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Spectral and spatial properties of polarized light reflections from the arms of squid (*Loligo pealeii*) and cuttlefish (*Sepia officinalis* L.)

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Summary

On every arm of cuttlefish and squid there is a stripe of high-reflectance iridophores that reflects highly polarized light. Since cephalopods possess polarization vision, it has been hypothesized that these polarized stripes could serve an intraspecific communication function. We determined how polarization changes when these boneless arms move. By measuring the spectral and polarizing properties of the reflected light from samples at various angles of tilt and rotation, we found that the actual posture of the arm has little or no effect on partial polarization or the e-vector angle of the reflected light. However, when the illumination angle changed, the partial polarization of the reflected light also changed. The spectral reflections of the signals were also affected by the angle of illumination but not by the orientation of the sample. Electron microscope samples showed that these stripes are composed of several groups of multilayer platelets within the iridophores. The surface normal to each group is oriented at a different angle, which produces essentially constant reflection of polarized light over a range of viewing angles. These results demonstrate that cuttlefish and squid could send out reliable polarization signals to a receiver regardless of arm orientation.

Key words: cephalopod, polarized light, squid, cuttlefish, signal, iridophore, structural color.

Introduction

Polarized light patterns of reflected light appear on the surfaces of many animals, including insects, crustaceans and cephalopod molluscs, where they are often used in biological signaling (Cronin et al., 2003). The surfaces of many cephalopod species are unusual in that they reflect changing patterns of polarization (Shashar and Hanlon, 1997; Hanlon et al., 1999; Mäthger and Denton, 2001; Shashar et al., 2001). In both squid (*Loligo pealeii*) and cuttlefish (*Sepia officinalis* L.), iridophores are responsible for the reflection of this polarized light (Shashar et al., 1996; Hanlon et al., 1999; Mäthger and Denton, 2001; Shashar et al., 2001; Mäthger et al., 2004). Such iridophore cells are distributed all over the bodies of these coleoid cephalopods (Hanlon, 1982; Hanlon and Messenger, 1996), although there are different types and arrangements of iridophores that have not yet been inventoried with precision. In general, iridophore cells are passive reflectors that are quite angle-dependent for viewing. Curiously, reflections from some of the arm iridophores on squid and cuttlefish are less dependent on the angles of illumination and viewing, and this provides one of the rationales for the present study.

The multilayer platelet structures within iridophores, thought to be responsible for the reflection of iridescent structural colors as well as the polarized light, have been described by several investigators (Land, 1972; Cloney and Brocco, 1983; Hanlon and Messenger, 1988; Cooper et al., 1990; Hanlon et al., 1990; Shashar et al., 1996; Shashar et al., 2001). Light reflected from a multilayer reflector of this kind is almost always colored, and it is polarized as well when the illumination is at oblique angles of incidence. Two prerequisites for spectrally favored reflection are that (1) there is a difference in refractive index between the platelets and the spaces separating them, and (2) the platelets and spaces have thicknesses on the order of wavelengths of light (Land, 1972). Moreover, since it is well known that when a light beam is reflected from a stack of plates, the spectrum of the reflected light is a function of their spacing, orienting the iridophore plates to a different angle is expected to result in a change of reflectance. Changes in the relative angle between a light source and the surface normal of a multilayer reflector produce a sequence of color changes known as Newton's series. For color-blind animals like cuttlefish and squids (Marshell and Messenger, 1996; Gleadall and Shashar, 2004; Mäthger et al., 2006), the changes in wavelength would be perceived as different brightnesses. Because brightness information can be easily affected by the lighting environment, this type of reflectance change is unlikely to be a good visual signal for communication.

In most animals, body coloration and achromatic patterns function in heat exchange, radiation protection, communication or camouflage (e.g. Cott, 1940). In contrast, polarization body patterns apparently function primarily for intraspecific communication (Cronin et al., 2003). The 'iridescent arm stripe' described in squid (*Loligo pealeii*) (Hanlon et al., 1999) and the 'pink iridophore arm stripes' described in cuttlefish (*Sepia*

officinalis L.) (Hanlon and Messenger, 1988) reflect highly polarized light (Shashar et al., 1996; Shashar and Hanlon, 1997; Hanlon et al., 1999). It has been hypothesized that the main function of these arm stripes is to act as visual signals for intraspecific communication (Shashar et al., 1996; Mäthger and Hanlon, 2006). Since light underwater generally arrives from nearly overhead, the polarized light reflected from the arm stripes has a somewhat horizontally oriented electric vector (e-vector) that is observable from before sexual maturation through adulthood (Shashar et al., 1996; Shashar and Hanlon, 1997; Gleadall and Shashar, 2004). Cuttlefish and squid are capable of turning the polarization on or off voluntarily (Hanlon and Messenger, 1988; Shashar and Hanlon, 1997), and there is recent behavioral evidence that polarization signals are used among cuttlefish (Boal et al., 2004). Changes of the e-vector orientation of the reflected polarized light have also been reported to occur instantaneously in both animal species (Shashar et al., 1996; Shashar and Hanlon, 1997; Hanlon et al., 1999; Shashar et al., 2001).

Iridophores that exhibit physiological alteration of the reflection properties (also known as active or motile iridophores) have only been found in a few types of animals. In several species of squid (including *L. pealeii*, *L. vulgaris*, *Lolliguncula brevis*, and *Alloteuthis subulata*), the common neurotransmitter acetylcholine (ACh) induces an ultrastructural change in the protein of iridophore platelets, changing their ability to reflect light; increased quantities of ACh (in physiologically normal quantities) change the thickness of the iridophore platelets as well as the space between them, producing a color shift (Cooper et al., 1990; Hanlon et al., 1990; Mäthger et al., 2004; Mäthger and Hanlon, 2007). Active iridophores in fishes and lizards are also capable of changing the spacing between reflecting platelets. The physical mechanisms that activate the iridophores include mechanical force, osmotic pressure and temperature (Lythgoe and Shand, 1982; Oshima et al., 1985; Clothier and Lythgoe, 1987; Lythgoe and Shand, 1989; Nagaishi et al., 1990; Morrison et al., 1996).

There are two questions that this paper addresses concerning the polarized-light signaling system in cuttlefish and squid. First, we would like to know how the surface orientation of these flexible animals affects the polarization signals they reflect. In other words, since the multilayer reflectors are located in unusually flexible appendages, it would be of interest to know whether arm orientation affects the reflected signal. By manipulating the relative angles of skin samples and the direction of illumination, we measured changes of the polarization signal generated by the pink iridophore arm stripes of cuttlefish and the iridescent arm stripes of squid. Second, we examined how the above-mentioned signals are optically produced. To determine the physical basis of the optical properties of the arm stripes, the fine structures of the iridophores were studied using electron microscopy.

Materials and methods

Animals

Wild-caught adult squid *Loligo pealeii* Verrill 1873 and laboratory cultured cuttlefish *Sepia officinalis* L. were maintained by the Marine Resources Center at the Marine Biological Laboratory, Woods Hole, MA, USA. Squids and cuttlefish were euthanized by decapitation or by overanaesthetizing them (10% ethanol in seawater). The arms and/or tentacles were ablated and pinned down onto a silicone elastomer (Sylgard 184, Dow Corning Co., Midland, MI, USA)-coated disc, and this preparation was then mounted onto a tilting table, as described by Denton and Nicol (Denton and Nicol, 1965). Due to the tendency of the dermal tissue to lose its transparency over time, the collection of spectra and images occurred within 5 h following the sacrifice of the animal. Throughout the experiment, the tissues were submersed in freshly collected seawater.

