act as light lures for attracting their planktonic crustacean prey, which then become trapped in the arm webs.

Chromatic Pulse

Chromatic pulses are dynamic skin patterns consisting of a single band or spot of color contrast sweeping across part of the animal in a particular direction. Past studies have referred to some of these displays as "*passing cloud*" or "*wandering cloud,*" with some authors proposing that this display mimics the movement of dappled light from surface waters (Packard and Sanders, 1969; Mather and Mather, 2004; Huffard, 2007). In this work, we prefer the term "chromatic pulse" as it does not presuppose the functional mechanism for the display. Data are presented here on six new reports and two previous reports of cephalopod species that employ chromatic pulse displays.

While foraging nocturnally, the tropical octopus, *Octopus laqueus*, produces a chromatic pulse display in which a dark patch passes from the posterior part of the mantle to the arms. Starting at the posterior mantle tip, the pulse diverges to pass bilaterally around the sides of the mantle, and then converges into a single patch at the head. From here the patch continues down to the tips of the dorsal arm pair (**Figure 3a**). A continuous 140 s video sequence of *O. laqueus* foraging off the Philippines (Supplementary Video 3.3.1) recorded 33 pulses, each lasting 0.55 ± SD 0.11 s, produced at a variable frequency of around 0.25 Hz. The deployment of this display is closely associated with the movement of the animal over the substrate. *O. laqueus* forages by moving in a stop-start pattern across coral rubble, swimming or crawling from one location to the next, then stopping to probe under rubble and into crevices. The chromatic pulse display is synchronized with the "stop" part of the locomotory pattern, each time the animal ceases movement to probe crevices with the arm tips. This is evidenced in the recorded sequence by the animal moving significantly faster (3.5 times) in the moments before each chromatic pulse compared to afterwards (**Figure 3b**; speed before: 12.1 ± SD 6.0 pixels.s^{-1}; speed after: 3.5 ± SD 2.1 pixels.s^{-1}; *t*-test: $t = 8.1$, $p < 0.001$). Given the precise behavioral context of the display, we can hypothesize three possible functions. One possibility is that the display acts as a conspicuous warning signal to ward off potential predators as the octopus forages among the coral rubble. The second possibility is that the display acts as a form of motion camouflage during low-light conditions. It may disguise the precise moment when the animal stops moving by continuing a false motion cue in the direction of travel after the animal has stopped. Thirdly, the display may help to flush out prey from the coral rubble, startling them into evasive behavior. It is important to note that this description was based on a single individual in the only known video of *O. laqueous* chromatic pulse patterns, and so further observations and experiments are required to study this in detail.

A very similar dynamic skin pattern was observed in several other octopuses, including an *Abdopus* species, the Caribbean two spot octopus *Octopus hummelincki*, and possibly the Caribbean reef octopus *Octopus briareus*. Observations of an as-yet undescribed species of *Abdopus* were obtained from a series of videos of a single individual moving around shallow rock pools at night near Broome, Western Australia. The chromatic pulse originates along the dorsal midline of the mantle and head, and then spreads laterally across the mantle, extending to the ventral part. The pulse then flows anteriorly along the webs and dorsal arm pair and the dorsal halves of the second arm pair (**Figure 3c**; Supplementary Video 3.3.2). A single chromatic pulse took 1.2 ± SD 0.07 s to complete ($n = 13$ pulses observed over 93 s from a single individual) with irregular intervals. The pattern was performed while the octopus was raised on its arms, with the mantle held parallel to the substrate. The overall effect is of the chromatic pulse passing from the highest point on the body, down to the lowest part. The chromatic pulses of *O. hummelincki* and *O. briareus* are very similar, but were only observed in individuals housed in personal aquaria (Supplementary Information 3.3; a, b, c, d, e, f, g).

Another variant of the chromatic pulse pattern was observed in the reef-dwelling Broadclub cuttlefish, *Sepia latimanus*. The display was filmed in the daytime on the Great Barrier Reef, Australia (Supplementary Video 3.3.3) during an interaction between a small male and a larger mate-guarding male. The small male assumed a mottled body pattern with a whitened head and arms, and slowly approached the rival male in a direct head-on posture with its arms tucked closely together. The small male then produced a dark blush around the head over a period of about 1 s, then quickly expelled a small cloud of ink, while simultaneously expanding the dark blush down the head and arms (**Figure 3d**). The latter part of the display was relatively fast (<0.25 s). Given the behavioral context, as well as the nature of the synchrony between the chromatic pulse and the expelled ink, we suggest that this display is used as an aggressive or territorial signal between rival males. It is possible that smaller males incorporate jets of ink to enhance the visual impact of the display.

A similar (although inkless) display has been filmed in the bigfin reef squid *Sepioteuthis lessoniana* (**Figure 3e**). A video, reportedly from the waters around the United Arab Emirates,

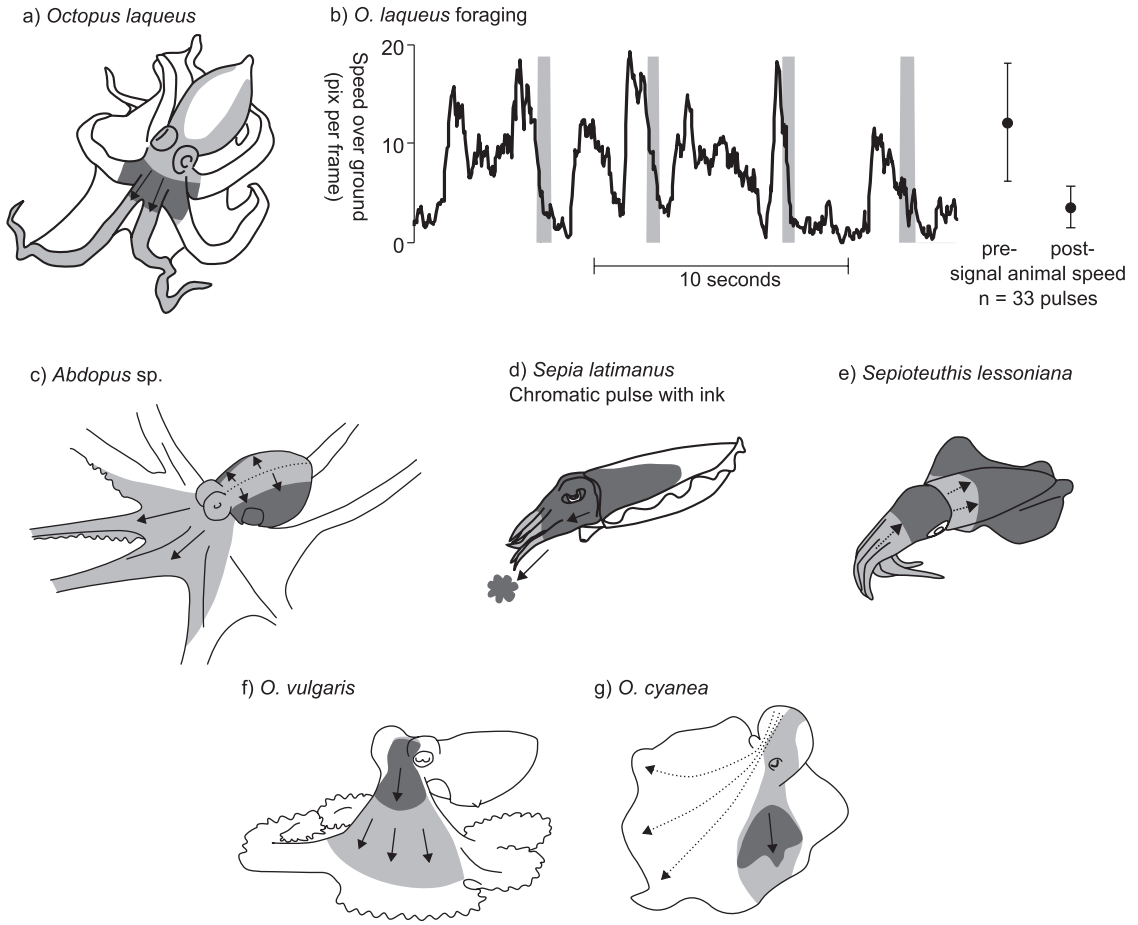

FIGURE 3 | Examples of chromatic pulse patterns. In panels **(a)** and **(c–g)**, light gray indicates the sweep area of the chromatic pulse, and dark gray indicate the position of the pulse at a fixed point in time. **(a)** *Octopus laqueus*. **(b)** Timing of chromatic pulse during *O. laqueus* foraging. Graph presents a subsection of 33 chromatic pulses recorded in a continuous video sequence. Gray bars indicate pulse timing; black line indicates approximate speed over ground of the foraging octopus. Subsequent data points and error bars indicate mean speed ± SD prior to display and after each display is finished. **(c)** *Abdopus* sp. Dotted line indicates line of chromatic pulse origin. **(d)** *S. latimanus* chromatic pulse with coordinated ink jet. **(e)** *Sepioteuthis lessoniana*. **(f)** *O. vulgaris* (redrawn from Packard and Sanders, 1971). **(g)** *O. cyanea* (redrawn from Mather and Mather, 2004). Dotted arrows indicate alternative possible routes for the chromatic patch.

shows a day-active *S. lessoniana* performing occasional chromatic pulses, in which the body is first darkened all over, then pulses of white are passed simultaneously from the tips of the arms toward the head, and from the anterior edge of the mantle toward the mantle center (Supplementary Information 3.3). The display appears to be performed in response to the presence of the camera or diver, and may represent a threat signal.

Finally, two other examples of chromatic pulse displays have been previously described in the literature and these show many similarities to those of *O. laqueus*, *Abdopus* sp., and *O. hummelincki*: Packard and Sanders (1969) described the chromatic pulse display of *O. vulgaris* as "*dark flushes of color that pass as a wave outwards from the head and then fade into the general background mottle*" (**Figure 3f**). The display tended to be produced during foraging behavior, specifically when pursued crabs stopped moving. These authors proposed that this signal functions to startle the prey into moving (conveying the message "*move you other animal*"). No information on the time of day or ambient lighting conditions under which the display was observed was recorded (Packard and Sanders, 1971; Wells, 1978). Mather and Mather (2004) described the display in *Octopus cyanea* as a "*dark cloud*" moving in a posterior–anterior direction from the mantle, down over the head, and down the arm web (**Figure 3g**). The duration of the display lasted on average 0.85 s. These authors noted that the exact placement of the moving patches on the body was not fixed, and displays could vary in their patch trajectory. Furthermore, on some occasions, two bilaterally symmetric patches 'moved" across the body rather than just a single patch on one side. The relative contrast of the dark patch in the *O. cyanea* display was also enhanced by a paling of the surrounding area. The display was only observed during periods of foraging activity within artificial enclosures.

Rhythmic Passing Waves

This type of body pattern involves the movement of rhythmic bands of contrast across the skin surface in a single, constant

direction. Here, we report four new examples of the display and one from the literature.

The unidirectional passing wave display of *Sepia officinalis* has been frequently mentioned in the literature, but not described in detail. One of the earliest descriptions comes from Holmes (1940), "*...the color change seems to result from the passage over the head and arms of waves of contraction and expansion of the chromatophores.*" More detail was provided by Hanlon and Messenger (1988) who described it as, "*a kinetic pattern, lasting only a second or two, characterized by broad transverse bands of chromatophore expansion moving rapidly forward from the posterior mantle tip across the dorsal body surface to the anterior tip of the arms*" (**Figure 4a**; Supplementary Information 3.4). The stripes move across the body at a frequency of about 1 Hz, and the pattern is often produced by young cuttlefish as they move across a substrate. This display is reported primarily for juvenile cuttlefish during hunting behavior (Holmes, 1940; Hanlon and Messenger, 1996; Adamo et al., 2006), although there is some suggestion that it may also function as a defensive signal in response to approaching predators (Hanlon and Messenger, 1988).

A very similar pattern has been filmed in young giant cuttlefish *Sepia apama* (Supplementary Videos 3.4.1 and 3.4.2). In this species, each wave of contrast takes around 1.5–2 s to pass along the length of the mantle. Although the exact ecological context is not clear, the pattern appears to be produced by camouflaging animals under low light conditions as they locomote in a posterior direction across a substrate.

The dwarf cuttlefish, *Sepia bandensis*, produces a very similar passing wave pattern to *S. officinalis* and *S. apama*, with the difference that the direction of the wave is reversed—from the anterior to the posterior mantle. (**Figure 4b**; Supplementary Information 3.4; a, b, c, d, e). Unfortunately, little is known about the natural ecology of this species and the contexts in which this pattern may occur.

