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RESEARCH ARTICLE

How does the blue-ringed octopus (*Hapalochlaena lunulata*) flash its blue rings?

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SUMMARY

The blue-ringed octopus (*Hapalochlaena lunulata*), one of the world's most venomous animals, has long captivated and endangered a large audience: children playing at the beach, divers turning over rocks, and biologists researching neurotoxins. These small animals spend much of their time in hiding, showing effective camouflage patterns. When disturbed, the octopus will flash around 60 iridescent blue rings and, when strongly harassed, bite and deliver a neurotoxin that can kill a human. Here, we describe the flashing mechanism and optical properties of these rings. The rings contain physiologically inert multilayer reflectors, arranged to reflect blue–green light in a broad viewing direction. Dark pigmented chromatophores are found beneath and around each ring to enhance contrast. No chromatophores are above the ring; this is unusual for cephalopods, which typically use chromatophores to cover or spectrally modify iridescence. The fast flashes are achieved using muscles under direct neural control. The ring is hidden by contraction of muscles above the iridophores; relaxation of these muscles and contraction of muscles outside the ring expose the iridescence. This mechanism of producing iridescent signals has not previously been reported in cephalopods and we suggest that it is an exceptionally effective way to create a fast and conspicuous warning display.

Key words: iridescence, cephalopod, warning display, communication, color change, chromatophore.

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INTRODUCTION

As one of the world's most venomous marine animals, the blue-ringed octopus (*Hapalochlaena lunulata*), a small and generally docile tropical marine animal, spends most of its time hiding in-between shells and rubble where it puts on a very effective camouflage pattern. What gives this octopus its name are the approximately 60 iridescent blue rings, which the animal flashes in an aposematic warning display when disturbed or harassed (Roper and Hochberg, 1988). Following the warning display (and if given the opportunity), the blue-ringed octopus will bite and inject the venom tetrodotoxin, which can quickly kill a human (Sheumack et al., 1978). To date, the exact flashing mechanism remains undescribed.

Cephalopods (squid, cuttlefish, octopus) can produce a variety of body patterns for camouflage and signaling using neurally controlled pigmented chromatophores as well as structural light reflectors (Hanlon and Messenger, 1996; Mäthger et al., 2009b). Chromatophores are organs that contain a pigment sac filled with dark brown/black, red or yellow pigments. Attached to the pigment sac are a series of radial muscle fibers that are innervated directly by the brain, and contraction or relaxation of these muscles expands or retracts the pigment sac. In cephalopods, there are generally two types of structural reflectors: leucophores and iridophores. Leucophores are broadband structural reflectors responsible for whiteness in cuttlefish and octopus (Hanlon and Messenger, 1996). All cephalopod iridophores studied to date have been described as multilayer reflector cells and their reflective plates have been assumed to consist of a protein interspersed by spaces of cytoplasm, each differing in refractive index. This creates constructive interference colors covering the entire visible spectrum, including the near ultra-violet and infra-red (Denton and Land, 1971; Land, 1972; Crookes et al., 2004; Mäthger et al., 2009b).

In this paper, we describe the flashing mechanism and optical properties of the iridescent blue rings that give the blue-ringed octopus its name.

MATERIALS AND METHODS

Spectrometry

Hapalochlaena lunulata (Quoy and Gaimard 1832) were killed by an overdose of anesthetic (3–5% ethanol in sea water). We measured reflectance spectra of skin samples using a fiber optic spectrometer (QE65000; Ocean Optics, FL, USA). Measurements were taken with bare fibers (polarization and UV) or by attaching the fiber to the c-mount of a Zeiss Discovery V20 microscope (physiology). For polarization/oblique angles of incidence, a broadband (UV, visible, IR) light source was used (HPX2000; Ocean Optics). To analyze polarization, we used two pieces of linear polarizing filters (LEE Filters, CA, USA). All measurements were standardized against an Ocean Optics WS-1 standard, which is >98% reflective from 250 to 1500 nm; reflectance is given as a percentage relative to the white standard. Samples were pinned onto the Sylgard-coated base of a Petri dish.