Sample setup and spectral measurements

All measurements were made under a Zeiss dissecting microscope with a polarization filter attached (Quantaray; Ritz Camera Centers Inc., Irvine, CA, USA). After marking the polarization axis of the filter, it was mounted under the objective lens of the microscope with a custom-built adaptor (Fig. 1A). Rotating this polarization filter permitted the analysis of the polarized light reflected from the preparation. Using the tilting table, we could freely change the angle of the preparation while the positions of light source and detector or camera remained stationary. We used a right-handed three-dimensional Cartesian coordinate system to describe the relative position and angles of the sample, the light source, the observer and the e-vector. We assigned the origin to the point of measurement, with the positive z-axis pointing up, x-axis pointing right, and the y-axis pointing away from the observer (Fig. 1B). The angle of the sample is defined as the angle in the xz-plane between the surface normal of the arm stripe and the (vertical) z-axis. Starting from 0°, at which angle the surface normal was parallel to the z-axis, the angle was increased by bringing the surface normal toward the positive arm of the x-axis (i.e. rotation occurred only on the y-axis). We used the relation between the longitudinal axis of the arms and the y-axis to denote the orientation of the arms, measuring two sets of data by setting the longitudinal axis of the arm either parallel or perpendicular to the y-axis. If the arm was perpendicular to the y-axis, we changed the angle of the sample by tilting it in the xz-plane; if the arm was parallel to the y-axis, the angle was changed by rotating it (around the arm's axis).

Samples were illuminated with a 150 W halogen light source through a fiber-optic light guide. Since the direction of observation was constant (e.g. on the z-axis), we set the light source at two positions to simulate observers located at different angles. First we set the light source pointing at the sample parallel to and from the positive side of x-axis. In the second setup, the light source was set on the axis that bisects the positive x- and z-axes (i.e. 45° up from the horizontal; Fig. 1). Since, in nature, the light is typically refracted at the surface of the sea to arrive nearly vertically, the first illumination setup represents the case where the signaler and receiver are located at nearly the same depth, while the second indicates a situation in which the receiver is located at a position ~45° above the signaler.

Spectral and imaging polarimetry

The spectral properties of the light reflected from samples were measured using a spectrometer (USB 2000, Ocean Optics Inc.,

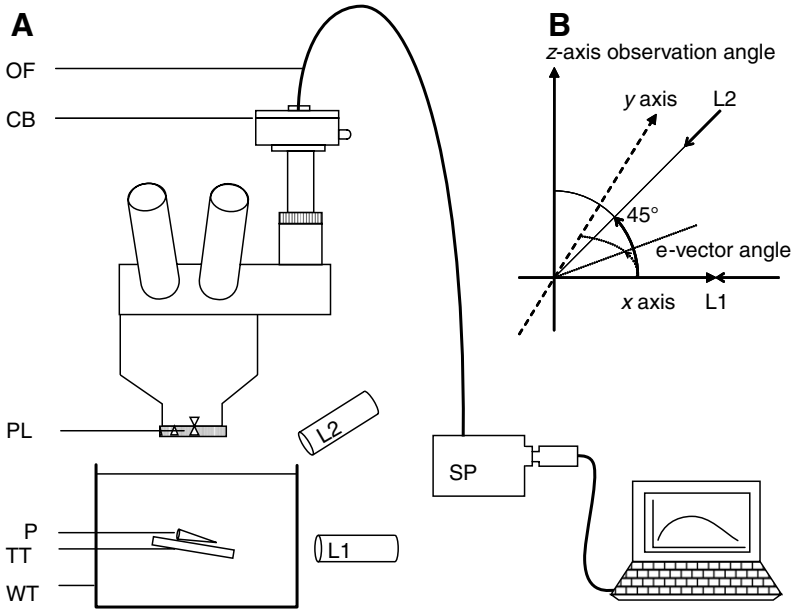

Fig. 1. (A) A diagrammatic view of the setup used to measure the spectral properties of the polarization reflection and to obtain images for imaging polarimetry. OF, optic fiber; CB, the attached film camera body; PL, linear polarizer; P, preparation; TT, tilting table as described by Denton and Nicol (Denton and Nicol, 1965); WT, water tank; L1, first illumination setting (Position 1); L2, second illumination position (Position 2); SP, spectrometer. (B) Geometry of the setup. The arrows marked L1 and L2 indicate the two illumination directions (at 90° and 45° to the observation axis, respectively). The dotted lines illustrate how the e-vector angles (which fall in the *xy* plane) were defined in terms of the *x*- and *y*-axes. All rotations of the tilting table were made about the *y*-axis. Thus, when the arm was perpendicular to the *y*-axis (i.e. it extended along the *x*-axis), changes in angle caused the arm to tilt upwards towards the light. When the arm axis was parallel to the *y*-axis, changes in angle produced rotation of the arm.

Dunedin, FL, USA) attached to an optic fiber (P1000-2-UV/VIS, Ocean Optics Inc.) that was connected to a camera mounted on the microscope. The tip of the optic fiber was adjusted to the image plane of the camera to permit precise positioning and focus of the object being measured. Because the e-vector of light is always perpendicular to the direction of propagation, in our setup it was always parallel to the *xy*-plane. We define the angle of the e-vector to equal 0° when it is parallel to the *x*-axis, and it increases counter-clockwise from the observer's point of view (Fig. 1B). Therefore, at a 90° angle, it is parallel to the *y*-axis.

Sets of four spectra, including a dark reference measurement and with the polarization filter positioned at 0°, 45° and 90°, were collected from each particular mounting position of a sample. We took another four sets of spectra recorded in the same conditions from a strip of Teflon tape used as a diffuse white standard having a reflection value of 100% at all wavelengths. From these we calculated, at 1-nm intervals from 400 nm to 800 nm, the e-vector angle and the partial polarization of the light reflected from sample as well as the spectral reflectance of the sample. The calculation was performed by a custom-written program that is based on the equations derived by Wolff and Andreou (Wolff and Andreou, 1995).

In addition to the spectral measurements, images of each preparation were taken with a digital camera (C5050 Zoom, Olympus America Inc., Center Valley, PA, USA) that was attached to the microscope with a universal digital camera microscope coupler (Edmund Optics, Barrington, NJ, USA). The camera was set to the manual exposure mode to maintain a constant shutter speed and aperture size for successive images. We also set the camera to the manual focus mode to prevent any change of sample position or magnification within sets of images caused by lens movements due to auto-focusing. The camera was calibrated for its response-intensity functions as described by Cronin et al. (Cronin et al., 2006). Sets of three images were taken for each sample, with the polarization filter positioned at 0°, 45° or 90°. After transferring the images to a computer, we averaged the values of the three color channels (red, green and blue; 8 bits per channel), weighted for linearity, and calculated the e-vector angle, partial polarization and the relative reflectance for each pixel with a custom-written program based on the same equations as for the spectral measurements. The e-vector angle and partial polarization value of each pixel were then coded with color and weighted by the relative intensity value of the corresponding pixel to display the signal clearly. For example, if part of the sample was highly polarized but had a very low reflectance value, the area would be shown with a darker color of the same hue in comparison with another part that was equally polarized and having higher reflectance.

Transmission electron microscopy (TEM)

To ensure that the orientation of iridophores did not change during fixation, the arms were pre-fixed with 2.5% glutaraldehyde in artificial seawater (ASW, in mmol l^{-1}: NaCl 425, KCl 10, $CaCl_2$ 10, $MgCl_2$ 25, $MgSO_4$ 25, Hepes 40, pH 8.0) for at least 30 min while they were still pinned to the disc on the tilting table. No color changes were observed in the iridophore cells after transferring the samples from seawater to the prefix solution. The arms were subsequently transferred to individual tubes containing the same fixative and placed at 4°C overnight. Cross sections less than 2 mm thick were taken from pre-fixed arms near the location where polarization measurements had been taken. Although glutaraldehyde renders the dermal tissue somewhat opaque, at this stage the iridescent color was still visible through the incision surface. The sample slices were post-fixed with 2% osmium tetroxide in ASW for 2 h at 4°C followed by dehydration, infiltration and embedding in Epon-812 epoxy resin. Thin sections (~60 nm) were cut from the blocks with a diamond knife on a Reichert-Jung Ultracut E microtome. Sections were examined under an electron microscope (EM 10CA, Zeiss, Gottingen, Germany) without further staining.