Perhaps the most striking example of a unidirectional passing wave display in cephalopods is produced by the Broadclub cuttlefish *S. latimanus* during hunting behavior. On sighting a prey item, the cuttlefish will tentatively approach in full camouflage, typically in the "branched coral" pose. Once within 0.5–1 m of its prey it switches to the following, highly conspicuous passing wave pattern (Supplementary Video 3.4.3). The camouflage pattern is replaced by a light uniform whitish color and the first three arm pairs are thrust forwards into a tight cone, while the two ventral arms are splayed outwards and flattened so that the arm/web margin surface is perpendicular to

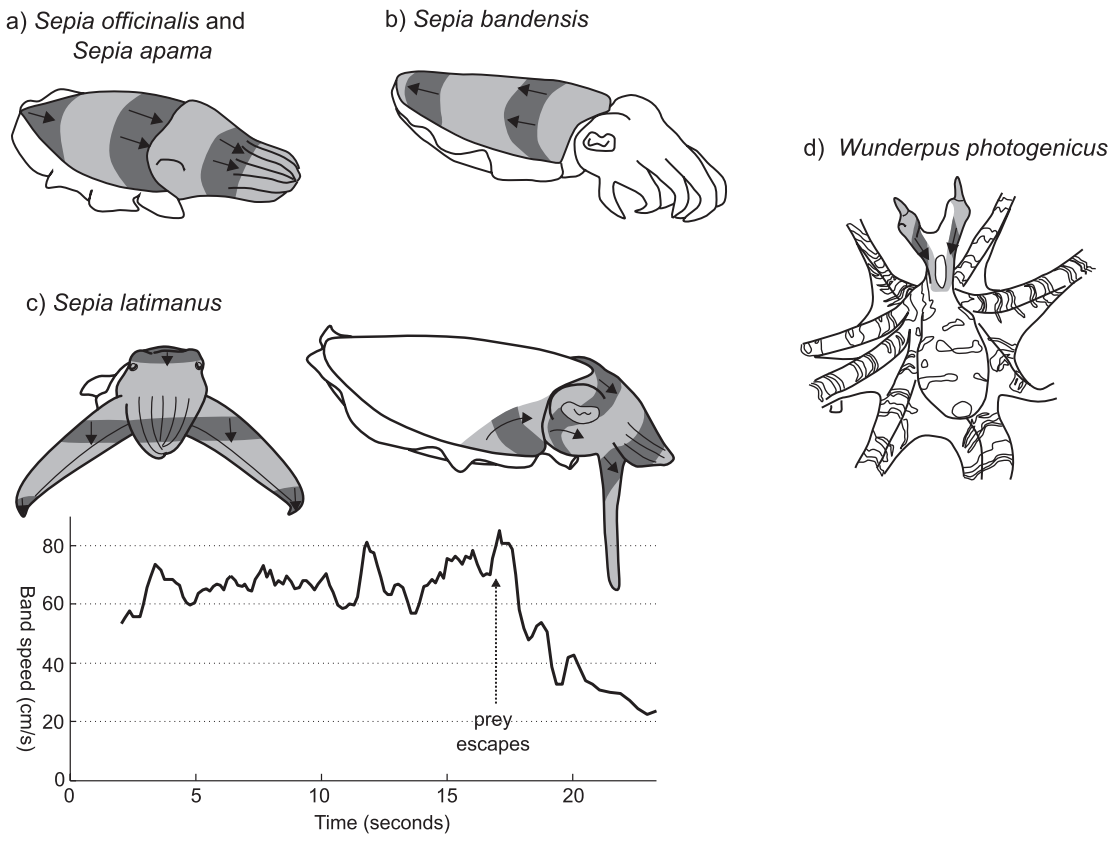

FIGURE 4 | Rhythmic passing wave patterns. **(a)** *S. officinalis* (redrawn from Hanlon and Messenger, 1996) and *S. apama*. **(b)** *S. bandensis*. **(c)** *S. latimanus* front view (left); lateral view (right). Graph shows the approximate change in speed of the banding pattern before and after aborting a predation attempt. Vertical arrow indicates the timing of prey escape. **(d)** *W. photogenicus*. Image shading conventions as per **Figure 3**.

the viewing direction of the prey animal (**Figure 4c**, left). Moving bands of dark contrast are then generated, passing quickly across the head and anterior mantle at speeds of 40–80 cm/s. These bands originate at the anterior lateral margins of the dorsal mantle, then rotate upwards and anteriorly toward the eye, merging on the dorsal head, then passing down the four arm pairs (**Figure 4c**, right). When viewed directly from the perspective of the prey animal, the moving bars are oriented so that, despite passing over the unevenly curved head of the cuttlefish and anteriorly projecting arm cone, they appear horizontally straight, moving in a uniformly downwards direction. The speed of the moving bars can be adjusted, and seems to be linked to the behavioral context. The inset graph in **Figure 4c** presents the downward speed of moving bars during a single prey-approach event recorded on video. Bar speed appears relatively stable around 65 cm/s, while approaching the prey item. But once the prey escapes (arrow at 17 s), the cuttlefish slows the speed of its display to 20–25 cm/s before reverting to static skin patterns. Despite being well-known among divers in Indonesia, as well has having been presented in several high-profile natural history documentaries (Supplementary Information 3.4), we could find no reports of this behavior in the scientific literature.

A final example of a rhythmic passing wave pattern can be found in the octopus *Wunderpus photogenicus*. This Indo-Pacific species produces a rhythmic, unidirectional wave pattern down the eye stalks. Originating at the distal tip of the eye stalks, the chromatic waves pass downwards, over the head and to the junction of the mantle and arm crown, at a frequency of approximately 1–2 Hz (**Figure 4d**; Supplementary Video 3.4.4; source J. Finn). This display was observed both while the animal was foraging and when within its burrow with only the head protruding, but the ecological function of the pattern is unclear.

Multidirectional Passing Wave Displays

Multidirectional passing wave displays are similar to the rhythmic passing wave displays described above. However, the moving stripe patterns occur in multiple directions in different parts of the animal's body. We identified several examples of multidirectional passing waves, exclusively within cuttlefish. Most of these patterns are bilaterally symmetric, so that each field is paired across the body midline. We define the number of display fields according to the subunits containing passing waves per side of the mantle. Data is presented here on two-, three-, and five-field dynamic patterns across five cuttlefish species. Some species also display additional rotating bands on the lateral head and arm bases.

One of the most conspicuous examples of this type of dynamic pattern is produced by the giant cuttlefish *S. apama*. During reproductive activity, males can be observed producing a striking dynamic pattern toward competing males (Norman et al., 1999; Hall and Hanlon, 2002). This display is directed toward the recipient cuttlefish during close interactions by tilting the lateral mantle toward the opponent (**Figure 5a**, left, Supplementary Video 3.5.1). The arms are extended and flared to maximize their visual surface area. The mantle is almost white and repeated dark bands are passed across the nearest lateral half of the mantle surface. The display is relatively slow moving, with each band taking 7.0 ± SD 1.0 s to travel across the mantle and at a low frequency of 0.38 ± SD 0.08 Hz ($n = 5$ individuals). Because the display tends to be viewed by rival males at close range (∼10 cm), it occupies a large proportion of the receiver's visual field (visual angle of moving band width ∼20° and interval ∼30° when viewed from 10 cm). The wave patterns originate along a diagonal line that stretches from the anterio-lateral mantle border with the fin to the midpoint of the medial dorsal mantle (dotted line **Figure 5a**, left). Waves of contrast are initially propagated synchronously in two different directions, one moving diagonally toward the anterior midpoint of the dorsal mantle (**Figure 5a**, right, field A), and the other moving diagonally toward the opposite posterior midpoint of the dorsal mantle (**Figure 5a**, right, field B). As these bands diverge, they remain in contact along the line of divergence, producing the impression of an expanding arch. Occasionally, a third passing wave field is visible on the head in the region of the eye nearest to the rival male (**Figure 5a**, right, field C). In this region, the waves of contrast commence from behind the eye, rotate over the brow of the eye and onto the base of the arms in synchrony with the other two contributing skin fields. Due to the predominantly lateral orientation and presentation of this display, individuals often contract the skin on the non-signaling side of the mantle to stretch the dynamic display over a larger proportion of the mantle surface (**Figures 1b**, **5a**, left). This display type is typically restricted to a single side of the body. However, in situations where rivals are located on both sides of the displaying male, the dynamic signals can be presented symmetrically on both sides of the body.

The second context for use of this display does not appear to relate to reproduction. Juveniles of this species are occasionally observed amongst moving weed at night, over a light-colored sand substrate, producing a strong, bilaterally-symmetric dynamic pattern almost identical in form and timing to the agonistic display (Supplementary Videos 3.5.2 and 3.5.3). The pattern appears to match the motion of dark sea grass over light sand patches and could represent a form of dynamic camouflage.

Another striking multi-field dynamic pattern is produced by two species of the genus *Metasepia*: the Flamboyant cuttlefish, *Metasepia pfefferi,* and the Paintpot cuttlefish, *Metasepia tullbergi*. As the patterns of these species are very similar, we will describe them together. For a more detailed analysis of *M. pfefferi* see Thomas and MacDonald (2016), and for *M. tullbergi* see Laan et al. (2014). These species inhabit subtidal soft sediments and are typically benthic, employing the fourth arm pair and ambulatory flaps on the ventral surface of the mantle to amble along the seafloor with a quadrupedal walking gait (Roper and Hochberg, 1988). The species hunt by stalking small fishes and crustaceans on the seafloor. When disturbed, the species displays a high-contrast pattern of white, yellow, red, and dark brown. This display often includes a multi-field dynamic pattern (Supplementary Information 3.5; a, b, c, d, e). In some situations, animals produce a two-field display composed of field A—anterior third of the dorsal mantle generating a posteriorly moving vertical bar of contrast—and field B—posterior third of the dorsal mantle generating an anteriorly-moving diagonal

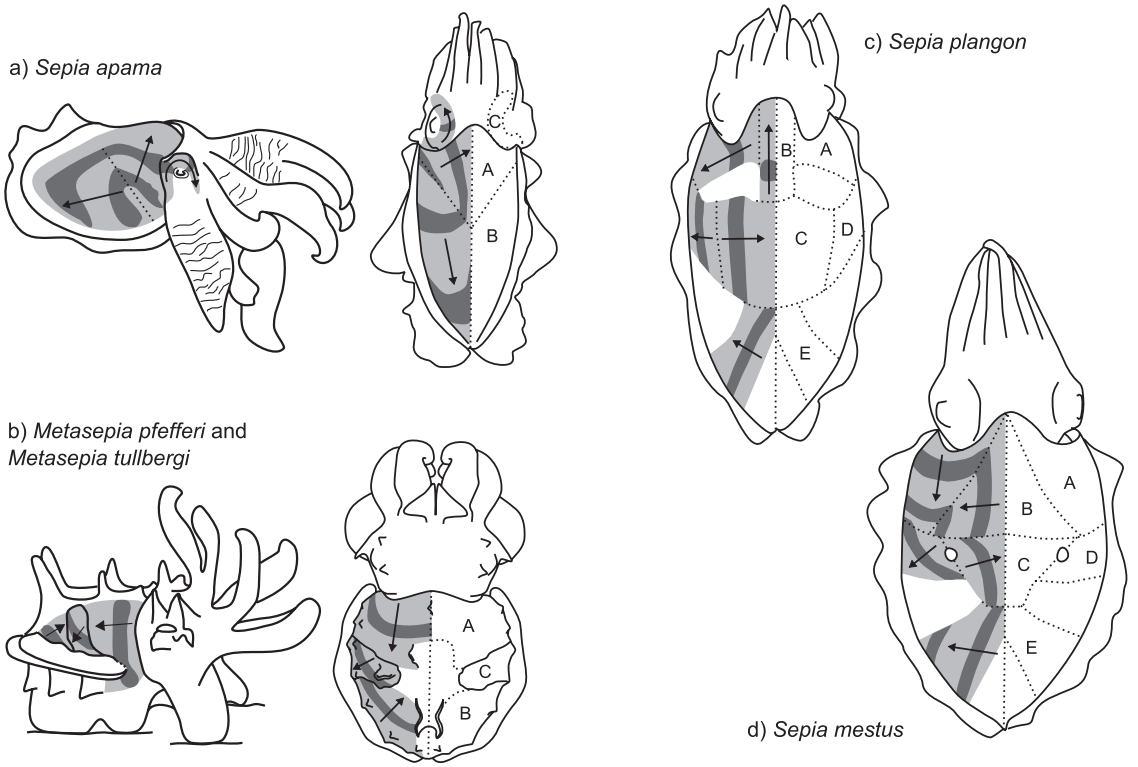

FIGURE 5 | Multidirectional passing wave patterns. **(a)** *Sepia apama*: left—competitive display produced by rival males during the spawning aggregation; right—dorsal view of the two motion fields (without skin stretch). **(b)** The dynamic pattern of the two closely related species, *Metasepia pfefferi* and *M. tullbergi*: left—lateral view; right—dorsal view. **(c)** *S. plangon* (dorsal view). **(d)** *S. mestus* (dorsal view).

bar of contrast (**Figure 5b**). In some individuals, a third motion field was observed within the central part of the dorsal mantle (field C), consisting of a diagonal band of contrast moving in a ventro-posterior direction (**Figure 5b**). The combined effect of these three motion fields along with the flamboyant color pattern is a highly conspicuous visual signal. The precise function of the signal remains unknown, although there is some suggestion that it may represent an aposematic signal of toxicity in the flesh of the animal.

In several examples, five or more distinct fields of passing waves could be seen in a single animal. Mourning cuttlefish, *Sepia plangon*, housed in aquaria under low light conditions can be observed producing a striking dynamic pattern (Lee, unpublished data; Supplementary Video 3.5.4). As these displays only occurred during low-light conditions, the exact structure of the signal is difficult to record. However, preliminary examination indicates at least five separate motion fields (**Figure 5c**, Fields A–E). The movement of the bars of contrast in each field appear to be temporally synchronized with each other, so that in some areas the pattern seems to transfer continuously over into a different field. For example, as the central bar in field C reaches the midline, the patch in field B starts moving toward the head. Then as it reaches the head region, the bar in field A starts moving laterally away from the head. The combined effect of these areas working in synchrony produces the illusion of a continuous movement of contrast, spiraling from the center of the mantle, around toward the head, then laterally to the mantle edge. A similar effect is achieved to the posterior end of the animal, with the temporal correlation of fields C and E. Overall, the display is relatively slow moving, with a repeat frequency of around 0.3–0.5 Hz, although there seems to be variation both within and between individuals.

A similar, but more conspicuous dynamic pattern can be found in the Reaper cuttlefish, *Sepia mestus*. Our data is based on a small number of video clips (Candace McBride, unpublished data; Supplementary Information 3.5; a, b, c, d). In this example, a day-active individual cuttlefish, reacting either to the presence of the observing SCUBA diver or to another individual, positions itself near a small clump of dark weed, then performs a striking dynamic display with five distinct fields of motion (**Figure 5d**, fields A–E). The movement of the high contrast pattern is combined with the pair of "dorsal mantle white spots" (Packard and Sanders, 1971) in the center of the mantle, from which the pattern in fields C and D emanate. When passing over these spots, the dark bands occlude the white pattern, so that these spots appear to "blink" between black and white. The contrast pattern moves much more quickly than that of *S. plangon*, at a rate of around 1.5 Hz.

Simultaneous Displays

Several species of cephalopod are notable in that they are able to produce more than one distinct type of dynamic skin

pattern. Above, we described two different patterns produced by the Australian giant cuttlefish, *S. apama*: the single field passing wave display, in which waves of contrast pass from the posterior end of the mantle toward the head, and the multi-field display, in which waves emanate from a line midway along the mantle (**Figures 4a**, **5a**). We have observed an individual of this species switching quickly between these two patterns, potentially in response to the presence of observing (filming) SCUBA diver. On several occasions the animal expressed both patterns simultaneously for a period of several seconds (Supplementary Video 3.4.1).

DISCUSSION

Body patterning for camouflage and communication is a well-studied aspect of animal biology (Cott, 1940; Stevens, 2013). Many of these static patterns incorporate movement of the animal to enhance the effect. Unusually, in cephalopods we have the unique opportunity to see how evolution can shape body patterns that incorporate intrinsic dynamic components. All patterns described in this comparative study have several design features in common. Firstly, they tend to be high contrast, involving dark patches moving on light backgrounds. This is most extreme in the *Metasepia* species (Supplementary Information 3.5; a, b, c, d, e) and the essentially "black and white" color of the signals may be linked to the color-blind nature of the cephalopod species described here (Chung and Marshall, 2016). Secondly, they have a relatively narrow range of motion speed or frequency. Several species showed displays outside the typical range of motion speed, including the hunting pattern of the Broadclub cuttlefish, *S. latimanus* (whose display is directed toward prey species rather than conspecifics), and the flashing pattern of *D. gigas* (whose display does not contain intrinsic motion, rather repeated on/off switching between pattern components). It is well-known that motion detectors in animals are contrast and speed sensitive (Borst and Egelhaaf, 1989) and so perhaps, in the absence of color, these design features are likely to be adaptations for increased saliency of the pattern.