Physiology

We tested physiological activation of iridophores using the following pharmacological solutions: acetylcholine (ACh; up to 30 mmol l^{-1}), L-glutamate (L-Glu; up to 6 mmol l^{-1}), serotonin (5-HT; up to 3 mmol l^{-1}), potassium chloride (KCl; 30 mmol l^{-1}) and noradrenaline

(NA; up to 1 mmol l^{-1}) (all chemicals were purchased from Sigma, St Louis, MO, USA). Solutions were applied separately to tissue samples and were prepared in cooled artificial seawater (ASW, in mmol l^{-1}: NaCl 470, KCl 10, $CaCl_2$ 10, $MgCl_2$ 60, Hepes 10; pH 7.8). Samples were exposed for a minimum of 5 min. Images and spectral measurements were obtained every 20 s.

Electron microscopy

Tissue samples were fixed in a modified Karnovsky's fixative containing 3% glutaraldehyde, 1.5% formaldehyde (from paraformaldehyde) in 0.1 mol l^{-1} cacodylate buffer (pH 7.4) plus 5 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} EGTA and 0.3 mol l^{-1} sucrose (final tonicity≈1100 mOsm), for 24 h at 4°C. Tissue was post-fixed in 1% osmium tetroxide in cacodylate buffer (30 min, 4°C), dehydrated in 50% methanol and 2,2-dimethoxypropane (DMP; Sigma), followed by propylene oxide as a transition solvent, then epoxy embedded. Sections were cut on a Reichert Jung Ultracut microtome and viewed on a JEOL JEM 1010 transmission electron microscope.

For scanning electron microscopy (SEM), polyester wax (Stepan PEG 400 distearate, melting point 36°C)-embedded blue-ring tissue was cross-sectioned (6 µm thickness). Sections were de-waxed in two changes of absolute ethanol and dried in a low dust environment with three changes of hexamethyldisalizane (HMDS, Sigma). The third change of HMDS was allowed to evaporate overnight. Dried samples were sputter-coated with 7.5 nm of platinum using a Leica EM MED020 with platinum target. Copper tape was applied to both long edges of the slide to reduce charging effects and images were obtained using a Zeiss Supra SEM (Carl Zeiss Microimaging, Thornwood, NY, USA).

Brightfield microscopy

Blue-ring tissue was infiltrated with and embedded in polyester wax. Tissue was mounted on blocks and serially cross-sectioned (6 µm thickness) using a rotary microtome (Leica). Sections were mounted on glass slides pre-coated with Weaver's subbing solution (Weaver, 1955) using 2% paraformaldehyde. Sections were de-waxed in an ethanol series and stained with Mallory's triple connective tissue stain (acid fuchsin, Aniline Blue, Orange G) (Humason, 1967; Kier, 1992). After staining, slides were dehydrated, cleared with toluene and cover-slipped with Histomount. Brightfield microscopy was performed using a Zeiss AxioSkop and sections were photographed with a Canon 5D-Mark 2 digital camera.

Confocal microscopy

Polyester wax cross-sections of the blue ring were dewaxed in an ethanol series and cover-slipped using Prolong Gold Antifade with DAPI (Invitrogen, Carlsbad, CA, USA). *Z*-stacks and tiled *Z*-stack images were collected using the DAPI label (405 nm excitation) in combination with the autofluorescence signal from the 488 and 633 nm laser lines. Sections were imaged using a LSM 780 confocal microscope (Carl Zeiss Microimaging), with optical sectioning and tile scans achieved using the appropriate functions in the Zen software. The tile scan was stitched to form a composite image using the Zen stitching algorithm set for 70% stringency. All images derived from *Z*-stacks were displayed using a maximum intensity *Z*-projection (FIJI software).