Results

Properties of the polarization reflection

In both squid *L. pealeii* and cuttlefish *S. officinalis L.*, bright iridescent stripes extend the entire length of the dorsal side of

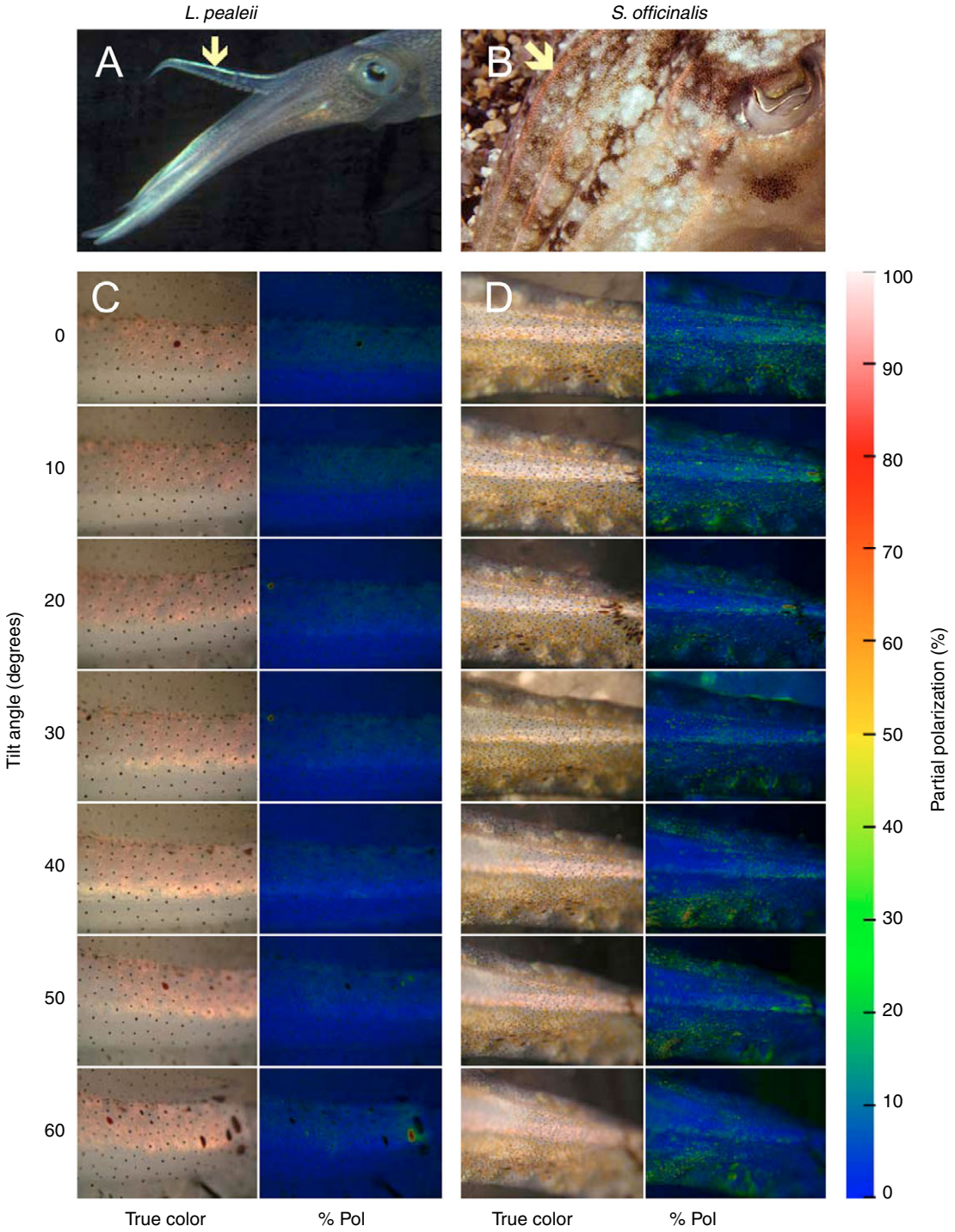

Fig. 2. Images of a squid (A) and a cuttlefish (B) showing the position of the arm stripes (as indicated by arrows). The differences in colors of the arm stripes are caused by differences in illumination of the animals in the photographs. (C,D) Close-up images of the squid (C) and the cuttlefish (D) arms tilted at various angles under the second illumination setup (Position 2, incident light from 45° above the horizontal). The left panels in C and D show the arm stripes in true-color images, while the right ones illustrate partial polarization values (%Pol) coded as in the key on the figure's right edge. The brightness of each color is proportional to the relative reflectance of the pixel in the original image (see Materials and methods for details of how relative reflectance values were obtained). The number at the left of each row of images indicates the tilt angle of the arm.

each arm (Fig. 2A,B). Similar polarization reflections were also found near the tip of the squid tentacle but not in the cuttlefish. When the illumination arrived from 45° above the horizontal plane (e.g. Position 2), the arm stripes of both animals clearly showed their characteristic pink color, especially at higher tilt angles (Fig. 2C,D left panels). Under these conditions, the

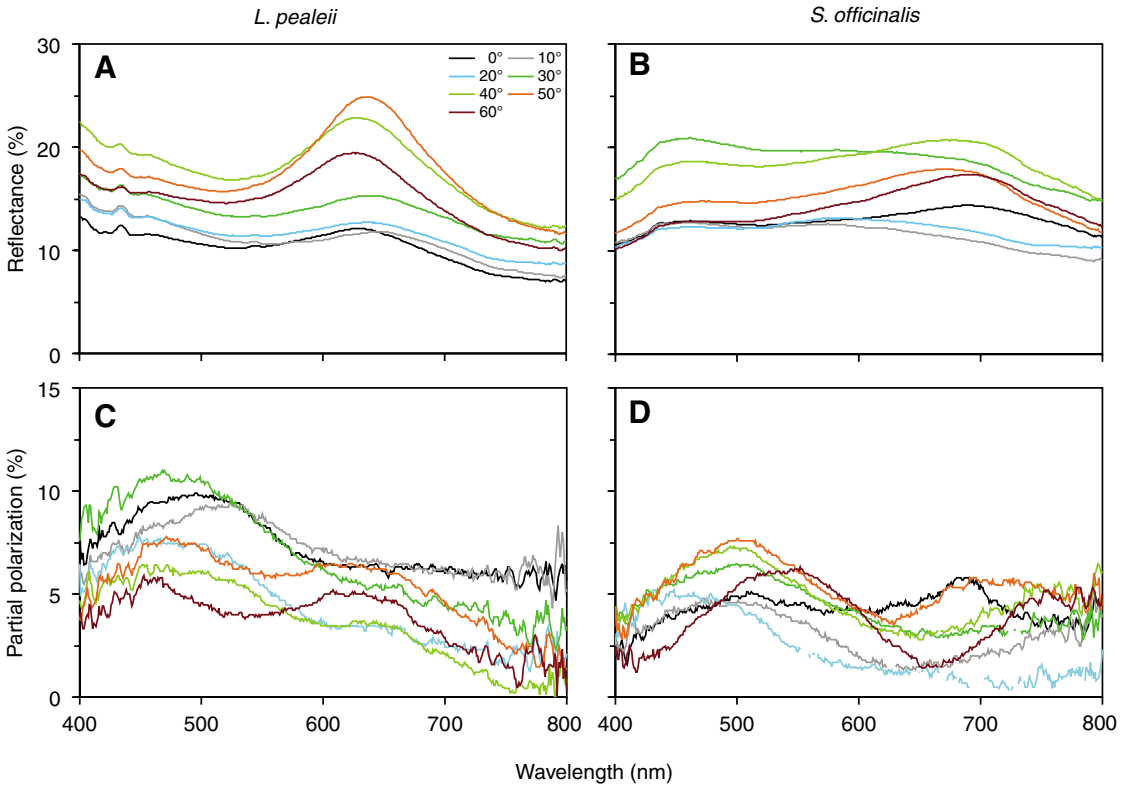

Fig. 3. Averaged reflectance spectra of the arm stripes of the squid (A) and the cuttlefish (B) and corresponding partial polarization spectra from the squid (C) and the cuttlefish (D). Data collected in Position 2 (see Fig. 1). Different colored curves in each figure represent spectra obtained from a sample tilted from 0° to 60° in increments of 10° (the key in A also applies to B–D). Note that identical reflectance values between spectra occurred in some cases (which are mathematically not possible to analyze); hence, some of the values in partial polarization curves are not plotted.