Interestingly, the dynamic patterns described herein have striking parallels with some research methods in visual ecology. For example, moving gratings (similar to the Broadclub hunting display) and visual playback of looming patterns (similar to *S. apama* agonistic displays and *Octopus* chromatic pulses) have been used extensively to study the visual capabilities of a wide range of animal species, including cephalopods themselves (e.g., Talbot and Marshall, 2010; Temple et al., 2012). These experimental methods are designed to stimulate the motion detection system of the animal viewing the stimulus, and it seems likely that the natural dynamic displays of cephalopods have evolved for a similar purpose.

Neural Control

The comparative analysis of so many diverse dynamic patterns across the Cephalopoda allows us to expand upon some of their suggested control mechanisms. It has previously been established that motor neurons are responsible for the synchronous control of multiple chromatophores in discrete fields on the skin of cephalopods (Packard, 1974; Froesch-Gaetzi and Froesch, 1977; Packard and Hochberg, 1977). These chromatophore motor units (Boycott, 1961; Dubas and Boyle, 1985) are controlled centrally from the chromatophore lobes and stellate ganglion (Young, 1976; Dubas et al., 1986; Williamson and Chrachri, 2004). How these discrete, yet overlapping skin fields are coordinated to elicit specific patterns remains a complex and unsolved problem.

One possible mechanism for generating the dynamic patterns of cephalopods is through endogenous processes in the skin, otherwise termed "myogenic" control. The muscular units responsible for expanding individual chromatophore sacs are known to be electrically coupled to neighboring units (Florey, 1969; Florey and Kriebel, 1969; Reed, 1995) and, under certain experimental conditions, randomly moving passing waves of expanding and contracting chromatophores can be induced in cephalopod skin in the absence of any central control (Sanders and Young, 1974; Messenger, 2001). However, it seems unlikely that this mechanism could be behind the complex and highly controlled dynamic patterns reported here.

Through a detailed analysis of the complex dynamic pattern of *M. tullbergi*, Laan et al. (2014) propose an alternative neural control system originating from the central nervous system. They suggest that passing wave patterns could be controlled via a set of oscillatory neurons analogous to the central pacemakers governing rhythmic locomotory movements. Indeed, control networks for skin chromatophores and swimming fin motor neurons are known to coexist in parts of the cuttlefish brain and stimulation of these areas can result in both patterning and locomotory behavior (Messenger, 2001; Osorio, 2014). This kind of central control could generate dynamic wave patterns in single skin fields, and, most interestingly, could be applied to multiple skin fields resulting in the synchronous activation of different pattern units, such as those in the *Metasepia* species (Laan et al., 2014). Furthermore, central control would permit the speed of passing waves to be adjusted depending on behavioral context, and for different dynamic and/or static patterns to be co-expressed (e.g., *S. latimanus, S. apama*, and *M. tullbergi*; Laan et al., 2014).

As an interesting addendum to this, it must be noted that the octopods recorded in our study do not produce rhythmic passing wave patterns (with the exception of the eyestalk waves of *W. photogenicus*), rather single, non-rhythmic, chromatic pulses. It seems no coincidence that these species also lack the rhythmically controlled lateral swimming fin of *Sepia*. Instead, perhaps the chromatic pulse control system has its origins in different locomotory motor circuits, such as those governing mantle contraction for jetting behavior. Indeed, in some video sequences it appears that mantle contraction and chromatic pulses occur in synchrony (e.g., *Sepioteuthis lessoniana*, Supplementary Information 3.3; and the chromatic pulse/ink jet combination of *S. latimanus*, Supplementary Video 3.3.3) adding weight to this suggestion.

Dynamic Displays across Diverse Taxa

The dynamic skin patterns described here occur across a wide diversity of cephalopod groups, with some forms reported

across six cephalopod orders—squids (Teuthida), cuttlefishes and pygmy squids (Sepioidea), finned octopods (Cirrata), finless octopods (Incirrata), and vampires (Vampyromorpha).

The breakdown of forms of display by taxonomic group reveals some patterns. Strobing and flashing are primarily associated with squids, in the form of strobing in the oegopsid Humbolt squid, and parallels in light flashing in other oegopsid squids and iridescent flashing in loliginid squids.

Chromatic pulses appear to be the domain of benthic octopuses and cuttlefishes. The extent of these displays across the more than 350 octopod species and more than 100 cuttlefish species that exist is virtually unknown as the vast majority have not been observed live. Of the more than 70 shallow-water octopus species observed by two authors of the current study (M. Norman and J. Finn), such displays appear restricted to a small subset and primarily occurred in diurnal species. The pulse displays of *O. laqueus* while night hunting appears to be an exception. Dynamic displays were never observed for a number of common shallow-water genera, such as the night-active genus *Callistoctopus* nor the predominantly crepuscular genus *Amphioctopus*.

For cuttlefishes, the presence of such displays across distinct genera (*Sepia* and *Metasepia*) and multiple species suggest that this form of the display may occur more widely in the group. Due to their excellent crypsis and sudden flight from divers, observations of natural behaviors in shallow-water cuttlefishes are rare. Many species also occur beyond diving depths (e.g., >30 m) and are yet to be observed live.

By gender, dynamic displays are part of the repertoire of males in courtship displays for a number of cuttlefish species, particularly for the Australian giant cuttlefish, *S. apama*, where dynamic displays were not observed in females of the species in breeding aggregations (Norman et al., 1999). The more solitary octopuses lack elaborate courtship displays and we know of no evidence of gender -specific dynamic displays in this group.

Dynamic displays used as camouflage and/or as a component of hunting behaviors (e.g., *S. latimanus*) were observed in both juvenile and adult cuttlefishes and may represent a basal capacity from which reproductive display capacities are likely to have evolved.

Function of Displays

In many of the examples described in this paper, the precise behavioral function of the display is unknown or poorly studied. Based on the context in which the pattern was observed we can make some educated guesses as to the broad functional category that they fall into. In general, the dynamic patterns could be described as either fulfilling the function of (A) deceiving or (B) communicating with the target viewer, with most of the examples in this study falling into the latter.

(A) Deception

Using dynamic components of body patterns to deceive intended viewers is a novel area of study that has receive little attention in the scientific literature. Here we described the display of several species that seem to do just this.

The clearest example is the hunting display of the Broadclub cuttlefish *S. latimanus* (**Figure 4c**, Supplementary Video 3.4.3). This pattern is directed toward prey during the final moments of approach, and its highly conspicuous and unusual appearance has led many divers to use terms such as "mesmerizing" or "hypnotizing." Whether or not the pattern alters the behavior of the intended prey in some way remains to be demonstrated, but it would seem unlikely to have evolved this hunting strategy without some increase in predation success. One possibility is that the downward trajectory of the passing waves provides an overlaying motion cue that masks the expanding motion of the cuttlefish outline as it approaches, as a form of motion camouflage. A similar effect has been recorded from motion-detecting neurons in locusts, in which the sensitivity to localized looming cues is inhibited by broad-field motion cues (Simmons and Rind, 1997). Another hypothesis is that the passing wave motion is so unusual and beyond the standard repertoire of natural motion patterns experienced by the prey item that it causes a confused delay in the escape response. A final hypothesis is that the pattern may induce an optokinetic flow-field response in the prey that alters the position or posture of the animal, centering it between the pulsating arms and facilitating a tentacle strike from the cuttlefish. Further research is clearly necessary to determine the precise mechanism of action.

Another dynamic pattern that is directed toward prey is the chromatic pulse of *Octopus vulgaris* and *O. cyanea* (Packard and Hochberg, 1977; Mather and Mather, 2004). This display may deceive the prey item by simulating an approaching object, thus inducing the prey animal to move, presumably to facilitate capture in some way.

A further example of a dynamic display with a potentially deceptive function is the expanding waves of the juvenile giant cuttlefish, *S. apama*. Animals have been observed producing this usually conspicuous display while camouflaging among seaweed moving in the swell (Supplementary Videos 3.5.2 and 3.5.3). The motion characteristics of the display are not unlike the motion of the surrounding weed, leading us to conclude that this dynamic pattern is being produced to blend in to the movement of the environment. Interestingly, this behavior, as well as that of the Mourning cuttlefish, *S. plangon* (**Figure 5c**, Supplementary Video 3.5.4), was only observed at night or under low light conditions in aquaria, suggesting that it may be less effective during the daytime, when the motion may instead render the animal more conspicuous.

(B) Communication

Other dynamic patterns are produced during close interactions with conspecifics, implying a communication function. One of the clearest examples of this is the male-male threat display of *S. apama* performed during mate guarding (**Figures 5a**; Supplementary Video 3.5.1). This slow-moving, expanding display has several features that may enhance the signal's function. Firstly, the expanding motion cue originates from a lateral position on the anterior mantle edge, close to the location of the nearest eye of the observing rival. Although it is difficult to film the display from the precise position of the observing animal, it is possible to imagine that this expanding cue may appear intimidating, possibly even simulating the expanding motion of an approaching rival. The display is further enhanced by contracting the skin on the lateral half of the mantle away from

the rival, thereby stretching the signaling skin field across the midline to substantially increase the visual angle subtended by the display. Whether through just being a large, confusing, moving area or a more directed flow-field pattern giving an illusion of self-motion, the end result of intimidating or driving a rival away appears to be the same.

Another clear example of a dynamic pattern used for communication is the chromatic flashing display used by Humbolt squid, *D. gigas*, during group foraging or mating behavior. Rosen et al. (2015) observed individuals of this species performing the display in the presence of other displaying conspecifics. Given that this species is known to be highly cannibalistic (Markaida et al., 2008), presumably one of the main functions of the display is as a warning or identification signal to conspecifics in the area.

The chromatic pulse exhibited by small male *S. latimanus*, in combination with a jet of ink, represents another clear example of a directed communication signal (**Figure 3d**; Supplementary Video 3.3.3). We observed the display being performed repeatedly by a small male during full daylight as it tentatively approached a larger rival male, suggesting an antagonistic or bluffing signal. It is tempting to think that the coordination of chromatic pulse and ink jet has the overall effect of extending the motion cue of the moving dark patch beyond the borders of the animal's skin, as a sort of "bluff" signal. However, further research is needed to demonstrate this clearly.

Finally, a different function of a dynamic pattern is likely exhibited by the *Metasepia* species *M. pfefferi* and *M. tullbergi* (**Figure 5b**; Supplementary Information 3.5; a, b, c, d, e). These bold and striking patterns are produced strongly when the animal is startled by a diver or potential predator, showing clear parallels with other types of warning coloration (Cott, 1940; Ruxton et al., 2004). These species are slow moving and usually found walking with a quadrupedal gait across the sea floor. They are without obvious weaponry, so it is tempting to conclude that the warning display represents a form of aposematic signal. However, no toxicological study of the flesh of the animal has been published to date, so further study is required.

CONCLUSION

Cephalopods and their dazzling array of visual representations and behaviors continue to fascinate human observers. Given the generally shy nature of this animal group, the complexity of visual signaling reported here is likely to be a fraction of the potential behaviors yet to be discovered. As a result, the role of "pattern motion" in cephalopod visual displays remains a largely unexplored area of research and warrants greater investigation in both laboratory and field settings. In particular, further work is essential for cataloguing the displays and the fine-scale behavioral context in which they are performed in the natural environment. Furthermore, the identification of species that can be elicited to produce the displays in controlled lab environments would allow an experimental approach to investigate the form and function of these enigmatic patterns.

ETHICS STATEMENT

This study is based purely on observations of animals behaving naturally.

AUTHOR CONTRIBUTIONS

Study conceived by MH and NM. Data provided by MH, MN, JF, and WC. Manuscript written by MH. Comments and edits were contributed by all authors.

FUNDING

MH supported by the Royal Society (UF140558). MH and NM partially supported by the Air Force Office of Scientific Research (FA8655-12-1-2112). MN and JF supported by the Australian Biological Resources Study, Australian Federal Government.

ACKNOWLEDGMENTS

We would like to acknowledge the helpful advice and video contributions of Yi-Hsin Lee, Matteo Guardini, Chris Merritt, and Candace McBride.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00393/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 06 February 2017
doi: 10.3389/fphys.2017.00040

Visual Equivalence and Amodal Completion in Cuttlefish

I-Rong Lin[1] *and Chuan-Chin Chiao*[1,2]*

[1] *Institute of Systems Neuroscience, National Tsing Hua University, Hsinchu, Taiwan,* [2] *Department of Life Science, National Tsing Hua University, Hsinchu, Taiwan*

Modern cephalopods are notably the most intelligent invertebrates and this is accompanied by keen vision. Despite extensive studies investigating the visual systems of cephalopods, little is known about their visual perception and object recognition. In the present study, we investigated the visual processing of the cuttlefish *Sepia pharaonis*, including visual equivalence and amodal completion. Cuttlefish were trained to discriminate images of shrimp and fish using the operant conditioning paradigm. After cuttlefish reached the learning criteria, a series of discrimination tasks were conducted. In the visual equivalence experiment, several transformed versions of the training images, such as images reduced in size, images reduced in contrast, sketches of the images, the contours of the images, and silhouettes of the images, were used. In the amodal completion experiment, partially occluded views of the original images were used. The results showed that cuttlefish were able to treat the training images of reduced size and sketches as the visual equivalence. Cuttlefish were also capable of recognizing partially occluded versions of the training image. Furthermore, individual differences in performance suggest that some cuttlefish may be able to recognize objects when visual information was partly removed. These findings support the hypothesis that the visual perception of cuttlefish involves both visual equivalence and amodal completion. The results from this research also provide insights into the visual processing mechanisms used by cephalopods.