RESULTS

The blue-ringed octopus (*H. lunulata*) can simultaneously flash its ~60 rings, which cover the mantle, head and arms, in an aposematic warning display. We filmed six adult animals (sex not determined) and found that this display can be shown fully in as little as 0.3 s (full expression is accomplished within 10 frames; 30 frames s^{-1} recording rate; occasionally, the display took slightly longer, ~0.5 s). During the display, bright blue iridescent rings are exposed and dark brown chromatophores on either side of the rings are expanded to increase the contrast of the iridescence (Fig. 1). Michelson contrast to a typical octopus eye with monochromatic (color-blind) vision at λ_{max}=475 nm (Muntz and Johnson, 1978) is as high as 0.92 [on a scale from 0 to 1, with 1 being highest; methods for obtaining Michelson contrast are outlined elsewhere (Mäthger et al., 2006)]. In other body areas, chromatophores are typically retracted, giving the animal an overall pale appearance, drawing even more attention to the iridescence. Together, these skin elements create a highly conspicuous visual signal and the underlying flashing mechanism differs from iridescence changes reported in other cephalopods. The iridophores creating the blue–green iridescence are located in modified skin folds, or 'pouches', and are covered and exposed by fast movement of the skin surrounding the ring. Confocal microscopy and brightfield studies illustrate the overall morphology

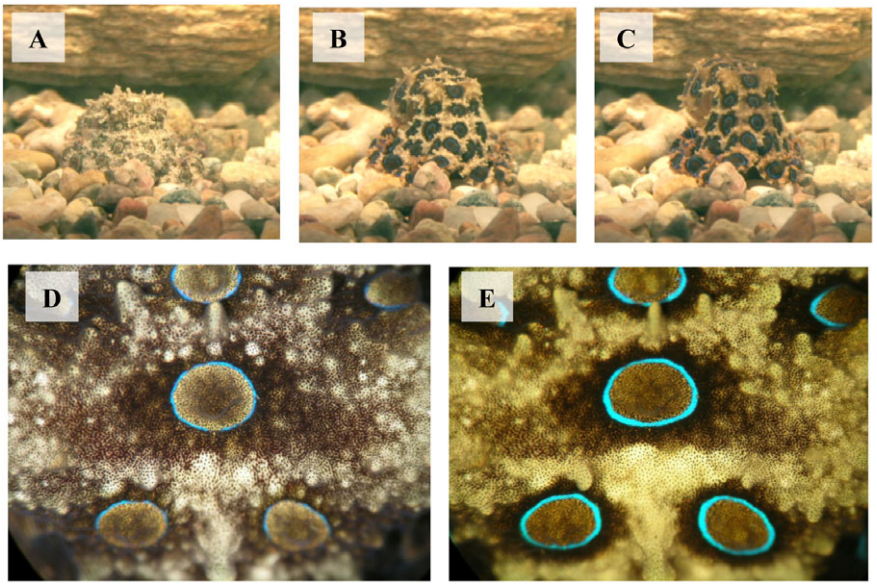

Fig. 1. (A–C) Blue-ringed octopus (*Hapalochlaena lunulata*; ~3 cm mantle length) with a camouflaged body pattern (A) and flashing rings (B,C). (D,E) Close-up images of semi-closed ring (A) and flashed ring (B). Blue-ring diameter ~0.5 cm.