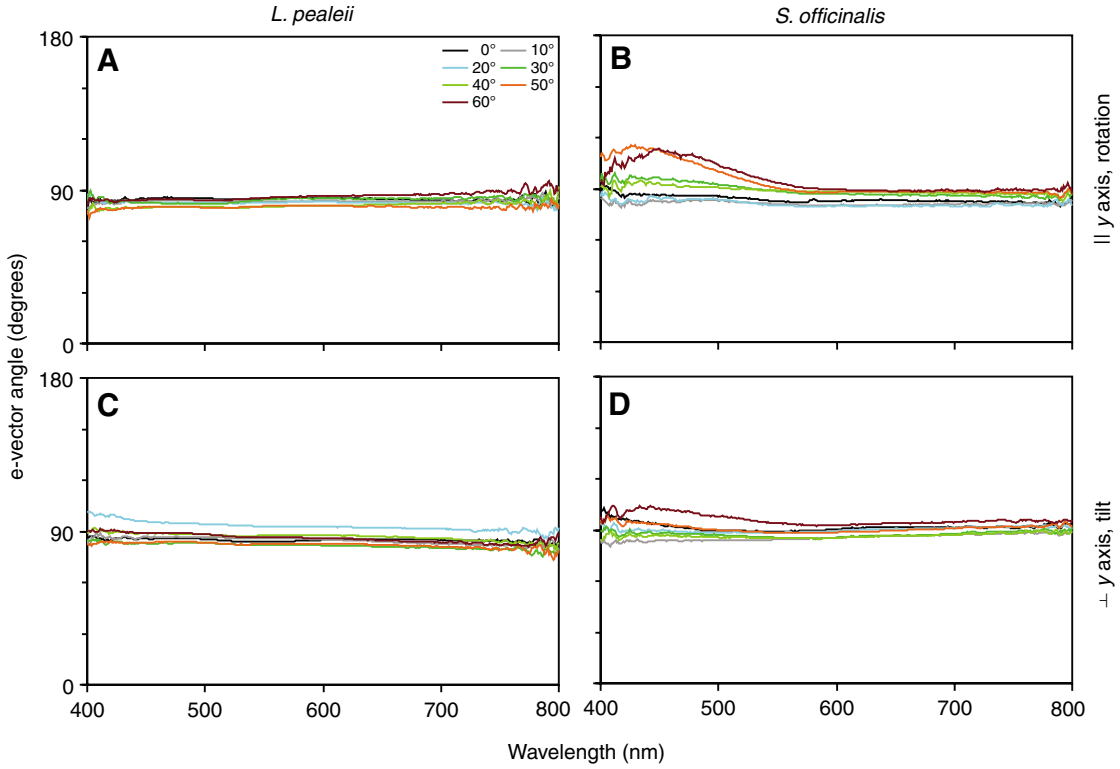

Fig. 4. Spectra of e-vector angles of the arm stripes of the squid and the cuttlefish oriented parallel (A,B) or perpendicular (C,D) to the *y*-axis. Data collected in Position 2 (see Fig. 1). As in Fig. 3, different colored spectra represent the calculated results from samples at various tilt or rotation angles (key in A also applies to B–D).

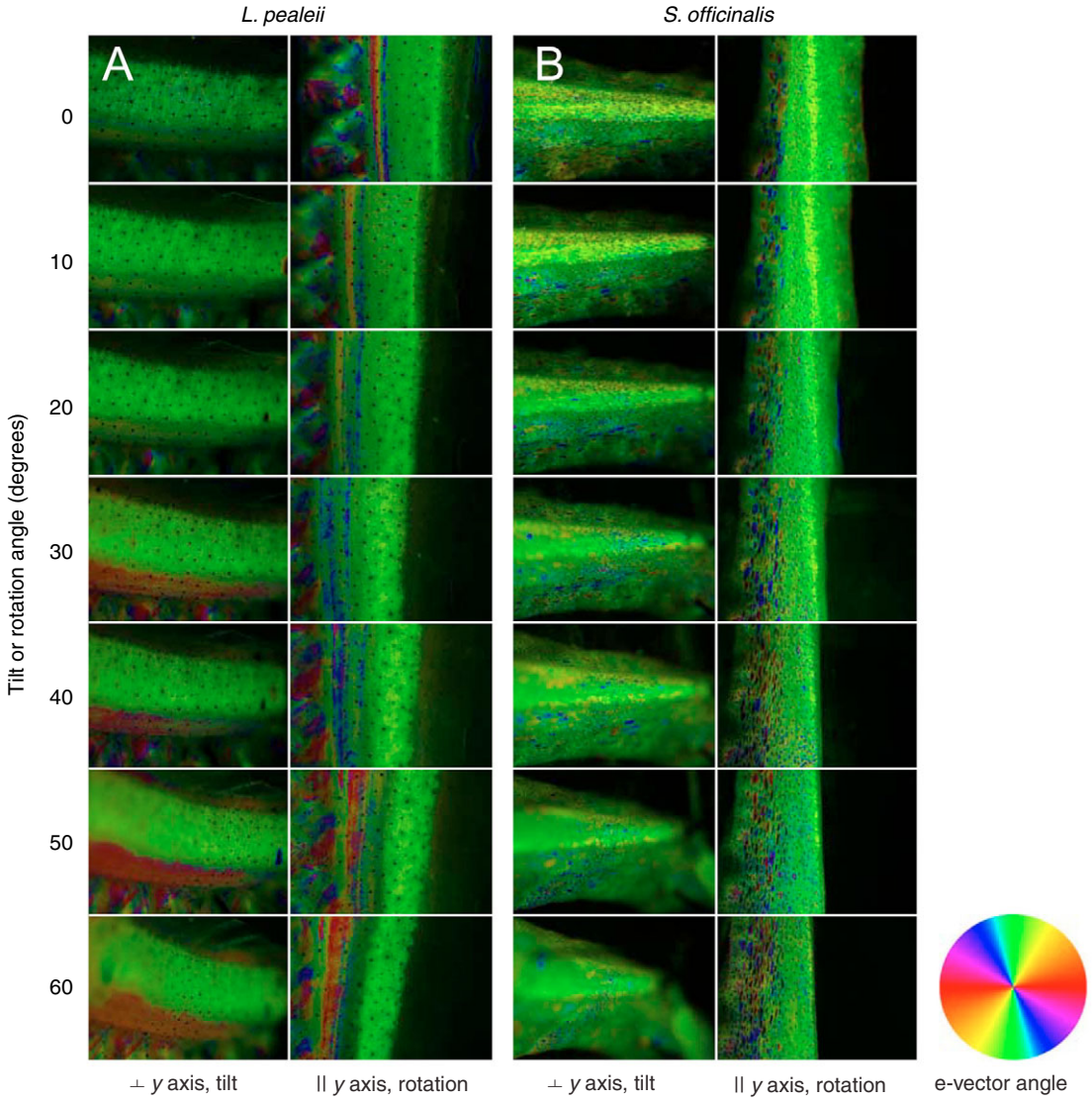

Fig. 5. Color-coded images of the arm stripes of the squid (A) and the cuttlefish (B) showing the e-vector angles of the arm stripes. The key for color coding of the e-vector angles is shown at the bottom right of the figure. As in Fig. 2C,D, the brightness of each coded color is proportional to the relative reflectance value at that point in the full-color image, and the arm stripes were tilted or rotated at different angles as indicated at the left side of each row. The original pictures were taken from the *z*-axis with illumination from right side of the image (Position 1; see Fig. 1).

reflectance spectra from squid and cuttlefish peaked at 634 nm and 671 nm, at tilt angles of 50° and 40° respectively (Fig. 3A,B). The spectral reflectances reached their highest values when samples were tilted at around 40° or 50° (Fig. 3A,B), gradually decreasing as the tilt angle became greater or smaller than the angle of peak reflectance; as the brightness decreased, the color of the samples appeared less saturated (Fig. 2C,D left panels). Despite the bright reflection of light, the partial polarization of the light reflected from both squid and cuttlefish was low under this illumination condition (see Fig. 2C,D right panels; % Pol <11%, Fig. 3C,D). The same low polarization values occurred whether the axis of the arm was parallel or perpendicular to the *y*-axis. Consequently, quantitative polarization spectra are reported only for the first illumination setup (Position 1, light at 90° to the microscope axis). Note that maximum polarization occurred near 500 nm, in a much shorter wavelength range than the reflectance maximum.