Keywords: visual discrimination, visual perception, object recognition, size constancy, visual completion

OPEN ACCESS

Edited by:
Daniel Osorio,
University of Sussex, UK

Reviewed by:
Akira Sakurai,
Georgia State University, USA
Jordanna Dorothy Henry Sprayberry,
Muhlenberg College, USA

***Correspondence:**
Chuan-Chin Chiao
ccchiao@life.nthu.edu.tw

Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *15 September 2016*
Accepted: *13 January 2017*
Published: *06 February 2017*

Citation:
Lin I-R and Chiao C-C (2017) Visual Equivalence and Amodal Completion in Cuttlefish. Front. Physiol. 8:40. doi: 10.3389/fphys.2017.00040

INTRODUCTION

Cephalopods possess the largest and most complex nervous systems in invertebrates (Nixon and Young, 2003). Their brains can be anatomically divided into 30–40 interconnected lobes that have similarities to the brain organization of vertebrates (Young and Boycott, 1971; Hochner, 2010). As highly visual animals, cephalopods exhibit a repertoire of sophisticated motor responses that are driven by their visual systems (Packard, 1972). Their keen vision assists them in executing a diverse series of complex behaviors such as camouflage body patterning and conspecific communication (Hanlon and Messenger, 1996). Therefore, it seems likely that vision has played an important role in shaping the evolution of cephalopod cognition (Darmaillacq et al., 2014). Although previous studies have demonstrated that cephalopods are capable of various types of visual discrimination, evidence indicating how the highly developed visual systems of cephalopods generate visual sensation and perception are lacking (Zylinski and Osorio, 2014).

Stimulus generalization is a fundamental cognitive ability that is characterized by an organism treating similar stimuli equivalently (Bruce et al., 2003). Basic generalization capacity is typically demonstrated by showing that animals with a learnt response to a given stimulus are able to transfer the established behavior to a novel stimulus that resembles the previous one (Shettleworth, 2009). Physical similarity between the perceived and stored information underlies stimulus generalization and therefore such transfer is both immediate and specific to a given stimulus (Marr, 2010). This adaptive response to new situations not only reduces the visual memory load of an organism, but also is likely to have the potential to increase the foraging success of the animal and to lower the threat from predators (Wynne and Udell, 2013).

Vertebrates and insects display high degrees of visual generalization (reviewed in Ghirlanda and Enquist, 2003; Horridge, 2009). For example, systematic studies using honeybees have shown that bees trained to recognize complex stimuli are able to transfer their choices to novel stimuli that preserved common features; these features include size, shape, orientation, pattern, and symmetry (Stach et al., 2004; Lehrer and Campan, 2005; Gross et al., 2009). However, visual generalization has seldom been investigated in cephalopods. Muntz (1961) studied interocular generalization in octopuses (*Octopus vulgaris*). Octopuses were trained to discriminate two visual stimuli using one eye, and then were tested using the untrained eye. Their results showed that the performance of octopuses in training had an impact on the degree of generalization. In a separate experiment, the same author also showed that octopuses trained to distinguish two complex shapes were able to transfer their responses to shapes that had different orientations to that of the original ones (Muntz, 1970). Similar to the aforementioned visual generalization, the ability of visual equivalence in cuttlefish was actually examined in the present study. Images are considered visually equivalent if they convey the same impressions of scene appearance, even if they are visibly different (Ramanarayanan et al., 2007).

Visual systems are known to engage in a process that allows active fill-in of absent details via connecting physically discontinuous image regions (Kanizsa, 1979; Michotte et al., 1991). This grouping mechanism allows the organism to perceive a complete rather than an incomplete form and is generally called "visual completion" (Bruce et al., 2003). This process has been divided into two types, modal and amodal. Visual completion by inducing a clear visual impression of a contrast border in an image region where there is no physical contrast border is known as "modal completion" (Snowden et al., 2012). The induced border is referred to as "illusory contour," since it is not present in the physical stimulus. A classic example of modal completion is the Kanizsa triangle, which appears to most observers as a white triangle superimposed on three black discs (Kanizsa, 1979). On the other hand, visual completion by inducing a visual perception of a partially occluded object as an integral unity without generating any local contrast and illusory contours, which means that the perceived object has the same "mode" as the whole object, is known as amodal completion (Marr, 2010; Snowden et al., 2012). Thus, amodal interpolation of the likely form when there is an obscured region is based on the visible portions of the object.

The ability to carry out visual completion is ubiquitous in humans, and has been demonstrated in a number of other vertebrate taxa including non-human primates (Sato et al., 1997; Deruelle et al., 2000), rodents (Kanizsa et al., 1993), and fishes (Sovrano and Bisazza, 2008; Darmaillacq et al., 2011). Furthermore, honeybees are able to complete objects modally rather than amodally (Hateren et al., 1990; Horridge et al., 1992), which implies the possibility that other invertebrates may also be equipped with the ability to carry out visual completion. Recently, Zylinski et al. (2012) provided the first evidence of contour completion in cuttlefish (*Sepia officinalis*) by showing that cuttlefish respond with similar camouflage body patterns to either a whole visual stimulus or a fragmented visual stimulus.

In the present study, our goals were to examine the visual recognition capacities of one species of cuttlefish (*S. pharaonis*). We trained the cuttlefish to discriminate between two images using a newly developed behavioral paradigm. The images used in the study were artificial images of fish and shrimp. The performance of the cuttlefish thus allows us to evaluate their ability to carry out visual equivalence and amodal completion. Studying whether cuttlefish have similar visual processing mechanisms to their vertebrate counterparts, namely visual equivalence and completion, should increase greatly our understanding of convergent evolution in the context of animal visual processing.

MATERIALS AND METHODS

Animals

Twenty-one cuttlefish (*S. pharaonis*) from three different sources were used in the present study. Three animals formed Group A (cuttlefish A1–A3; mantle length, 3–5 cm) and were reared from eggs (trawled from the sea southwest of Taiwan near Tungkang and hatched in April 2011) at the National Museum of Marine Biology and Aquarium in Pingtung; these animals were transported to the National Tsing Hua University (NTHU) in Hsinchu for the experiments during June 2011. Ten animals formed Group B (cuttlefish B1–B10; mantle length, 5–12 cm) and these were also reared from eggs (collected by local fishermen fishing from Penghu and hatched in April 2011) at the National Penghu University of Science and Technology in Penghu; these animals were transported to the NTHU for the experiments during July 2011. Eight animals formed Group C (cuttlefish C1–C8; mantle length, 9–15 cm); these were sub-adult animals caught in northeastern of Taiwan near Yehliu, and were kept in the National Taiwan Ocean University at Keelung before being transported to the NTHU for experiments during February 2012. At NTHU the cuttlefish were housed individually in plastic tanks (depending on their mantle length; ML $\leq$ 4 cm: 33 cm $\times$ 23 cm $\times$ 24 cm, 4 cm $\leq$ ML $\leq$ 9 cm: 50 cm $\times$ 29 cm $\times$ 29 cm, and ML $\geq$ 9 cm: 78 cm $\times$ 50 cm $\times$ 30 cm), in two close-circulation aquariums (700 L each; water temperature 21 $\sim$ 24°C). The cuttlefish were fed fish and shrimp twice daily and acclimated to the system at least 1 week prior to training. Training was started only when the cuttlefish showed signs of aggressive predation. All experiments

were conducted in the home tanks of cuttlefish between 10 a.m. and 6 p.m. from July 2011 to May 2012. Five animals died during the training sessions and 16 cuttlefish completed the training. Among the trained animals, two died soon after the training and thus only 14 cuttlefish underwent testing (**Table 1**).

Apparatus

The apparatus was constructed of white corrugated plastic sheets and included two separate regions (the choosing areas), where two different visual stimuli were presented on the front walls at a height of 5 cm above ground (**Figure 1**). The two lateral walls were flexible and could be swung toward or away from the central divider. This design allowed the visual stimuli to be covered before putting the apparatus in the tank for training or testing. The visual stimuli were revealed by slowly swinging out lateral walls for viewing only after the cuttlefish had settled down. This also ensured that the cuttlefish saw both visual stimuli simultaneously at the start of each trial. Both visual stimuli were illuminated equally during the experiment, though the central divider may sometimes cause slight shadows on visual stimuli.

Visual Stimuli

Pictures of fish and shrimp (length, 6.5 cm) were downloaded from the internet (**Figure 2A**). To investigate whether cuttlefish are equipped with the object recognition abilities to carry out visual equivalence and amodal completion tasks, several sets of paired images were modified from the originals using a graphic editing program (Ulead PhotoImpact X3). The reason that the images of fish and shrimp were chosen in the present study, instead of the simpler figures such as square and circle, is that cuttlefish were difficult to train to associate an abstractive stimulus with a reward. Since the cuttlefish were fed both fish and shrimp, it is unlikely that they have strong prey preferences. Furthermore, either a fish image or a shrimp image was randomly assigned to each individual cuttlefish before training (see below), thus the bias of their choice and learning ability due to the experience was reduced. To make the reduced size images, the original images of the fish and shrimp were resized to 60% of their original size (**Figure 2B**, up-left). To reduce the contrast of the fish and shrimp, the image contrast was adjusted to 50% of the original contrast (**Figure 2B**, mid-left). To create sketches of fish and shrimp, the sharpening effect of graphic editing program was used first to enhance edges and the image was thresholded to create a binary version (**Figure 2B**, bottom-left). To generate the contoured images, the outlines of animals were traced individually by hand (**Figure 2B**, up-right). To make the black silhouettes, the contoured region was filled with black (**Figure 2B**, mid-right). To make the white silhouettes, the contrast polarity was reversed from black to white (**Figure 2B**, bottom-right). The selectively occluded (amputated) images were generated by covering specific areas of the animals with white stripes (**Figure 2C**). These images consisted of partial occlusion (25% of the body covered by four stripes), tail occlusion (the posterior half covered), and head occlusion (the anterior half covered). The images were printed using a high quality laser printer (HP LaserJet P2055), then cut to give an 8.2 × 8.2 cm square with each pattern in center. Finally the images were laminated to make them waterproof.

Discrimination Training

The cuttlefish were trained to discriminate images of fish and shrimp (**Figure 2A**) using the operant conditioning paradigm. The goal is to train cuttlefish to strike reliably either a fish or a shrimp image with their tentacles. The reward image, a fish image or a shrimp image, was randomly assigned to each individual cuttlefish before training. Since cuttlefish do not naturally strike an object or image, the food (frozen shrimp) was initially presented in front of the reward image to draw animal's attention (i.e., the cuttlefish turned toward the reward image and showed convergence eye movement). During the visual attack of the cuttlefish (*S. officinalis*), it has been reported that attention is the first phase of the response (Messenger, 1968). Specifically, in attention there are color changes and movements of the eyes and head. The whole animal turns so that the prey comes to lie on a forward extension of the body axis. As soon as cuttlefish showed a sign of attention to the presentation of visual stimuli, swam into the reward image area, or carried out a strike on the image within 60 s, the food was delivered as a reward to motivate cuttlefish continuously performing this discrimination task. Each trial lasted 3 min, or until the cuttlefish made a correct choice. Each cuttlefish received five training trials per day. The position of the reward image was randomly assigned to the left or right in each trial. The discrimination training was considered complete only when cuttlefish achieved the learning criterion, which was an 80% correct response (that is choosing the reward image in 8 out of 10 trials over 2 consecutive days). To ensure the cuttlefish were able to discriminate the reward image from the non-reward image, after the training session a discrimination test was conducted. During this test the non-reward image was replaced by a novel image, such as a crab image, and the discrimination ability of each cuttlefish was then assessed again (data not shown).

Transfer Tests

A transfer test was conducted after animals passed the discrimination test to examine if cuttlefish are capable of visual equivalence and amodal completion. Each animal received 10 trials (five trials each day for two consecutive days) in a transfer

TABLE 1 | Number of discrimination training trials before reaching the learning criteria for each cuttlefish.

Cuttlefish	A1	A2*	B1	B2	B3	B4	B8	B9	B10	C1	C2	C4	C5	C6	C7	C8*
# of trials	80	105	50	85	90	95	20	90	25	40	110	90	55	130	45	65

**A2 and C8 died after training and did not take part in any of the later tests.*

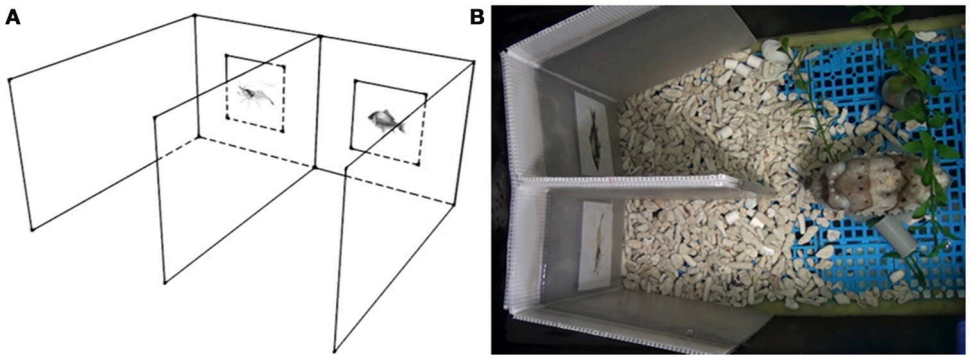

FIGURE 1 | The experimental setup. (A) A schematic diagram of the apparatus from a side view. The apparatus was constructed to include two separate regions (the choosing areas) where the two visual stimuli were presented on the front wall. **(B)** Top view of the apparatus in the home tank of the cuttlefish. Cuttlefish at the choice point could see both stimuli simultaneously.

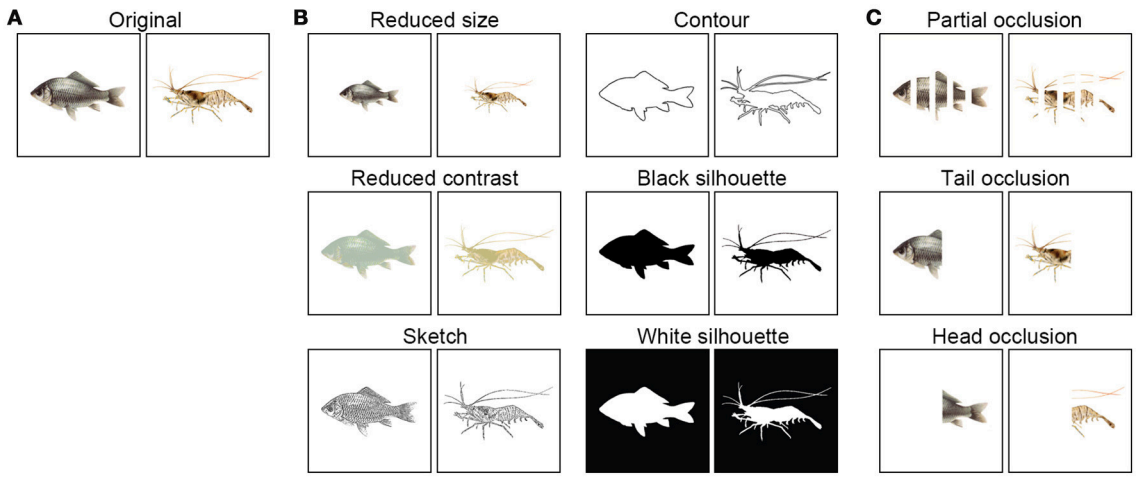

FIGURE 2 | Visual stimuli used in the present study. (A) Fish and shrimp images were utilized for the discrimination training. **(B)** Six versions of the original images were used in the generalization tasks. **(C)** Three variations of the original images were used for the amodal completion tasks.

test to retain the motivation of cuttlefish in performing the task. The position of the trained image was randomly assigned to the left or right in each trial, and the experimenter was not blind to the assignment of the previously rewarded image to each cuttlefish. To keep cuttlefish paying attention to the experimental apparatus, reward was offered for every correct response. If cuttlefish chose the previously non-reward image or did not respond at all in 5 min, the experimental apparatus was removed immediately, and the trial started again. To eliminate the effect of reinforcement and extinction, the image was covered during food delivery or before removing the apparatus. Between different transfer tests, an inter-test training session was held for cuttlefish to reinforce the conditioned response. Only when cuttlefish achieved the learning criterion of 80% correct response again, then a different transfer test was conducted. There were nine transfer tests (six for visual equivalence and three for amodal completion) that took place during the present study (**Figures 2B,C**).