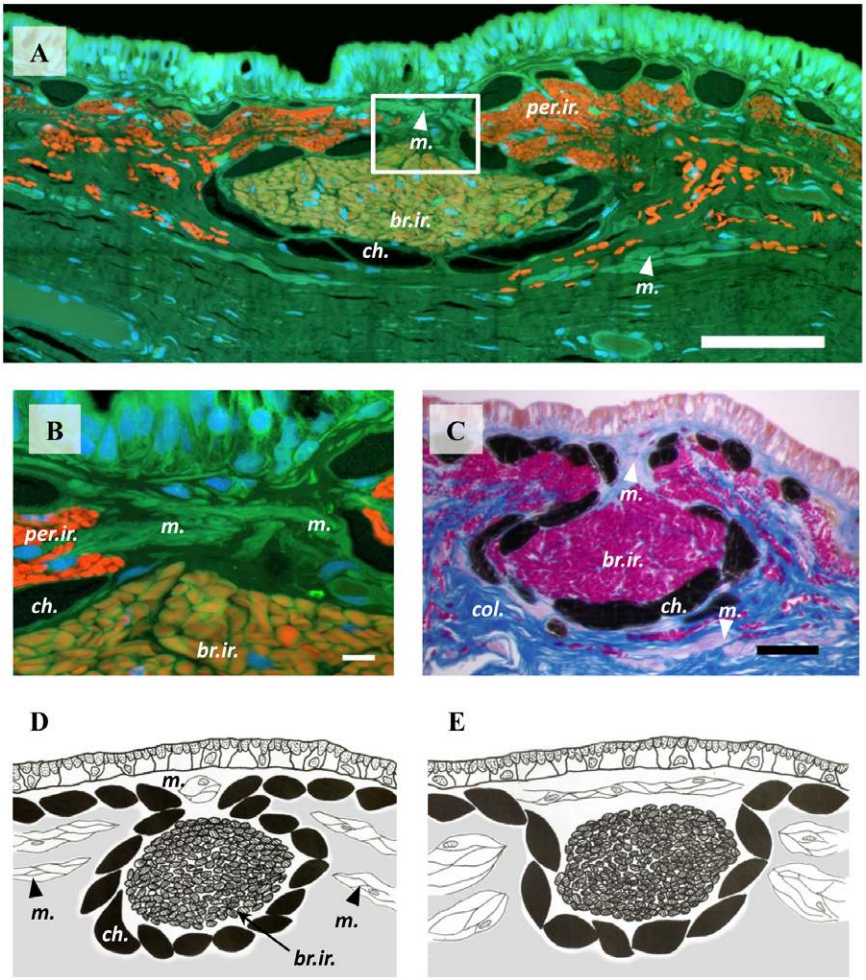

Fig. 2. (A) Confocal microscope image, showing the arrangement of iridophores, chromatophores and skin muscles of the blue ring. Colors indicate the following structures. Blue, nuclei (DAPI nuclear stain). Green, typical formaldehyde-induced autofluorescence for biological tissue. The bright green structures around the blue ring are muscle fibers (*m.*). Red, iridophores, which reflect red light to varying degrees. The blue-ring iridophores (*br.ir.*) reflect less strongly than iridophores around the periphery (*per.ir.*), which do not reflect blue light *in situ*. Black, chromatophores (*ch.*). Scale bar, 100 μm. (B) Higher magnification image of the area indicated in A, showing the muscle fibers responsible for keeping the blue ring concealed. Scale bar, 10 μm. (C) Mallory's triple connective tissue staining shows muscle fibers (light pink), connective tissue (collagen, *col.*, blue), iridophores (blue-ring iridophores and peripheral iridophores, dark pink) and chromatophores (black). Scale bar, 100 μm. (D,E) Drawings illustrating the flashing mechanism of the rings; (D) closed ring, (E) open ring. Contraction of transverse muscles, located above the blue-ring iridophores, covers iridescence. Relaxation of transverse muscles, combined with contraction of muscles around the perimeter, exposes iridescence.

of the blue ring and differentiate key structural elements, such as muscle fibers, iridophores and chromatophores (Fig. 2A–C). Contraction of transverse muscles, which are located above the iridophores of the ring and connect the center of each ring to the outside of the ring, covers the iridescence. Relaxation of these muscles, combined with contraction of muscles around the perimeter of each ring, exposes the iridescent flash (Fig. 2D,E).