Whenever the reflected light from the arm stripes was polarized, its e-vector angle was always perpendicular to the plane defined by the direction of illumination and the direction of observation (i.e. 90°, Fig. 4). In squids, the e-vector angle remained constant at 90° over the whole visible spectrum no matter how we tilted or rotated the sample (Fig. 4A,C). While the e-vectors of the polarized light reflections from the cuttlefish were generally similar to this, at short wavelengths the e-vector angle moved away from 90° (Fig. 4B,D). This variation in e-vector angle only occurred, however, when the partial polarization or reflectance (or both) were low. Imaging polarimetry also showed that the full widths of the arm stripes in both species reflected polarized light with a 90° e-vector angle (Fig. 5). The e-vector angle was not influenced by the tilt or rotation angle of the skin sample, being consistently perpendicular to the direction of illumination throughout the arm stripes at all orientations of the sample (Fig. 5).

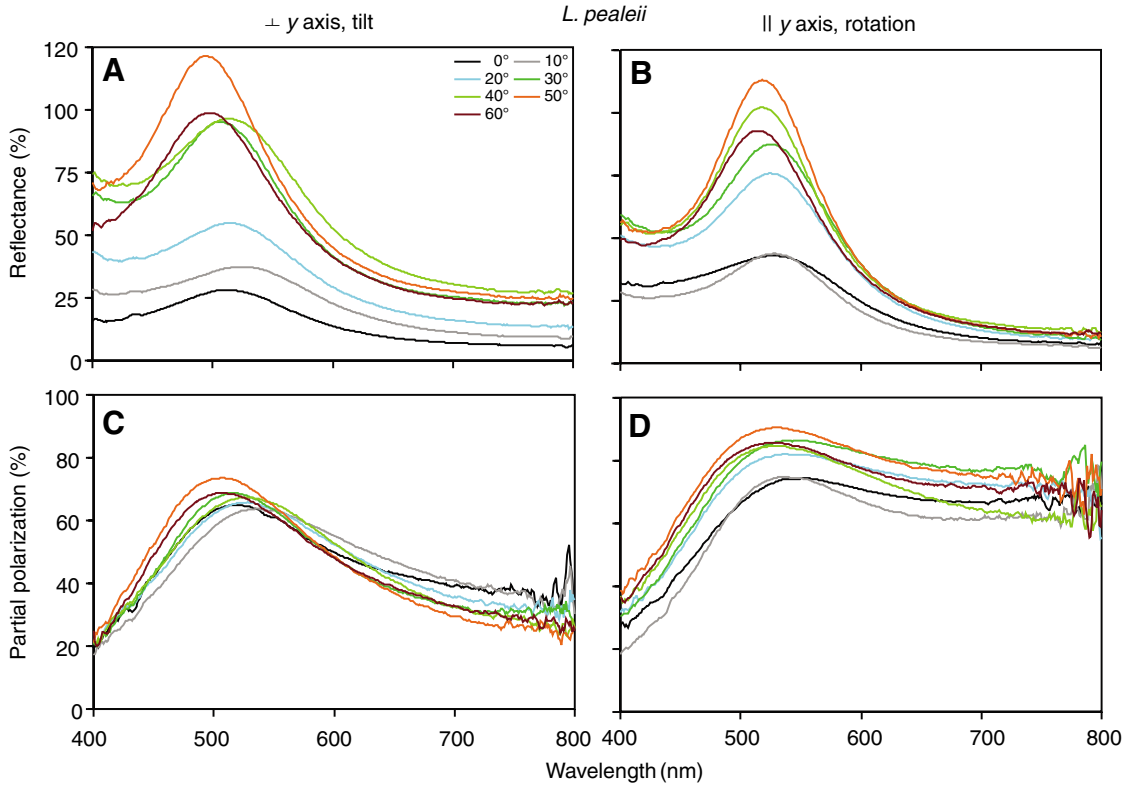

Fig. 6. Spectral reflectance curves of the arm stripe of the squid with the longitudinal axis of the arm perpendicular to the *y*-axis (A) or parallel to the *y*-axis (B) and their respective partial polarization spectra (C) and (D). Data collected in Position 1 (see Fig. 1). As in Fig. 3, seven sets of spectra were obtained from the sample in both orientations (key in A also applies to B–D).

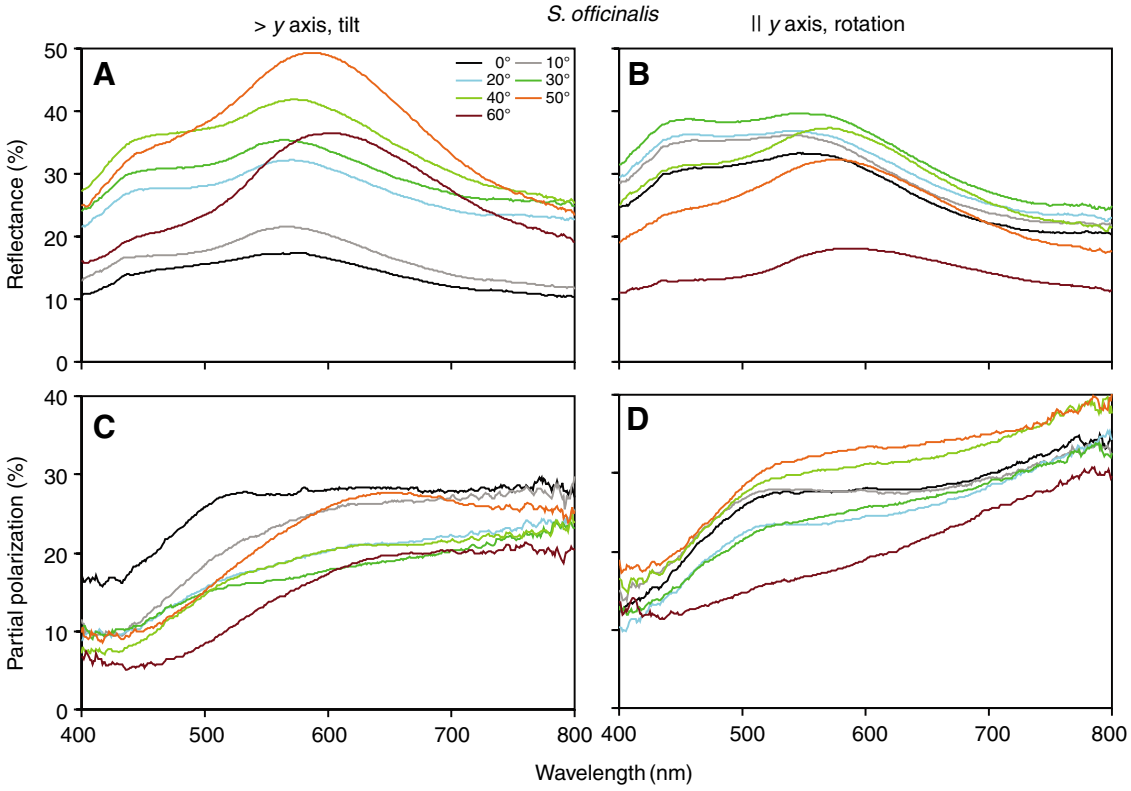

Fig. 7. Similar to Fig. 6, but obtained from the arm stripe of a cuttlefish (*Sepia officinalis*) oriented perpendicular to the *y*-axis (A,C) or parallel to it (B,D).