Scoring

The cuttlefish response in each task was graded at six levels (**Figure 3**): (0) no attention paid to the apparatus, (1) stared at the image with continuous attention (i.e., the whole animal turned so that the image came to lie on a forward extension of the body axis, subtending equal angles to the two eyes) for 1 min without entering the reward area, (2) stared at the image with continuous attention <1 min and entered the reward area, (3) stay in the reward area at a short distance from the previously rewarded image for 30 s, (4) touched the previously rewarded image with its arms, (5) struck at the previously rewarded image with its tentacles. Cuttlefish were considered making a correct choice when they showed any of the score above zero responses in a trial.

Data Analysis

The binomial test was used to examine the statistical significance of the difference between the numbers of correct choices and

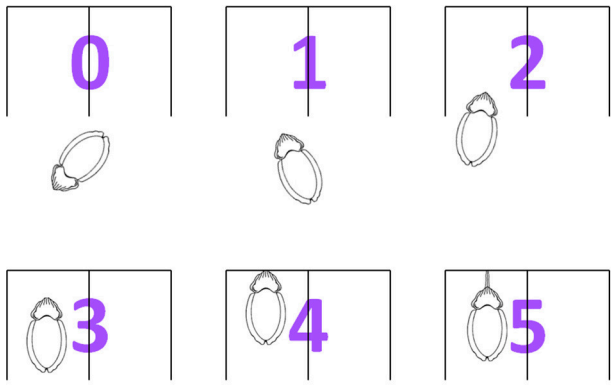

FIGURE 3 | The six levels of cuttlefish choosing response. The cuttlefish response in each task was graded into six scores: (0) no attention on the apparatus, (1) stared at the figure with continuous attention (i.e., the whole animal turned so that the image came to lie on a forward extension of the body axis, subtending equal angles to the two eyes) for 1 min without entering the reward area, (2) stared at the figure with continuous attention <1 min and enter the reward area, (3) stayed in the reward area at a short distance from the previously rewarded figure for 30 s, (4) touched the previously rewarded figure with its arms, (5) struck the previously rewarded figure with its tentacles.

incorrect choices for each animal over the nine transfer tests by comparing with the expected frequency of 50%. The score for each trial was normalized to the strongest response determined in the earlier discrimination training for each cuttlefish. The one-tailed Wilcoxon Signed Ranks test of the normalized scores was used to assess the choosing tendency of each animal over the nine transfer tests by comparing with the expected normalized score of zero. In addition, the one-tailed Wilcoxon Signed Ranks test was used to determine the choice tendency of all cuttlefish by analyzing the correct response percentages and the normalized scores obtained in each transfer task. All statistical analysis was conducted using SPSS.

RESULTS

Sixteen of the 21 cuttlefish finished discrimination training (**Table 1**), while five died during training and two died immediately after training (A2 and C8). Among these 16 trained cuttlefish, two animals (B8 and B10) reached the learning criteria in <25 trials, and another three animals (B1, C1, and C7) reached the learning criteria in <50 trials. These animals appeared to be faster learners. Discrimination learning was confirmed when the percentage of correct responses of the cuttlefish rose from below chance (50% correct) to a success rate ranging from 80 to 100% (**Figure 4**). After completion of discrimination training, all cuttlefish reached the response level of 5, except A1 and B9 which only attained the response level of 3 (see Supplementary Information). The performance of the cuttlefish improved over time and the learning curves for most of the cuttlefish were S-shape, though some animals showed few correct responses initially and followed by an extremely rapid improvement (**Figure 4D**). All data including the results from training sessions and transfer tasks (below) were provided as the Supplementary Information.

Visual Equivalence

When the transfer task involving the original fish and shrimp images being changed to reduce-scale images was carried out, the percentages of correct responses for seven cuttlefish (B1, B4, B8, B9, B10, C1, and C4) were higher than 80% (**Figure 5A**, left panel). For these animals, the numbers of correct choices were significant higher than those of the incorrect choices (binomial test, see **Table 2**). In terms of the cuttlefish average normalized responses, the scores of nine animals (B1, B2, B3, B4, B8, B9, B10, C1, and C4) were above 0.4 and seven of them were even higher than 0.7 (**Figure 5A**, right panel). Interestingly, cuttlefish B1, B4, and C4 obtained a score of +5 for all test trials. The same nine cuttlefish also showed a significant tendency to choose the rewarded images (Wilcoxon signed-rank test, see **Table 2**).

Using the low contrast version of the original images as stimuli, two cuttlefish (B4 and C1) exhibited 80% correct responses (**Figure 5B**, left panel). The correct choices made by these two animals were significant higher than the incorrect choices (binomial test, see **Table 2**). The average normalized scores were 0.475 and 1 (i.e., got +5 scores for all 10 test trials), respectively (**Figure 5B**, right panel). A significant tendency to target the rewarded image was also found (Wilcoxon signed-rank test, see **Table 2**).

When the initial images were replaced by sketches, the percentages of correct responses of three animals (B4, C1, and C4) reached 80% (**Figure 5C**, left panel). The correct choices made by cuttlefish C4 were significant higher than its incorrect choices (binomial test, see **Table 2**). Note that cuttlefish B1 preferred the non-reward image significantly ($p = 0.02$) for no obvious reason. In addition, the average normalized scores of two cuttlefish B4 and C4 were higher than 0.5 (**Figure 5C**, right panel). However, cuttlefish B4, C2, and C4 showed a significant tendency to choose the rewarded image (Wilcoxon signed-rank test, see **Table 2**).

The performance of all six cuttlefish toward the contoured original image was poor. The percentages of correct responses were lower than the 50% chance level (**Figure 5D**, left panel). None of these animals ever obtained a +5 score in a test trial and the average normalized scores were all <0.1 (**Figure 5D**, right panel). No significant trend was found (Wilcoxon signed-rank test, see **Table 2**).

When the stimuli were black silhouettes of original images on a white background, the percentages of correct choice of three cuttlefish (C1, C2, and C4) were higher than 80% (**Figure 5E**, left panel). The correct choices made by these three animals were significant higher than the incorrect choices (binomial test, see **Table 2**). The average normalized scores of four cuttlefish (B4, C1, C2, and C4) were higher than 0.5 (**Figure 5E**, right panel), and they also showed a significant tendency to choose the rewarded image (Wilcoxon signed-rank test, see **Table 2**).

In the case of white silhouettes of the original images on a black background, the percentages of correct choice were above 50% for four cuttlefish (B4, C1, C2, and C4; **Figure 5F**, left panel). However, only cuttlefish C4 made five correct choices and five undetermined responses and thus with this animal the number of correct choices was significant higher than its incorrect

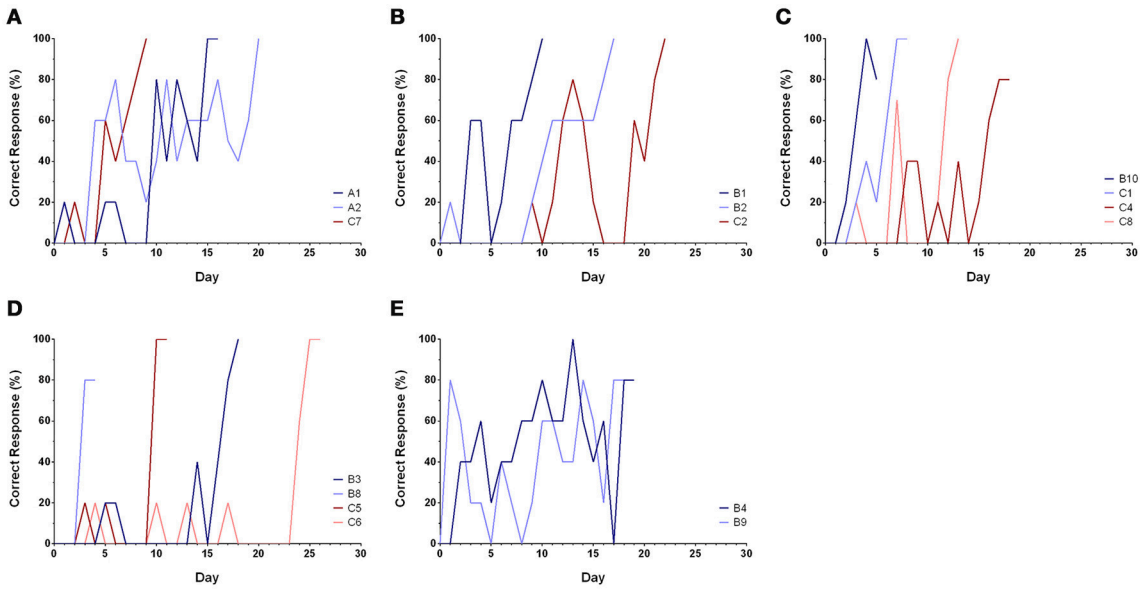

FIGURE 4 | Learning curves of cuttlefish in the discrimination training. (A–C) Learning behaviors of most cuttlefish were a typical S-shape, showing a relatively gradual improvement. **(D)** Few correct responses initially and followed by an extremely rapid improvement. **(E)** Early fast learning and followed by a slow improvement. It is apparent that some animals did not respond to the reward image at all in the first few days (i.e., scored 0 point) or chose the non-reward image at the beginning of the training.

choices (binomial test, see **Table 2**). The average normalized scores of cuttlefish C1 and C4 were 0.489 and 0.920, respectively (**Figure 5F**, right panel), and a significant tendency toward the rewarded images was also found (Wilcoxon signed-rank test, see **Table 2**).

In addition to assessing the responses of individual cuttlefish, we also consider the group performance for each task. Cuttlefish tended to respond to the rewarded images in the visual equivalence tasks when the images were reduced in size and sketches (**Figure 6A**; one-tailed Wilcoxon signed-rank test, see **Table 3**). Similarly, taking the strength of the responses into account, these animals also exhibited strong responses in tasks when the images were reduced in size and sketches (**Figure 6B**; one-tailed Wilcoxon signed-rank test, see **Table 3**). Even though the individual responses had at least one or more animals showed the statistical significance in five of six tasks (except in the contour test), due to the small sample size in some experiments, the population results only supported cuttlefish's capacity in two of six visual equivalence tasks (reduced size and sketch).

Amodal Completion

During the first amodal completion task, the fish and shrimp images were partially occluded by four 0.4 cm white stripes (25% of the body covered by four stripes) and under these conditions, the percentages of correct choices of four cuttlefish (B1, C4, C6, and C7) were above 80% (**Figure 7A**, left panel). For these four animals, the numbers of correct choices were significant higher than those of incorrect choices (binomial test, see **Table 2**). The average normalized scores of these four animals were higher than 0.4 (**Figure 7A**, right panel). A significant tendency toward the rewarded image was found for these animals (Wilcoxon signed-rank test, see **Table 2**).

In the second amodal completion task, fish and shrimp images were posteriorly occluded (head visible) and the results showed that the percentages of correct responses of all four cuttlefish were higher than 80% (**Figure 7B**, left panel). The correct choices made by three animals (C4, C6, and C7) were significantly higher than their incorrect choices (binomial test, see **Table 2**). The average normalized scores of all cuttlefish were above 0.6 (**Figure 7B**, right panel). In addition, cuttlefish C4 and C7 obtained +5 scores for all 10 test trials. All four animals had a significant tendency to choose the rewarded images (Wilcoxon signed-rank test, see **Table 2**).

During the final amodal completion task, the fish and shrimp images were anteriorly occluded (tail visible). In this part of the study, the percentage of correct choice of only one cuttlefish C6 was above 80% (**Figure 7C**, left panel), but no statistical significant was found (binomial test, see **Table 2**). Among these subjects, the average normalized score of cuttlefish C6 was 0.64 (**Figure 7C**, right panel), and it showed a significant tendency toward the reward image (Wilcoxon signed-rank test, see **Table 2**).

In addition to assessing the responses of individual cuttlefish, we also consider the group performance for each task. Due to the small sample size in the present study, cuttlefish tended to respond to the rewarded images in only the partial occlusion task (**Figure 8A**; one-tailed Wilcoxon signed-rank test, see **Table 3**). Even taking the strength of the responses into account, cuttlefish still exhibited strong responses only in the partial occlusion task (**Figure 8B**; one-tailed Wilcoxon signed-rank test, see **Table 3**).