The iridophores inside the blue rings have optical properties of multilayer reflectors. When illuminated by a directional beam of light, the typical features of this type of reflector can be observed. At normal incidence (i.e. measured perpendicular to the skin surface using a bifurcated reflectance probe, QR200-7-UV-VIS, Ocean Optics), the ring reflects at approximately 500 nm. When the angle of incident light and the viewing angle are increased, peak reflectance shifts towards the shorter (UV) side of the spectrum and the reflected light is linearly polarized (Fig. 3). The iridophores of the ring are not physiologically active. A series of neurotransmitters that activate cephalopod and fish color-changing structures were applied to blue-ring samples: ACh (1×10^{-4} mol l^{-1}; N=14), known to activate iridophores in squid; 5-HT (1×10^{-4} mol l^{-1}; N=4), known to relax cephalopod chromatophore muscles; L-Glu (1×10^{-3} mol l^{-1}; N=4), known to contract cephalopod chromatophores; KCl (3×10^{-3} mol l^{-1}; N=4), which depolarizes cell membranes and can cause physiological activity; and NA (1×10^{-3} mol l^{-1}; N=4), which activates fish chromatophores. No iridescence changes were observed with any neurotransmitter (data not shown).

Blue-ring iridophores contain a large number of densely packed plates (as many as 30 plates in some cells) with even plate thicknesses averaging 62 nm (Fig. 4). This is in the range required for the plates to act as ideal 1/4 wavelength reflectors, for which $\lambda_{max}=4nd$, where n is the refractive index of the plate and d the actual thickness (Land, 1972). Assuming a refractive index of 1.59 (Kramer et al., 2007) and tissue shrinkage of up to 20% (Bell, 1984; Lee, 1984), plates of this thickness would reflect at 499 nm, which correlates with spectral measurements of intact tissue (see Fig. 3). Individual stacks are arranged at multiple angles (see Fig. 4A,B), ensuring that the blue–green iridescence is seen from a range of angles.

DISCUSSION

To function as an aposematic warning display, the display colors have to be within the limits of the visual system of the relevant predator species (Endler, 1978; Endler, 1988; Endler and Mappes, 2004). The peak reflectance of the blue ring lies well within the range of mid- and long-wavelength sensitive opsins of potential marine vertebrate and invertebrate predators, including cetaceans, pinnipeds, birds, teleost fishes and other cephalopods (Hanlon and Messenger, 1996; Cronin et al., 2000; Hart, 2004; Levenson et al., 2006; Gačić et al., 2007; Theiss et al., 2007). Furthermore, the blue–green part of the visible spectrum is the most prominent ambient underwater light field (Tyler and Smith, 1970; Jerlov, 1976), so the iridescence is spectrally well tuned to be maximally visible. As the peak reflectance of the iridescence also matches the spectral absorbance of some of the known cephalopod visual pigments

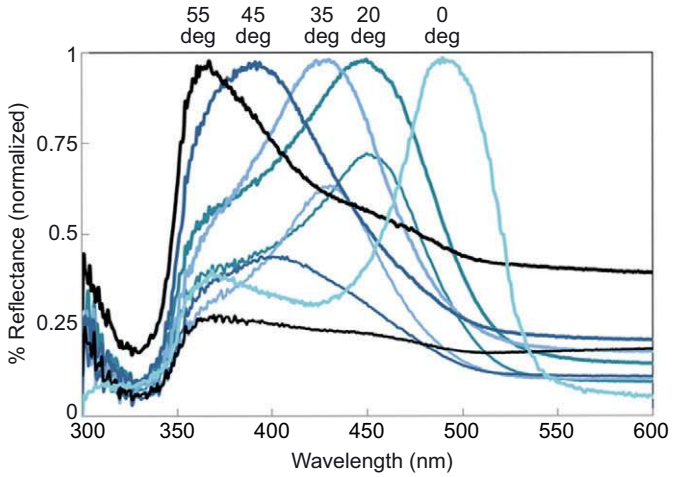

Fig. 3. Spectral reflectance measurements of iridophores from the blue ring of *H. lunulata* at increasing angles of incidence and viewing and in two planes of polarization, showing that reflected light shifts towards the short (UV) end of the spectrum and becomes linearly polarized with increasing angle of incidence.