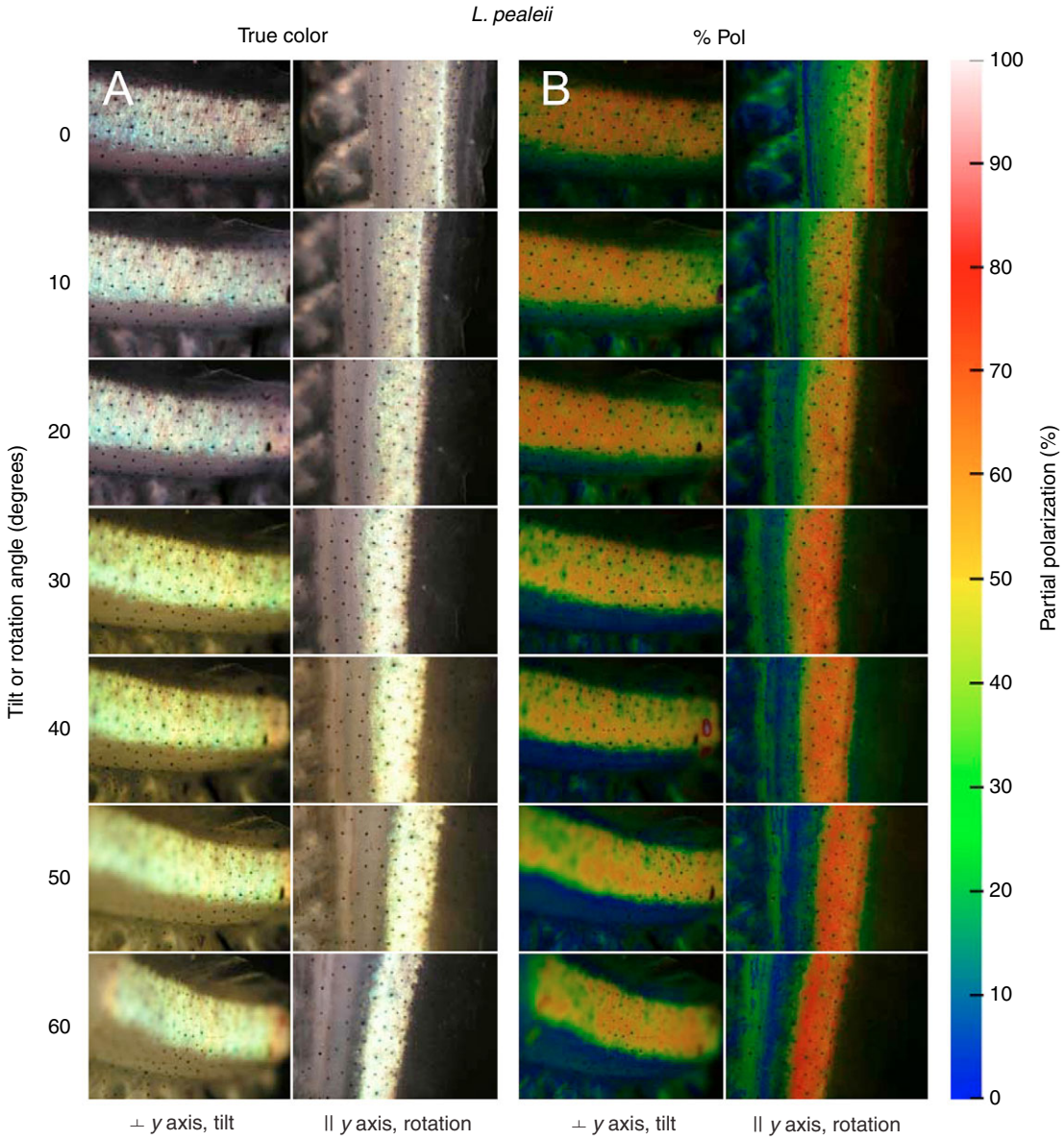

Fig. 8. Similar to Fig. 5A, but showing the real color images (A) and false color images (B) of the partial polarization (% Pol) of the arm stripes of the squid (*Loligo pealeii*). Data collected in Position 1 (see Fig. 1). The tilt or rotation angle is shown at the left side of each row. The color coding of the partial polarization values is shown at the right of the images. The color coded images were also weighted with relative reflectance values of the original images, as in the right panels of Fig. 2C,D. Note the apparent color change in the photos of squid arm stripes at greater angles of tilt. These changes are not seen in the reflectance spectra (Fig. 6), and the arms did not obviously change color as seen by eye; they apparently arise because of the extreme brightness of the reflections at these angles, which affected the digital camera's automatic white-balance setting.

When the longitudinal axis of a squid's arm was perpendicular to the *y*-axis, increasing the tilt angle (i.e. in the *xz*-plane) from 20° to 30° produced an increase of more than 75% in reflectance at the peak of the spectrum (Fig. 6A). Qualitatively similar results were obtained when arm stripes of squid were oriented parallel to the *y*-axis; changing the rotation angle from 10° to 20° caused a 60% increase in peak reflectance (Fig. 6B). The highest overall reflectance values were obtained at tilt or rotation angles of 50° in both arm orientations. Note that the peak of the spectral reflectance curve (at around 500 nm in both cases) can exceed 100% (the amount of light reflected by the diffuse white standard reflector; see Materials and methods), which indicates that specular reflection contributed to the measured light when samples were tilted or rotated near the 50° angle. Polarization spectra obtained from squid arms always reached polarization values greater than 60% at the maximum, between 500 nm and 550 nm. In both arm orientations, the tilt or rotation angle did not have much effect on the partial polarization values within the measured spectral range (Fig. 6C,D). On the other hand, when the longitudinal axis of the squid's arm stripe was oriented perpendicular to the *y*-axis (i.e. tilted in the *xz*-plane), the partial polarization values

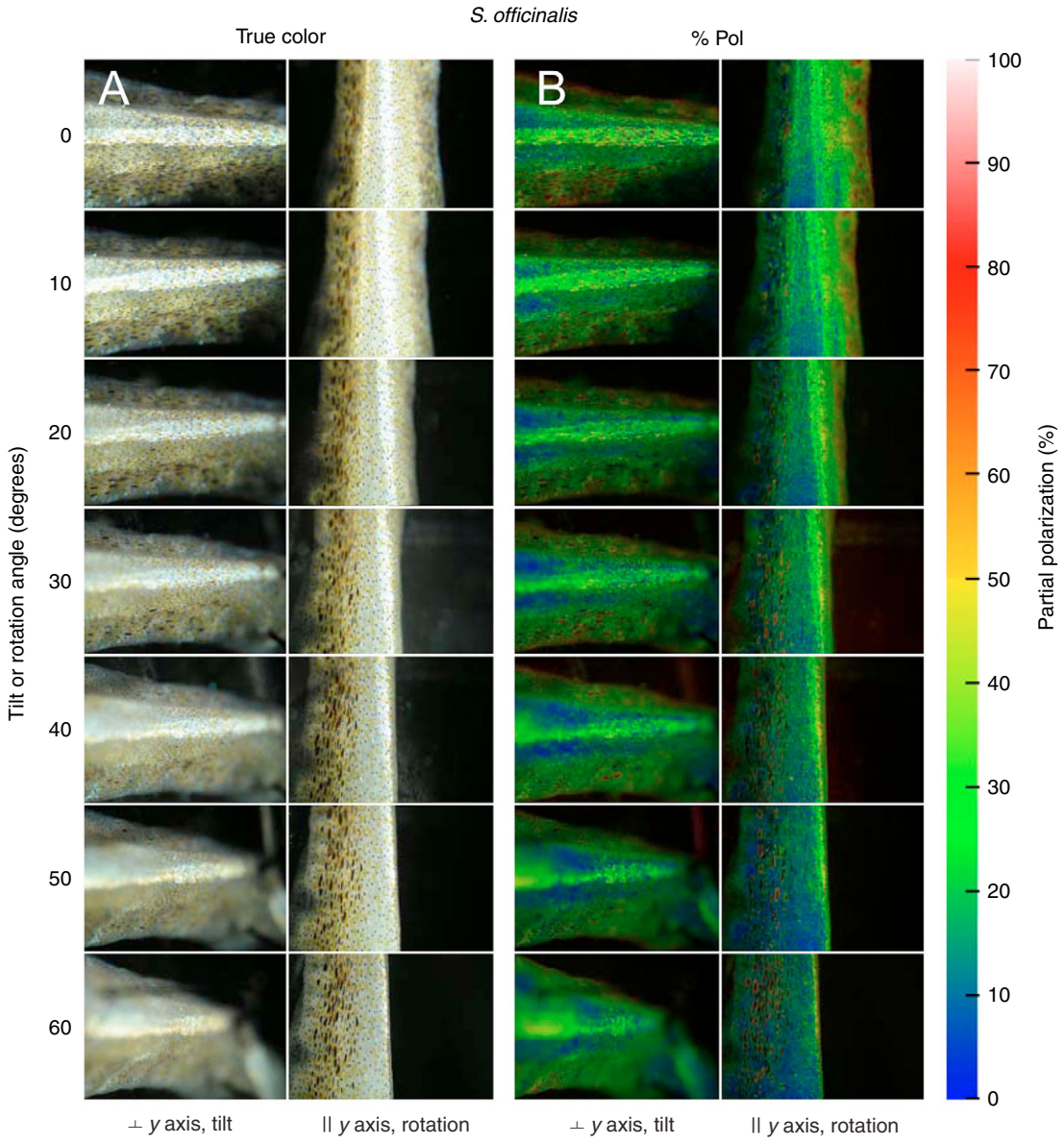

Fig. 9. Similar to Fig. 8, but from the arm stripes of the cuttlefish (*S. officinalis*) at various orientations and tilt or rotation angles.