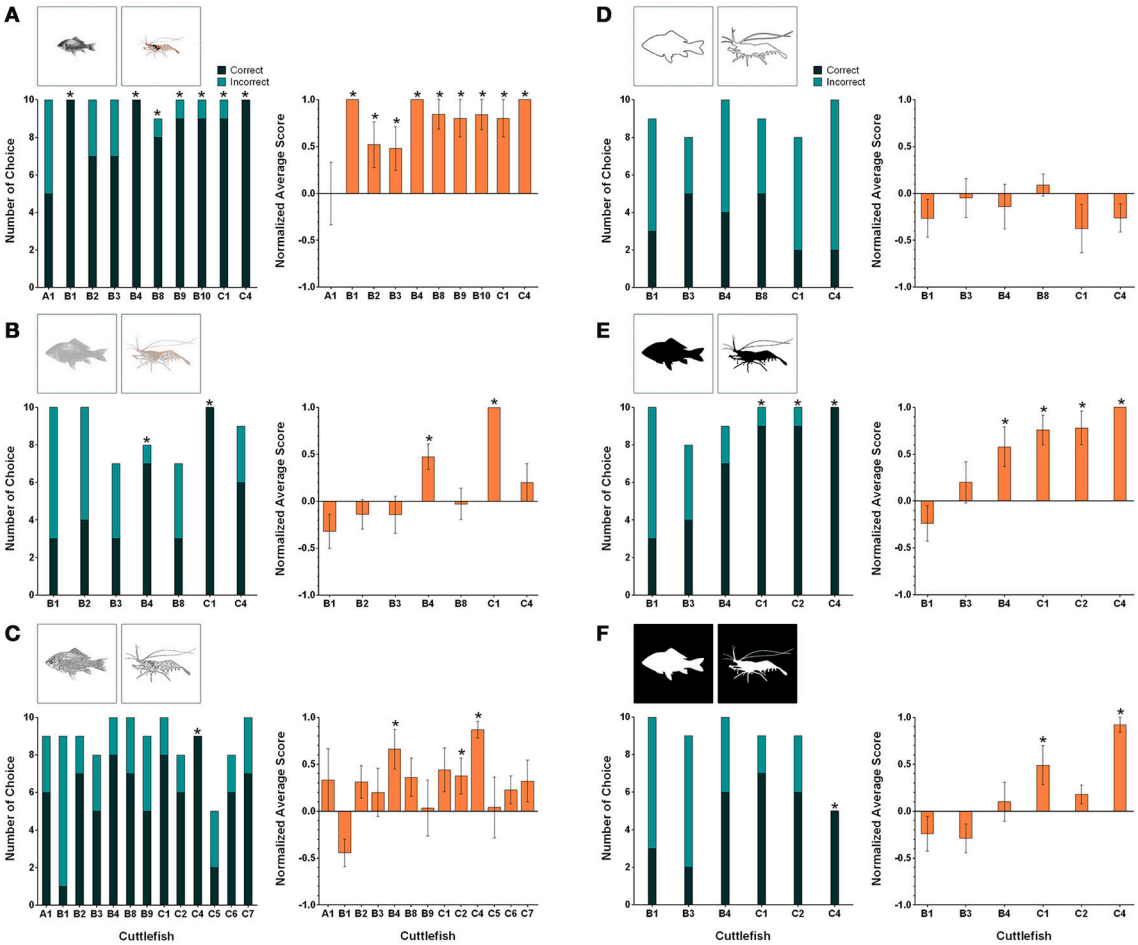

FIGURE 5 | The results for individual cuttlefish during the six visual equivalence tasks: (A) Reduced size, **(B)** Reduced contrast, **(C)** Sketch, **(D)** Contour, **(E)** Black silhouette, and **(F)** White silhouette. The left panels show the correct/incorrect number of choices made by individual cuttlefish during these tasks. The correct response was determined when cuttlefish showed any of the score above zero responses in a trial (see Section Materials and Methods for scoring). Asterisks indicate statistical significance for the correct choice ($p < 0.05$). The right panels show the average normalized scores of the individual cuttlefish for the same tasks. The scores were normalized against the strongest response in the training. Asterisks indicate a significant tendency toward the reward figure. Note that cuttlefish B1 was significant for the incorrect choice and the tendency toward the non-reward figure in the sketch task, but asterisks were not labeled. Error bars are SEM.

However, it is apparent that the individual responses had at least one or more animals showed the statistical significance in all three tasks, thus although the population results only supported cuttlefish's capacity in the partial occlusion task, it is likely that cuttlefish are also capable of amodal completion at least in the posterior occlusion task.

DISCUSSION

Visual Association Learning in Cuttlefish

Although some cuttlefish took a significant longer time to learn the association between the visual stimulus and the reward, once they had learnt, they could be tested using a range of different visual perception tasks. More importantly, the time cuttlefish spent learning (**Figure 4**) appears to be independent of their performance in these transfer tests. This suggests that there is variability between individual cuttlefish regarding visual association learning and reliability when carrying out visual perception tasks.

In addition to striking the rewarded target, some other behavioral features were observed during the training and these might be useful when assessing cuttlefish learning. For example, cuttlefish tended to approach the target image with a "stop-and-go" or stealth-type locomotion while raising and waving their first pair of arms in front of the target image and then changing their skin coloration on recognizing the target image. These signs suggest that the cuttlefish is paying attention to the target image or at least is able to recognize the visual stimulus during both training and testing. Interestingly, we also found that all cuttlefish responded to the reward image with a tentacle strike initially, but after a few trials, some animals begin to grab the images with their arms instead. This behavioral shift in their foraging strategy may result from pain when the tentacles strike (Messenger, 1973) and is another indication of learning by visual association.

TABLE 2 | Statistical results for each individual cuttlefish across all tasks.

Testing stimuli	Statistical analysis		Cuttlefish													
			A1	B1	B2	B3	B4	B8	B9	B10	C1	C2	C4	C5	C6	C7
Reduced size	Binomial test	P	0.623	0.001*	0.172	0.172	0.001*	0.020*	0.011*	0.011*	0.011*	–	0.001*	–	–	–
	Wilcoxon signed-rank test	W	0	55	43	39	55	43	44	53	44	–	55	–	–	–
		Z	0.000	−3.162	−2.282	−2.040	−3.162	−2.758	−2.530	−2.940	−2.530	–	−3.162	–	–	–
		P	0.500	0.001*	0.011*	0.021*	0.001*	0.003*	0.006*	0.002*	0.006*	–	0.001*	–	–	–
Low contrast	Binomial test	P	–	0.172	0.377	0.500	0.035*	0.500	–	–	0.001*	–	0.254	–	–	–
	Wilcoxon signed-rank test	W	–	−31	−17	−10	33	−2	–	–	55	–	15	–	–	–
		Z	–	−1.596	−0.889	−0.862	−2.328	−0.171	–	–	−3.162	–	−1.000	–	–	–
		P	–	0.056	0.187	0.195	0.010*	0.432	–	–	0.001*	–	0.159	–	–	–
Sketch	Binomial test	P	0.254	0.020*	0.090	0.363	0.055	0.172	0.500	–	0.055	0.145	0.002*	0.500	0.145	0.172
	Wilcoxon signed-rank test	W	15	−35	23	13	49	32	1	–	29	24	45	1	19	25
		Z	−1.000	−2.090	−1.475	−0.933	−2.595	−1.638	−0.064	–	−1.499	−1.703	−2.810	−0.137	−1.354	−1.287
		P	0.159	0.019*	0.070	0.176	0.005*	0.051	0.475	–	0.067	0.044*	0.003*	0.446	0.088	0.099
Contour	Binomial test	P	–	0.254	–	0.363	0.377	0.500	–	–	0.145	–	0.055	–	–	–
	Wilcoxon signed-rank test	W	–	−25	–	−5	−17	13	–	–	−16	–	−27	–	–	–
		Z	–	−1.530	–	−0.352	−0.900	−0.787	–	–	−1.206	–	−1.430	–	–	–
		P	–	0.063	–	0.363	0.184	0.216	–	–	0.114	–	0.077	–	–	–
Black silhouette	Binomial test	P	–	0.172	–	0.637	0.090	–	–	–	0.011*	0.011*	0.001*	–	–	–
	Wilcoxon signed-rank test	W	–	−22	–	13	37	–	–	–	52	51	55	–	–	–
		Z	–	−1.135	–	−0.923	−2.239	–	–	–	−2.716	−2.754	−3.162	–	–	–
		P	–	0.129	–	0.178	0.013*	–	–	–	0.004*	0.003*	0.001*	–	–	–
White silhouette	Binomial test	P	–	0.172	–	0.090	0.377	–	–	–	0.090	0.254	0.031*	–	–	–
	Wilcoxon signed-rank test	W	–	−22	–	−27	6	–	–	–	37	27	15	–	–	–
		Z	–	−1.188	–	−1.616	−0.318	–	–	–	−2.239	−1.634	−2.121	–	–	–
		P	–	0.118	–	0.053	0.376	–	–	–	0.013*	0.051	0.017*	–	–	–
Partial occlusion	Binomial test	P	–	0.020*	–	0.254	0.623	–	–	–	0.637	0.500	0.035*	–	0.016*	0.002*
	Wilcoxon signed-rank test	W	–	41	–	27	12	–	–	–	4	−1	31	–	21	45
		Z	–	−2.459	–	−1.616	−0.624	–	–	–	−0.302	−0.137	−2.226	–	−2.232	−3
		P	–	0.007*	–	0.053	0.267	–	–	–	0.382	0.446	0.013*	–	0.013*	0.002*
Posteriorly occlusion	Binomial test	P	–	–	–	–	–	–	–	–	0.055	–	0.001*	–	0.011*	0.001*
	Wilcoxon signed-rank test	W	–	–	–	–	–	–	–	–	33	–	55	–	44	55
		Z	–	–	–	–	–	–	–	–	−1.897	–	−3.162	–	−2.530	−3.162
		P	–	–	–	–	–	–	–	–	0.029*	–	0.001*	–	0.006*	0.001*
Anteriorly occlusion	Binomial test	P	–	–	–	–	–	–	–	–	0.377	–	0.172	–	0.055	0.254
	Wilcoxon signed-rank test	W	–	–	–	–	–	–	–	–	13	–	30	–	40	19
		Z	–	–	–	–	–	–	–	–	−0.690	–	−1.576	–	−2.160	−1.150
		P	–	–	–	–	–	–	–	–	0.245	–	0.058	–	0.016*	0.125

The binomial test was used to examine the correct responses from the individual animals for each task (p, p-value). The one-tailed Wilcoxon signed-rank test was applied to analyze the normalized scores obtained from the individual animals for each task (W, test statistic; Z, the Z statistic; p, p-value). Asterisks indicate statistical significance.

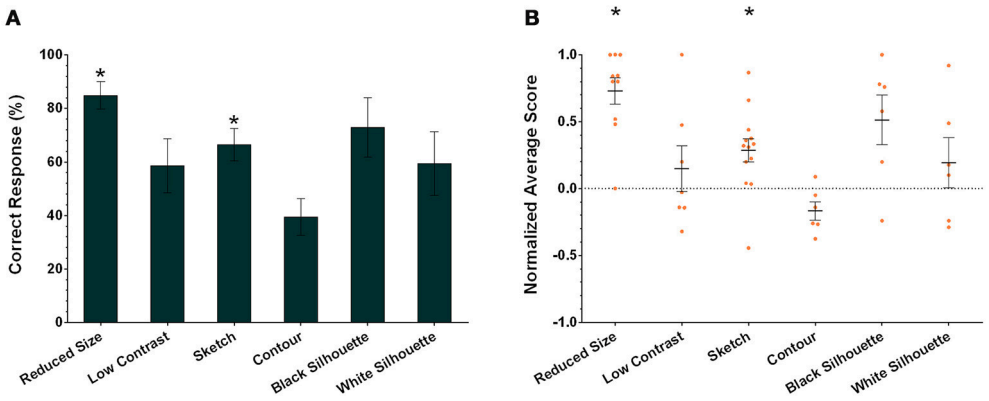

FIGURE 6 | The population results of cuttlefish in the six visual equivalence tasks. (A) Average correct response percentages of all cuttlefish in each task. **(B)** Average normalized scores of all animals in each task. Orange dots represent individual data. $N = 10$, 7, 13, 6, 6, and 6 for reduced size, reduced contrast, sketch, contour, black silhouette, and white silhouette, respectively. Asterisks indicate statistical significance ($p < 0.05$). Error bars are SEM.

Object Perception and Visual Equivalence

Species that live in rich and diverse natural environments need visual systems that work hard in order to process and organize the very large amount of visual information that is received by the organism's eyes (Land and Nilsson, 2012; Cronin et al., 2014). Visual generalization and equivalence is a fundamental ability that helps an individual to deal with similar visual events and helps the individual to make consistent responses without repeated information processing (Bruce et al., 2003). The ability to carry out generalization is found in a wide range of animals and is indispensable to survival in a constantly changing environment (Marr, 2010). This is because what has been learned from a limited experience is unlikely to recur in an identical form again. For example, bee foragers need to identify appropriate flowers regardless of their orientation, shape, color, illumination, etc. and therefore generalization of these features assists their forage success (Horridge, 2009).

In the present study, the strongest evidence of visual equivalence is presented by the data from the task with reduced size images, in which nine out of ten cuttlefish gave significant responses to the correct images. This result indicates that cuttlefish exhibit a highly degree of visual equivalence for size and it is not hard to understand why this is true. Specifically, there are abundant details of the prey preserved in the images and evolutionarily it seems likely that cuttlefish will want to know a larger prey and a smaller prey are both prey. Similar size equivalence has been demonstrated widely in vertebrates (Guttman and Kalish, 1956; Jenkins et al., 1958; Ewert, 1980; Dougherty and Lewis, 1991) and insects (Tinbergen et al., 1942). For instance, rats trained to open a door in the center of a white circle was able to transfer their responses with respect to opening doors in circles of a variety of different sizes. The ability to make a consistent judgment with respect to similar objects independent of its physical size resembles the concept of size constancy, which refers to the invariant judgment that occur with a particular object regardless of their size on the retina (Bruce et al., 2003; Marr, 2010; Snowden et al., 2012). Size constancy has been demonstrated in both vertebrates (Pastore, 1958; Lombardi and Delius, 1990) and insects (Jacobs-Jessen, 1959). For example, goldfish trained to discriminate between two similar objects of different sizes were able to exhibit successful discrimination when these objects are placed at different distances from the fish so as to subtend the same visual angle on the retina (Douglas et al., 1988). In the experiment using cephalopods, cuttlefish (*S. officinalis*) were trained to discriminate between squares of different sizes and were found to show size constancy (Messenger, 1977).

Visual generalization is not merely restricted to a single feature. Multi-feature generalization, which involves complex patterns, has been extensively studied in honeybees. Bees can be trained to discriminate circular patterns with differently oriented gratings in four quadrants and were able to transfer their choices to a corresponding simplified situation (Stach et al., 2004). Moreover, the degree of transfer was found to be dependent on the training length and prolonging the training length led to a promotion of both the generalization level and the discrimination strategy shift (Stach and Giurfa, 2005).

Well-experienced bees tend to extract only the minimum necessary information needed for discrimination since they cannot distinguish the original pattern from the simplified pattern. It has also been shown that the processing strategies involved in visual recognition include a shift from the elemental to the global as the trial numbers further increase, and this shift could decrease the bee's performance in recognizing the original image (Giurfa et al., 2003). In the present study, we found that tentacle strikes mainly occurred during the first one to two trials of the task with the sketched images, and the performance of some animals declined during the subsequent trials (see Supplementary Information). If we consider this in terms of the visual recognition strategy shift that occurs with bees, we suggest that cuttlefish use a similar strategy change for visual recognition. That is, cuttlefish might initially be concerned about the detailed information available, including structures, textures, and outlines, but subsequently they acquire a global view of the sketched image, the integral style of the image held by

TABLE 3 | Statistical results for all cuttlefish across nine tasks.