(Brown and Brown, 1958; Morris et al., 1993; Bellingham et al., 1998), the blue-ring iridescence may additionally function in intraspecific communication. Brief iridescence flashes were sometimes observed immediately before male–male and male–female mating attempts (Cheng and Caldwell, 2000). Furthermore, cephalopods have the visual hardware to be able to detect polarized light, and the polarized reflectance at oblique angles may function in communication (Mäthger et al., 2009a) but more work is needed to confirm such a role. It would also be interesting to know whether the blue-ringed octopus can show a partial display (e.g. unilaterally exposed rings may be advantageous under certain circumstances). We did not observe any variations in the actual iridescent flash but as chromatophores are under neural control, they can be expanded to varying degrees, resulting in various contrasts (Fig. 5). Furthermore, observations on a male *Hapalochlaena* [species no. 1 in the book by Norman (Norman, 2000)] from Northern Australia revealed the ability to expose only a part of a blue ring as well as control over the extent of blue-ring exposure on various parts of the body (R. Caldwell, personal communication).

There are several species within the genus *Hapalochlaena*. The data we report in this paper are for *H. lunulata*. In *H. fasciata*, the optical properties of the iridescent blue–green lines and rings, as well as the mechanism of the flashes, are the same as those reported here (L.M.M., personal observation). While it is unlikely that the iridescent flashing mechanism in other species within this genus differs from that of *H. lunulata*, it would nevertheless be interesting to compare our findings with those for other species of this genus, particularly because the arrangement of rings and lines differs between species.

The skin of cephalopods contains two types of structures that make color and pattern change possible: (1) pigmented chromatophores, which can be expanded in less than a second (Messenger, 2001; Hanlon, 2007) to create a wide range of almost instantly changeable body patterns for communication and camouflage; and (2) structural light reflectors (iridophores and leucophores), generally located in a layer beneath the chromatophores. While white leucophores (in cuttlefish and octopus only) are physiologically inactive, iridophores are physiologically tunable in some cephalopod species (Mäthger et al., 2009b).