were generally lower by about 20% than when oriented parallel to *y*-axis, especially at longer wavelengths (Fig. 6C,D).

Both the spectral reflectance and spectral polarization values measured from the arm stripes of cuttlefish were generally lower than those of squid (Fig. 7). Reflectance spectra peaked over a broad range between 550 nm and 600 nm (Fig. 7A,B). The rotation and tilt angles of the arm stripe of cuttlefish also brought about similar but more subtle changes in the spectral reflectance curves in comparison with those of squid. In cuttlefish, when the longitudinal axis of the arm stripe was perpendicular to the *y*-axis, increasing the tilt angle from 10° to 20° caused a 48% increase in the peak reflectance value (Fig. 7A). On the other hand, reflectance decreases were observed when the arm stripe was either parallel or perpendicular to the *y*-axis and the rotation or tilt angle increased from 50° to 60° (Fig. 7A,B). Within the measured spectral range (400 nm to 800 nm), the polarization spectra of cuttlefish arm stripes reached no sharp maximum; instead, the curves tended to flatten out at wavelengths above 500 to 600 nm (Fig. 7C,D). As observed in samples from squid, when the longitudinal axis of a cuttlefish's arm was oriented parallel to the *y*-axis (so that light struck the arm from the side), the reflected light had a higher partial polarization than when perpendicular to the *y*-axis (when light was parallel to the arm's axis) (Fig. 7C,D).

In Figs 8 and 9, we show the arms of squid and cuttlefish as true-color images together with false-color images of the partial polarization values. In these images, regions that reflected highly polarized light (orange to red in Fig. 8B and green to yellow in Fig. 9B) coincide with the areas where the iridophore arm stripes reside (Fig. 8A, Fig. 9A). The distribution of highly polarized reflections remained unchanged throughout most tilt or rotation angles (Fig. 8B, Fig. 9B). Exceptions were found in squid arm stripes when the sample was oriented parallel to the *y*-axis and rotated at 0° to 10°. In these cases, only a few of the iridophores were directly illuminated by the light, and these

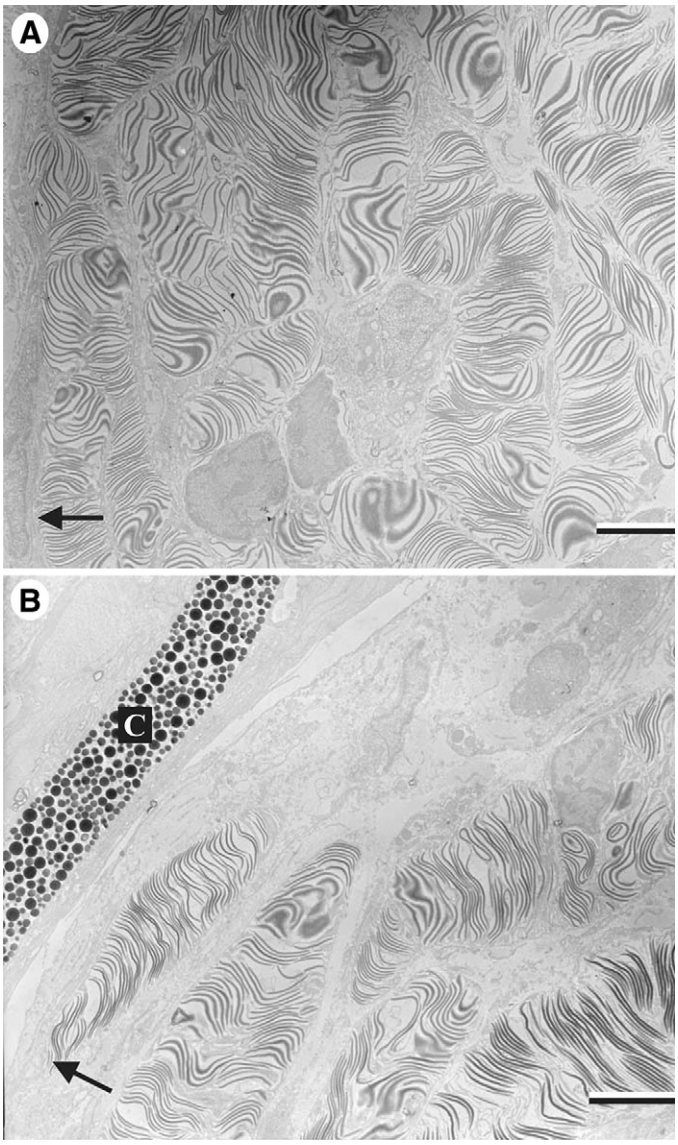

Fig. 10. Transmission electron micrographs of the iridophores in the arm stripes of the squid (*L. pealeii*) (A) and the cuttlefish (*S. officinalis*) (B). Sections were cut perpendicular to the longitudinal axis of the arm. The arrow in each graph indicates the direction toward the nearest skin surface. C, pigment granules of a chromatophore. Scale bars, 7.5 μm.

images illustrated lower brightness and partial polarization values in the regions of the arm stripes.

Structure of polarized light reflectors

Low-power electron micrographs of the iridophores within the polarized-light-reflecting arm stripes are shown in Fig. 10. The reflectors are composed of stacks of iridophore plates that, within each stack, are generally parallel to each other, as found in a variety of cephalopod species (Cooper and Hanlon, 1986; Hanlon, 1982; Shashar et al., 1996). In their overall architecture and shapes, the iridophores in the arm stripe regions of cuttlefish and squids closely resemble one another. Each stack of platelets forms a reflecting unit known as an iridosome (Mirow, 1972; Cloney and Brocco, 1983). In a given section, different iridosomes may show different thicknesses and spaces between plates (Fig. 10). These apparently variable thicknesses of iridosomal plates among iridosomes appear because the section plane was rarely perpendicular to the plates. As a result, neither the thicknesses of the plates nor the spaces between them can be determined accurately. For similar reasons, the number of platelets within each iridosome was also undetermined. Nevertheless, differences found between the species could affect the optics of the reflector. First, the number of reflecting units in the light path (a consequence of the thickness of the iridophore layer) appears to be different. In cuttlefish, we estimate that a light beam perpendicular to the skin surface may pass through up to eight iridosomes (Fig. 10B), while in squid more than twice as many reflecting units may be encountered (Fig. 10A). Including the spaces between iridosomes, the total thickness of the reflecting layer observed is between 41 μm and 60 μm in squids and 22 μm to 41 μm in cuttlefish. The two species also differ in the orientations of the reflecting units. While the iridophore plates in cuttlefish are generally more or less perpendicular to the skin surface, the ones in squid appeared to be oriented more irregularly (Fig. 10).