Testing stimuli	Statistical analysis		Population	
			Correct response percentage	Normalized average score
Reduced size	Wilcoxon signed-rank test	W	45	45
		Z	−2.687	−2.687
		P	0.007*	0.007*
Low contrast	Wilcoxon signed-rank test	W	6	6
		Z	−0.508	−0.508
		P	0.611	0.611
Sketch	Wilcoxon signed-rank test	W	63	63
		Z	−2.203	−2.203
		P	0.028*	0.028*
Contour	Wilcoxon signed-rank test	W	−13	−13
		Z	−1.363	−1.363
		P	0.173	0.173
Black silhouette	Wilcoxon signed-rank test	W	13	13
		Z	−1.761	−1.761
		P	0.078	0.078
White silhouette	Wilcoxon signed-rank test	W	6	6
		Z	−0.631	−0.631
		P	0.528	0.528
Partial occlusion	Wilcoxon signed-rank test	W	21	21
		Z	−2.207	−2.207
		P	0.027*	0.027*
Posteriorly occlusion	Wilcoxon signed-rank test	W	10	10
		Z	−1.841	−1.841
		P	0.066	0.066
Anteriorly occlusion	Wilcoxon signed-rank test	W	10	10
		Z	−1.826	−1.826
		P	0.068	0.068

The one-tailed Wilcoxon signed-rank test was used to determine the choices made by all cuttlefish (W, test statistic; Z, the Z statistic; p, p-value), and the same test was also applied to analyze the normalized scores obtained from all animals in each task. Asterisks indicate statistical significance.

the cuttlefish has now become very different from the original image.

Generalization is a process that involves feature extraction and therefore systematic studies on generalization should be able to provide a suitable way of identifying the visual cues utilized during visual recognition. Research on honeybee vision has a long tradition and generalization does indeed play an important role in understanding how the visual perception of bees operates (Ronacher, 1998; Horridge, 2009). In this cuttlefish study, the black and white silhouettes consist of the same area and both have a high-contrast edge; the difference is that the images have opposite contrast polarity. Interestingly the animals responded differently to the two types of images. This suggests that contrast polarity of a silhouette is a crucial cue during objection recognition. Black and white silhouettes from a biological perspective are related to two natural circumstances under which such high contrast is likely to be perceived. These are a shadow against a background light source and an object glowing in the dark, respectively. Cuttlefish perhaps view an images consisting of a black patch in the shape of prey on a white background as the silhouette of prey when they are looking upward in water toward the sun. On the other hand, an image involving a white patch on a black background might be prey with an extraordinarily high bioluminescence. The former is likely to be much more common in the cuttlefish's natural environment and this perhaps explains the animal's better visual equivalence in our study when it meets the former stimulus.

Object Recognition and Visual Completion

Amodal completion is a cognitive ability in animals whereby the viewing of a partially occluded object is treated by the animal as

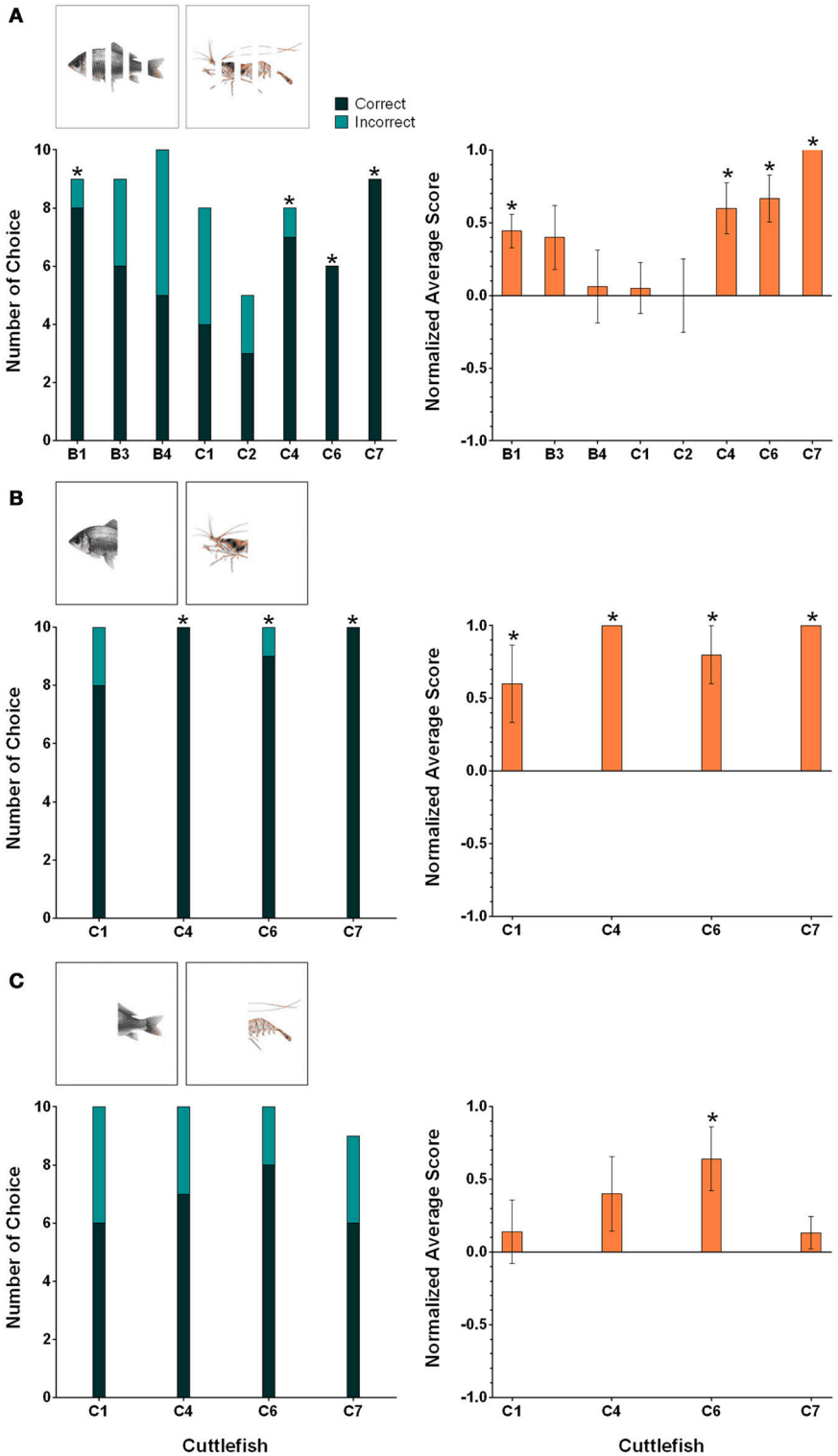

FIGURE 7 | The results for individual cuttlefish during the three amodal completion tasks: (A) Partial occlusion, **(B)** Tail occlusion, and **(C)** Head occlusion. The left panels show the correct/incorrect number of choices made by individual cuttlefish during these amodal completion tasks. The correct response was determined when cuttlefish showed any of the score above zero responses in a trial (see Section Materials and Methods for scoring). Asterisks indicate statistical significance for the correct choice ($p < 0.05$). The right panels show the average normalized scores of the individual cuttlefish for the same tasks. The scores were normalized against the strongest response in the training. Asterisks indicate a significant tendency toward the rewarded figure. Error bars are SEM.

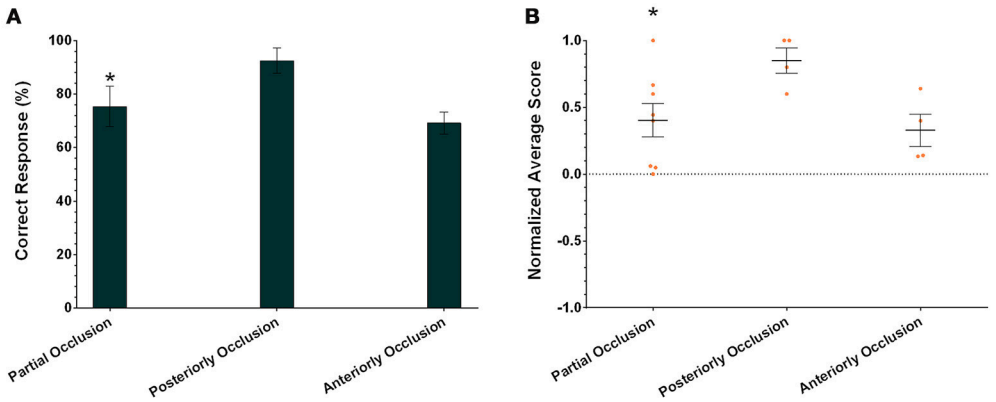

FIGURE 8 | The population results of cuttlefish in the three amodal completion tasks. (A) Average correct response percentages of all cuttlefish in each task. **(B)** Average normalized scores of all animals in each task. Orange dots represent individual data. $N = 8$, 4, and 4 for partial occlusion, tail occlusion, and head occlusion, respectively. Asterisks indicate statistical significance ($p < 0.05$). Error bars are SEM.

the entire entity; this is particularly important when detecting prey or predators. For instance, in the complex structures such as coral reefs, the visual stimuli that invoke territorial behavior in the coral reef fish may be a fragmented one (Darmaillacq et al., 2011). Darmaillacq et al. showed that two species of reef fishes, *Variola louti* and *Scarus niger*, exhibited territorial behaviors toward arrays of mirrors by responding as if they recognized an intruder. In another field experiment, two species of tits, *Poecile palustris* and *Poecile montanus*, tended to keep away from the partially occluded dummy of their natural enemies (Tvardíková and Fuchs, 2010). Our results also support the hypothesis that cuttlefish are able to complete a fragmented image of prey amodally. However, alternatively, all the experiments described above can also be interpreted as the outcome of recognizing specific bodily features rather than amodal completion of the image. The fact that there was different performances by the cuttlefish when the tasks involved half-body occluded images of either the front or back of the prey implies that the anterior part of the body may be more important to amodal completion than the posterior part or, alternatively, the critical features needed for recognition are located in the anterior part of prey. The presence of these specific features may influence the outcome of amodal completion. Thus we suggest that amodal completion leading to the image entity that is related to the original images may depend on the successful recognition of one or perhaps more key features.

In previous studies the ability to carry out contour completion by cuttlefish (*S. officinalis*) via their innate behavior, namely camouflage body patterning, was examined (Zylinski et al., 2009, 2012). Cuttlefish were found to respond to either full circles or fragmented circles with similar disruptive patterns, but showed a different body pattern in response to the rotated and scattered fragments (Zylinski et al., 2012). This result suggests that cuttlefish are able to complete the broken circles and recognize them as whole objects, whereas rotated and scattered fragments are interpreted as small and individual objects in the scene. It also supports that cuttlefish can reconstruct fragmented information and perform modal completion when presented with incomplete boundary information.

Individual Difference Exists Regarding Visual Processing by Cuttlefish

In the present study, we found that the performance of individual cuttlefish with each task varied somewhat and there is no general way of distinguishing the degree of difficulty of a given task with respect to an individual animal. That is, although all cuttlefish seem to be equipped with the ability of visual equivalence, the performance regarding this ability seems to vary quite a lot. This may be a universal phenomenon across all animal cognition. Previous studies of cephalopod behavior have also provided evidence of individual differences (Darmaillacq et al., 2014). One example is that each individual cuttlefish has a specific side-turning preference and another is that they employ one of the two strategies, response learning or place learning, during a spatial learning paradigm (Alves et al., 2007). Performance differences between individual animals have also been observed during a conditional discrimination test (Hvorecny et al., 2007). Furthermore, episodic personality has been found in gloomy octopuses (*Octopus tetricus*) in a playback study (Pronk et al., 2010). These octopuses could either behave in a shy or bold manner consistently across different experimental contexts over the same day, but this personality trait was not repeatable over a longer time, that is multiple days. Taken together, these findings support that individual variations observed in the present study may result from individual differences in their visual processing abilities.

ETHICS STATEMENT

The animal subjects used in the present study are cuttlefish, which are invertebrates and are exempt from this requirement.

AUTHOR CONTRIBUTIONS

IL conceived, designed, carried out the work, and drafted the manuscript. CC helped plan experiments, interpreted data, and revised the manuscript.

ACKNOWLEDGMENTS

We thank Yi-Hsin Lee and Chih-Chiang Lee for assistance of animal care. We also appreciate Drs. J. -P. Ueng and M. -F. Lee of the National Penghu University of Science and Technology for collecting cuttlefish eggs. This work was supported by the Ministry of Science and Technology of Taiwan (NSC-98-2628-B-007-001-MY3 and NSC-102-2628-B-007-001-MY3 to CC).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00040/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

MINI REVIEW
published: 23 December 2016
doi: 10.3389/fphys.2016.00618

Pioneering Studies on Cephalopod's Eye and Vision at the Stazione Zoologica Anton Dohrn (1883-1977)

Ariane Dröscher *

Department of Letters and Philosophy, University of Trento, Trento, Italy

From the late nineteenth century onwards, the phenomena of vision and the anatomy and physiology of the eye of marine animals induced many zoologists, ethologists, physiologists, anatomists, biochemists, and ophthalmologists to travel to the Zoological Station in Naples. Initially, their preferred research objects were fish, but it soon became evident that cephalopods have features which make them particularly suited to research. After the first studies, which outlined the anatomical structure of cephalopods' eyes and optic nerves, the research rapidly shifted to the electrophysiology and biochemistry of vision. In the twentieth century these results were integrated with behavioral tests and training techniques. Between 1909 and 1913 also the well-known debate on color vision between ophthalmologist Carl von Hess and zoologist Karl von Frisch took place in Naples. Largely unknown is that the debate also concerned cephalopods. A comparative historical analysis of these studies shows how different experimental devices, theoretical frameworks, and personal factors gave rise to two diametrically opposing views.