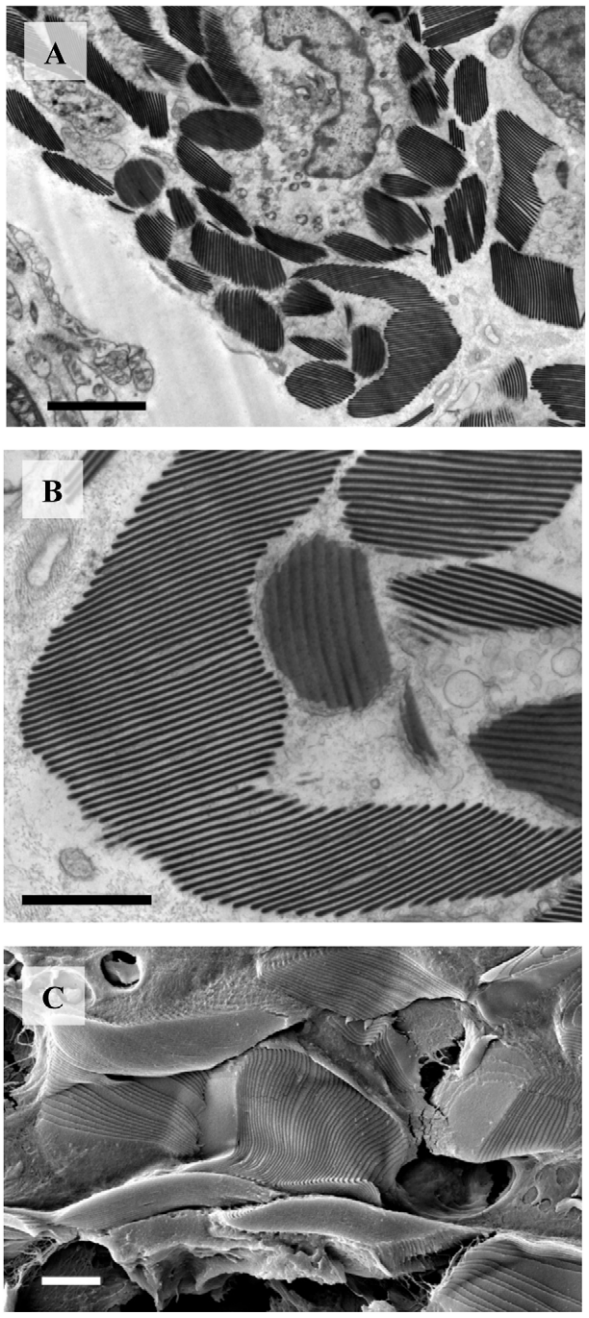

Fig. 4. (A,B) Transmission electron micrographs of the iridophores of the blue ring, showing varying orientations of stacks (A, scale bar 5 μm) and the parallel arrangement of plates within stacks (B, scale bar 2 μm). (C) Scanning electron micrograph of cross-sectioned blue-ring iridophores, showing the parallel arrangement of plates (scale bar 2 μm).

Cephalopods can tune iridescence in two ways: (1) by the action of muscarinic cholinergic receptors, which affect the spectrum and intensity of iridescence directly, and/or (2) the action of chromatophores, which indirectly block or spectrally filter iridescence (the second method also works to modify reflectance from physiologically inactive leucophores). While tuning iridescence directly (i.e. physiologically) takes seconds to minutes, spectral change *via* chromatophores is much faster because the surrounding muscles are under direct neural control, so chromatophores would in principle be able to create a very fast visual signal. However,