Discussion

The properties of polarized light reflections

Partial polarization and spectral reflectance

In our initial attempts to record the polarization signals produced by the arm stripes of cuttlefish and squid, we tested several combinations of sample placement and illumination angle. In both species, when the arm stripes produced polarization reflections, they always appeared iridescent blue or had a sparkling appearance, while when non-polarized, they looked red or pinkish. Previous reports on the polarization reflections of these arm stripes have not described this phenomenon; in fact, there have been no previous descriptions of polarization reflections from red or pinkish colored arm stripes (Shashar et al., 1996; Shashar and Hanlon, 1997; Shashar et al., 2001; Gleadall and Shashar, 2004; Mäthger and Hanlon, 2006). Nevertheless, similar results were found in pink or red iridophores found in the mantle of squids (Mäthger and Denton, 2001), where the reflected light was polarized only when the iridophores appeared bluish green in color.

Theory predicts that varying the angle of incidence of light falling on a multilayer reflector should result in a gradual shift of the dominant reflected wavelengths (Huxley, 1968; Land, 1972; Deparis et al., 2006). It has been shown in several squid species that, at least for the mantle area, altering the illumination angle does shift the reflectance and polarization spectra in a predictable way (Mäthger and Denton, 2001; Mäthger and Hanlon, 2006). When the illumination angles were changed (i.e. between Positions 1 and 2) we also observed changes in reflectance and polarization spectra similar to those of the mantle iridophores as described previously (Mäthger and Denton, 2001).

Both imaging polarimetry and spectral measurements showed that at any given orientation, the light reflected from the arms of cuttlefish always had a lower partial polarization than that from squid. Our data are consistent with previously reported values of the partial polarization found in squid (Shashar and Hanlon, 1997; Shashar et al., 2001). No quantitative data of the partial polarization from the arms of cuttlefish have been

published previously. Based on the ultrastructure of the iridophores, it is most likely that the relatively lower reflectance and polarization values found in cuttlefish compared to squids were caused by a thinner iridophore layer and comparatively fewer iridosomal plates in the light path.

e-vector angle

The e-vector angles of the polarization reflections of the iridophore arm stripes have variously been reported to be either parallel to the longitudinal axis of the squid arm (e.g. Shashar et al., 2001) or perpendicular to it (e.g. Hanlon et al., 1999; Shashar and Hanlon, 1997). As mentioned earlier, both squids and cuttlefishes can reportedly control their polarization reflections (Shashar and Hanlon, 1997; Shashar et al., 1996), so the differences between previous reports could result from experiments on animals of different physiological states. Since we did not measure polarizations from living animals, we cannot rule out the possibility that the e-vector angle can be actively changed by the animals. However, because the e-vector of the polarized light reflections is highly dependent on the illumination angle (Figs 4, 5), it is most likely that differences in illumination orientation alone are responsible for these conflicting reports.

The dependency of e-vector orientation on illumination angle implies that under illumination from two perpendicular light sources of similar brightness, reflected light should be weakly polarized, at best. Similarly, polarization reflections from arm stripes may be weak under diffuse illumination such as in a turbid environment. The cuttlefish and squid species that we studied inhabit from the surface of the sea to around 150 m and 400 m in depth, respectively, but in most cases, they spend their time at relatively shallow depths of the sea (<50 m), especially when they are active (Cargnelli et al., 1999; Sherrard, 2000). In this kind of shallow-water environment, when looking at the arm stripes of nearby fellows, no matter how their arms are oriented, the cuttlefish or squid should be able to discern clear and nearly constant patterns of polarization.

Physical basis of the polarization reflections

Multilayer reflectors occur in a number of animal species. Such devices produce bright, colorful reflectance with a ‘metallic’ appearance. This metallic impression is caused by constructive interference of light reflected from different layers (Land, 1972). When a beam of light reaches a dielectric surface, at an interface between two media of different refractive indices, the proportion of light reflected depends on the refractive indices of the media as well as the wavelength, incident angle and e-vector angle of the incoming light. In principle, a dielectric surface has the highest efficiency of separating light of different e-vector angles when light is incident and reflected at Brewster’s angle (the angle at which reflected light is fully polarized), and the partial polarization of the reflected light changes as the angle of incidence (and therefore reflection) changes. Furthermore, while Brewster’s angle varies with the wavelength of light, multilayer reflector-based polarizers are usually wavelength selective (Kliger et al., 1990). Therefore, a multilayer device could reflect highly polarized light in a particular wavelength range and relatively un-polarized light for all other wavelengths of light. This effect could explain why we found maximum polarization reflections from iridophores primarily at medium to short wavelengths (e.g. bluish), but when the arm stripes preferentially reflected long-wavelength light (when they appeared to be pink and when light arrived from 45°), polarization was extremely weak. The reflectance spectra of the two types of reflection complement each other (compare Fig. 3A with Fig. 6B or Fig. 3B with Fig. 7B). That is to say, whatever mechanism the reflector might be based on, it separates the incoming light into polarized light of shorter wavelengths and non-polarized light of longer wavelengths. Considering the ultrastructure of cephalopod skin (Cloney and Brocco, 1983), it is most likely that the non-polarized pink reflection is the result of light reflected or scattered from tissues underneath the iridophore layer.

It has long been suspected that, at least in squid, light reflections from arm stripes are based not purely on multilayer reflections but also on wavelength-specific scattering of light (Hanlon and Cooper, 1983). In addition, and contrary to the properties of typical multilayer reflectors, we found that the light reflected from the arm stripes of cuttlefish or squid did not show any obvious relationship between orientation and polarization properties. In both species, neither the peak wavelengths of the reflectance spectra, nor the partial polarization values, nor the e-vector angles of the reflected light are greatly affected by varying the orientation of the arm stripes (Figs 4–7).

While these results are qualitatively similar to the polarization of light caused by Rayleigh scatter, Rayleigh scattering is strongly wavelength-dependent; the shorter the wavelength, the stronger the scatter. As a result, higher reflectance values are to be expected at shorter wavelengths. Obviously, our results (Figs 6, 7) do not comply with this prediction. Our EM work instead suggests that the iridosomes in the arm stripes act as multilayer reflectors (Fig. 10). Although it is unclear what refractive indices the iridophore plates have, they are almost certainly made of a protein called reflectin (Crookes et al., 2004). Reflectin has a refractive index of 1.59, which is the highest refractive index ever found in any naturally occurring protein (Kramer et al., 2007). When light is reflected from the interface between a layer of reflectin and cytoplasm (refractive index=1.33), Brewster’s angle occurs at 50.09°. If there are other proteins present in the reflecting plates in addition to the reflectin, the refractive index of the plates could be slightly lower than predicted above, making Brewster’s angle nearer to 45°. Thus, the greatest values of partial polarization will always occur when the incident light is nearly perpendicular to the reflected light, so that the angles of incidence and reflection are both near 45° (i.e. as in illumination Position 1). In principle, one could use Fresnel’s equations (Feynman et al., 1963) together with measurements of polarization at many angles of incidence and reflection to compute the true values of the refractive indices of these structures; however, these properties of the iridophore plates are beyond the scope of this paper. Furthermore, because of the stacking of large numbers of plates and their complex orientations, it is unlikely that the results of such measurements would be meaningful.

Characteristic multilayer reflection has been found from iridophores in the mantles of several squid species (Mäthger and Denton, 2001; Mäthger and Hanlon, 2007). However, our results from the iridophores of cephalopod arm stripes do not entirely

reproduce the properties of typical multilayer reflections. Plates within the iridophores of the mantle surface or other iridescent regions on the bodies of squids and cuttlefishes are generally parallel throughout the entire cell, thus producing characteristic features of standard multilayer reflectors (Cooper et al., 1990; Hanlon et al., 1990). In contrast, we found that the orientations of the plates (as well as the number and thicknesses of the plates) in iridophores of the arm stripes vary. It seems likely that the arm stripes of cuttlefish and squid use multilayer reflectors with their surfaces arranged over various angles to produce uniquely constant polarized light reflection properties. Thus, while the tilt or rotation angle of the sample changes, one particular subset of the iridosomes (and their sets of parallel plates) is always favorably illuminated. As a result, the incident angle and thus the properties of the reflected polarized light are essentially constant across various tilt and rotation angles. In this way, any changes in the position of the arms of signaling squid of cuttlefish will affect the signal's polarization appearance only minimally. Consequently, a unique, reliable, and highly conspicuous signal can be produced.

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