Keywords: cephalopod vision, history of vision research, Karl von Frisch, Carl von Hess, Zoological Station Anton Dohrn, color discrimination, history of experimentalism

OPEN ACCESS

Edited by:
Frederike Diana Hanke, University of Rostock, Germany

Reviewed by:
Hans-Joachim Pflueger, Free University of Berlin, Germany
Binyamin Hochner, Hebrew University of Jerusalem, Israel

***Correspondence:**
Ariane Dröscher
coraariane.droscher@unibo.it

Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *20 September 2016*
Accepted: *25 November 2016*
Published: *23 December 2016*

Citation:
Dröscher A (2016) Pioneering Studies on Cephalopod's Eye and Vision at the Stazione Zoologica Anton Dohrn (1883-1977). Front. Physiol. 7:618. doi: 10.3389/fphys.2016.00618

INTRODUCTION

Of all the senses, visual perception has received by far the greatest attention. The main reason is that our human encounter and exchange with the environment mostly relies on optic stimuli. Another reason is that humans usually look into each other's eyes in order to access the other's emotional and mental sphere. In the twelfth century, Hildegard von Bingen expressed this desire with the aphorism "The eyes are the windows of the soul." The considerable advancement of notions and techniques of sensory physiology in the second half of the nineteenth century raised expectations that it might be possible to penetrate also the minds of animals. Excited by John Lubbock's book *On the senses, instincts and intelligence of animals with special reference to insects* (Lubbock, 1888), on 3 January 1892 Baron Farrer wrote to Lubbock from Naples: "it is clear that the thing now to do is to try to find out, as you have done, what animals really do see, hear and feel, rather than what their organs ought to enable them to do. What a world of possibilities the subject opens to us" (quoted from Hutchinson, 2014, p. I, 322).

From the first decades of the twentieth century cephalopods became a favorite object of vision research. Probably no other invertebrate depends so heavily on visual information. Vision is indispensable for their moving and hunting, as well as for their diurnal rhythm and the correct functioning of their hormonal glands (Wells and Wells, 1959; Wells, 1960). Although cephalopod

eyes are significantly distinguished from vertebrate eyes, they have also important analogies (Ogura et al., 2004), and they are particularly suited to vision research. Fröhlich (1913a) already listed the following favorable features: (1) the eye is of large size; (2) it survives long after its extirpation and (3) has only one type of receptor cell; (4) the optic nerves route directly behind the photocells; (5) the optic nerves are very long (in *Octopus*: 18 mm) and end in a separate part of the nervous system posterior to the eyeball, the optic lobe. In addition, (6) cephalopods are well suited to learning experiments and conditioning.

In the second half of the nineteenth century still little was known about the life of cephalopods. Matters changed with the creation of marine biology stations, first the *Stazione Zoologica Anton Dohrn* in Naples (1872), the two French *Station biologique* of Roscoff (1872) and the *Station marine* of Wimereux (1874), and then many others. Most of these marine stations had the two-fold purpose of (i) promoting knowledge about marine animals and (ii) "renaturalizing" biological research, which had become increasingly confined to urban laboratories. Yet whereas the French stations largely maintained their "field station" nature, Dohrn's *Stazione* developed into a research institution at which many important laboratory techniques were devised (Bont, 2014). Moreover, because the Station was an international research facility, it hosted scholars from many different countries and working in almost all the biomedical disciplines. This greatly favored interdisciplinary exchange; yet it made the Neapolitan research output very heterogeneous and, as we shall see, it sometimes led to conflicting research projects being carried out simultaneously at the same site.

PIONEERING STUDIES ON THE ANATOMY AND PHYSIOLOGY OF THE CEPHALOPOD EYE

The first studies on cephalopods carried out at the Stazione Zoologica provided the basic knowledge on the anatomy, physiology, development, habitat and phylogeny of these then still mysterious animals. They culminated in the two fundamental works by Jatta (1896) and Naef (1923)[1]. Quite soon, however, some very special features of this animal group became evident and led to the development of specific laboratory techniques. One of them concerned the visual organ.

Due to its large size and relatively simple anatomical structure, the retina of cephalopods was soon appreciated for comparative studies and as a model for the photoreceptive mechanism. As early as 1884, Hermann Grenacher showed that the octopus retina, despite its superficial similarity with those of vertebrates, is organized differently. These results were confirmed by his embryological studies. Octopus rhabdomes, in fact, are quadratic like those of arthropods, and they are formed of four rhabdomeres from four different cells (Grenacher, 1884). The Hungarian anatomist Michael von Lenhossék described a simple layer of long palisade-like rods whose terminal part consists of a "Stäbchenspindel" (spindle region) filled with pigments. His splendid illustration and his scheme of the fine anatomy of the retina and the optic nerves of *Eledone* served as a model for many decades (Lenhossék, 1894). After World War II, John Zachary Young and his numerous collaborators resumed and refined the study of the cephalopod retina by applying electron microscopy (summarized in Young, 1971). The retinal ultrastructure was investigated also by Jerome J. Wolken, M. F. Moody, and J. R. Parriss, who demonstrated that the rhabdomere tubules show a dichroism and that the orientation of the rhodopsin molecules is geometric, thus providing a plausible explanation for the sensitivity of octopods to polarized light (Wolken, 1958; Moody and Robertson, 1960; Moody and Parris, 1961; Young, 1962).

The functioning of the photoreceptors aroused particular interest. Rawitz (1891) demonstrated that the pigments of the octopus eye migrate from an inner to an outer layer, and vice versa, when exposed to different conditions of illumination. Carl von Hess confirmed this movement (Hess, 1905). In 1902, he was the first to detect rhodopsin in *Loligo,* thus demonstrating that it is not exclusive to vertebrates. Yet, he guessed that its physiological behavior is different (Hess, 1902). Hess's idea that the level of pigment metabolism is of great importance in order to understand the process of phototransduction was soon confirmed by Bauer (1911). However, more than half a century passed before his intuition about rhodopsin conversion was confirmed by Paul and Patricia Brown, who provided biochemical proof that in *Octopus* and *Sepia* the rhodopsin produces a stable metarhodopsin (Brown and Brown, 1958).

Despite the uniqueness of the visual apparatus of cephalopods, great expectations were raised by the opportunity to transform them into experimental animals for the general understanding of the process of vision in camera-like eyes. Taking advantage of the neat arrangement of the eye's elements and the optic nerves, Adolf Beck succeeded in inquiring receptor sensitivity, obtaining simple response curves on exposure to light flashes for *Eledone* (Beck, 1899). Repeating Beck's work, a few years later, Hans Piper was the first to succeed in measuring the magnitude of the retinal electric response of *Eledone alta* (Piper, 1904). Cephalopods became definitively established as experimental objects for the electrophysiological research of vision when, in 1913, Friedrich Wilhelm Fröhlich obtained the first electroretinogram (ERG) with isolated *Eledone* and *Octopus* eyes (Fröhlich, 1913a,b). About half a century later, Brian Boycott resumed this Neapolitan research tradition and obtained electroretinograms in living and intact animals (Boycott et al., 1965). These successes raised concrete hopes that for the first time insights could be gained into the functioning of a complex neural and sensory system, inducing Stuart Sutherland, W.R.A. Muntz, N.J. Mackintosh and other psychologists to use *Octopus* to elaborate models of "visual pattern recognition" and the neurophysiological bases of learning (Sutherland, 1954; Sutherland and Muntz, 1959; Sutherland and Mackintosh, 1971).

COLOR VISION IN CEPHALOPODS

Between 1909 and 1914, parts of one of the most famous disputes on whether animals are able to perceive and discriminate

[1]For a more complete bibliography see Ponte et al. (2013); and http://www.cephalopodresearch.org/cephs-science-history

colors took place at Dohrn's Station. It started with fish, then switched to cephalopods—a still largely unknown episode—and finally to honeybees. Its protagonists—the then already established ophthalmologist Carl von Hess (1863–1923) and the then still unknown zoologist Karl von Frisch (1886–1982)—followed profoundly different approaches, so that the debate was transformed into more than just a scientific dispute (Autrum, 1963, 1990; Dröscher, 2005).

Hess's greatest achievement was the devising of a first reliable experimental system with which to study color discrimination and its application to a broad range of animal classes. In 1902 he came to Naples for the first time, in order to investigate the anatomy and physiology of the cephalopod eye (Hess, 1905), in particular rhodopsin, the pigment called "Sehpurpur" (visual purple) back then (Hess, 1902). Four years later, he made the "first attempt to systematically reveal how fish see" (Hess, 1909). For this purpose, he modified a technique, developed in Naples by Werner Krause, recording the reaction of *Amphioxus* in a tank exposed to lights of different brightness (Krause, 1897). Observing that in a dark room the fish *Atherina hepsetus* always swims toward the brightest part of the aquarium, Hess exposed them to monochromatic lights, and noted that their behavior resembled that of achromatopsic (colorblind) humans, when asked to move toward the brightest place in the room.

In order to investigate the color-brightness interaction, Hess then put *Atherina* in aquaria illuminated at one side by white light and by a certain color light at the other. Gradually modifying the brightness of the white light, he determined the exact moment when the fish stopped showing any preference. Again, the resulting graph turned out to be almost perfectly identical to the one obtained with achromatopsic humans. Hess concluded that fish are unable to distinguish different colors; rather, they react only to brightness (Hess, 1909, 1910c, 1912a). Extending his research to other vertebrate and invertebrate species (Hess, 1910a,b), he summarized his results in his famous monograph *Vergleichende Physiologie des Gesichtssinnes* (Hess, 1912b) establishing the by then dominating paradigm of the colorblindness of fish.

The strongest attack against Hess's results and his entire experimental system came from Karl von Frisch. Because Frisch was a zoologist and naturalist, he approached the question from a different standpoint. He considered the coincidence between the behavior of fish and achromatopsic humans to be a mere analogy. In this doctoral thesis he had investigated the control of body coloration and the chromatic matching of fish to the background (Frisch, 1910, 1911a, 1912b,c, 1913a). Then traveling to Naples, he experimented with the matching behavior of *Phoxinus laevis*. By varying the color of the background, he showed that the body coloration reaction differed even if the two colors had the same level of brightness (Frisch, 1911b). Frisch then devised learning experiments in which he trained the fish to react to saffron yellow. Thus, he created an association of a reward with a certain color. When exposed to little yellow cards stuck on a greater gray card having the same brightness, the fish reacted equally to the yellow cards (Frisch, 1912a). For Frisch this was proof that they were able to discriminate objects on the basis of their chromatic difference.

Before it reached its climax with the dispute on color vision in honeybees (Frisch, 1913b; Hess, 1913; Frisch, 1915; Menzel and Backhaus, 1989; Munz, 2016, pp. 32–50), the polemic between Hess and Frisch passed through a partially unknown episode that regarded cephalopods. Hess assumed their colorblindness. Unable to train them to swim toward lights, as he had done with fish, he had to develop a new experimental set-up. Some years previously, Rudolf Magnus had worked in Naples on the pupillary reaction of octopods, discovering that the closure of the eyelid is accompanied by a dilation of the pupil (Magnus, 1902). He also demonstrated that the pupillary reflex is not spontaneous but controlled by two distinct centers in the central ganglia. Based on these findings, Hess exposed the animals to lights of different colors and measured their pupillary reflex (*Sepia*) or their phototactic response (*Loligo*) in a tank so small that they could move only slightly forwards or backwards when trying to avoid the most disturbing lights. Again he noted a correspondence between the responses of cephalopods and achromatopsic humans (Hess, 1912b, pp. 331–345).

A few years later, Frisch again set out to contest Hess's results. On January 14, 1913 he wrote a letter to Reinhard Dohrn, ordering several marine species for his next stay at the Stazione, among them cephalopods. He revealed that he wanted "to train the animals to certain colors, in order to see with what other colors or gray papers they confound the color they had been trained for, a method very successfully applied to bees." He then explained that he intended to train them,

> "making double-walled test tubes, with colored paper between the tubes that are then fused in order to obtain colored, water-proof glass tubes. Then one feeds cephalopods several times a day (is this possible?) e.g., always with a crab leg, put inside the red test tube (obviously in a way that the animal does not see it) and shows him contemporaneously several differently colored tubes, the others are empty, so that it learns that only in the red one it will find something. Then, later, one shows it an empty red tube, instead of one filled with food, in order to see if it has learned to discriminate the colors and to see with which gray or colored papers it confounds the red, a procedure easy to manage with an appropriate positioning (Reinhard, 1914)[2]."

Frisch never published his results. Consequently, we do not know if he actually carried out these experiments and how successful they were. Octopods show a great capacity of learning. Therefore, it is possible that Frisch performed them but that he did not obtain the desired results, and that he did not publish them, because his controversy with Hess had already reached a point where none of them could admit a failure. In fact, not only the results opposed Hess and Frisch. Their polemic was based on profoundly different approaches. Hess applied ophthalmological techniques, whereas Frisch acted as a naturalist. In Hess's sophisticated experimental system the animals were kept in precisely the conditions required to display the desired reactions, whereas Frisch tried to keep them in an environment that was as natural as possible. Frisch did this because he wanted to pose

[2]Letter from Karl von Frisch to Reinhard Dohrn, January 14, 1914, Historical Archives, Stazione Zoologica Anton Dohrn (Classified as Frisch 2013.A).

biologically meaningful questions, namely the adaptation of the animal's body color to the background or feeding preference. Hess, on the other hand, acted as an experimentalist, measuring reactions and drawing reductionist conclusions. The fact that, in the long run, Frisch's biological approach was awarded the Nobel Prize should not obscure that both ignored the role of the specific context in which their experimental objects displayed their behavior (Menzel and Backhaus, 1989). By placing the fish in a completely dark tank with sudden flashlights, Hess had created an emergency situation in which the animals did not care about colors and just swam toward the possible rescue, that is the brighter light. In Frisch's aquaria, instead, the animals were not fearing for their lives and had all the time necessary to make more nuanced choices.

Far from being definitively settled, the dispute on color vision continued to concern other researchers, who tried different experimental approaches. Based on his electroretinograms, Fröhlich demonstrated that octopods' retina reacted differently to different colors and brightness, and interpreted these responses as "the physiological basis of color discrimination." For Fröhlich, *Octopus* was able to distinguish among red, yellow, green, and blue (Fröhlich, 1913b). The Dutch animal psychologist J.A Bierens de Haan failed in his attempts to train *Octopus* to discriminate colors (Bierens de Haan, 1926). Alfred Kühn, instead, hit *Octopus* with a stick after three brief monochromatic flashlights until the animal had learned to respond with an immediate flight, as soon as it perceived the colored light. When the octopod was then exposed to flashlights of another color but the same brightness, it did not flee, and Kühn deduced that it was able to distinguish colors (Kühn, 1930, 1950). Finally, between 1973 and 1977, John B. Messenger demonstrated with still other learning experiments that *Octopus* does not distinguish different colors. The animals were successfully trained to discriminate between rectangles differing in brightness, but failed to give the same response to rectangles differing in hue (Messenger et al., 1973; Messenger, 1977). However, octopods recognize the plane of polarized light, as John Z. Young had assumed on the basis of his studies on the geometry of octopus rhabdomeres (Young, 1960), a hypothesis then experimentally confirmed by Moody and Parris (1961).

Over the last 150 years, research on cephalopod vision has yielded many path-breaking specific and general insights, yet it has also shown that the initial expectation that it would be possible to understand how animals see, hear and feel, was vain and misleading. Today, less ambitious goals and more pragmatic definitions prevail (Kelber and Osorio, 2010).

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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