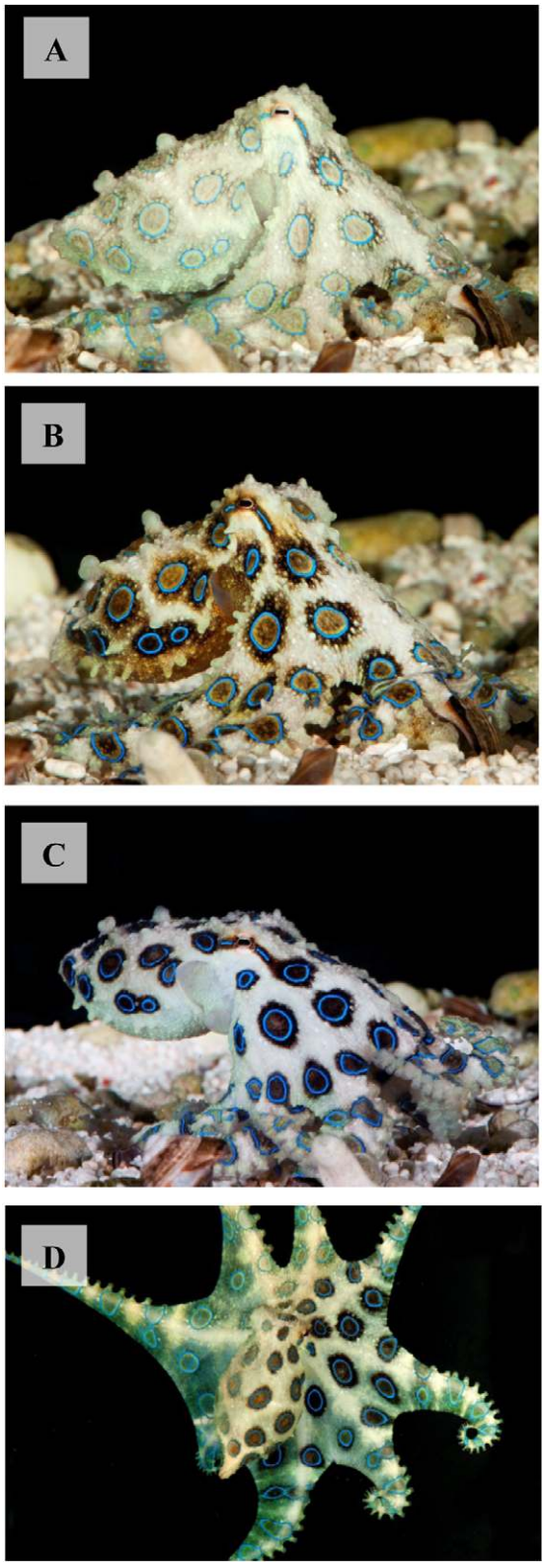

Fig. 5. Blue-ringed octopus (*H. lunulata*) with exposed blue rings, showing how varying states of expansion of chromatophores surrounding each ring can dramatically change the conspicuousness of the blue rings from relatively pale (A) to very conspicuous (B,C). (D) As a result of neural control of chromatophores, *H. lunulata* can enhance contrast of the blue rings unilaterally. In this image, only chromatophores surrounding the rings of the animal's right side are expanded. Photo credit for all four images: Roy Caldwell.

cephalopod chromatophore pigments generally reflect longer wavebands of light (red, orange, yellow, brown) that alone are insufficient to create high-contrasting patterns and, because of absorption properties of water, are ineffective wavebands for underwater communication, at least at greater depths, where long wavelength light is quickly absorbed. It is therefore not surprising that a high-contrast communicative signal is created by recruiting structural reflectors, such as iridophores, which add short wavelength colors to the color repertoire of cephalopods (these colors are more efficient at a range of water depths), as well as white leucophores that are found in some cuttlefish and octopus. However, when high-contrast signals are not desired, structural reflectors must be physiologically turned off or covered by chromatophores. There are disadvantages to both of these strategies because physiological control of iridophores takes time, and using chromatophores to mask a reflective signal means these chromatophores cannot be recruited to any other camouflage or signaling body pattern.

The system of the blue-ringed octopus, where highly reflective iridophores are covered with modified skin pouches, has so far not been reported in any other cephalopod. Hiding structural reflectors in a pouch is an ingenious way to make a highly visible short- to mid-wavelength signal immediately accessible while freeing all chromatophores to contribute to body patterning. It takes *Octopus vulgaris* 2 s to show a full threat display (Hanlon, 2007), involving textural, locomotor, chromatic and postural changes, half of which are carefully orchestrated at the skin surface. In contrast, the blue-ringed octopus can expose iridescence and expand chromatophores for maximal contrast simultaneously, increasing the speed of its warning display. A fast, conspicuous display would certainly be beneficial to both the predator and the octopus.

Aposematic warning colors often involve conspicuous iridescent patterns because iridescence can be extremely bright and saturated, and it is generally not possible to produce such signals using pigmented colors (Doucet and Meadows, 2009). *Morpho* butterflies, for example, use their wings to give a blue iridescent warning flash that deters predators (Young, 1971) and there are other examples of birds and butterflies pairing iridescent colors with dark pigmented colors to maximize contrast (e.g. Frith and Beehler, 1998; Stavenga et al., 2004; Wilts et al., 2012). Cephalopod skin, in particular that of cuttlefish and octopus, is highly elastic and muscular, lending these animals the unique ability to give their skin 3-dimensional texture for camouflage and signaling (Hanlon, 2007). Given the intense network of muscles and nerves in their skin, it is therefore not too challenging to conceive how the blue-ring flashing mechanism evolved in *H. lunulata*. Cephalopods have an enormous advantage over many other animals in that their skin is adaptable. The vast majority of animals must move part of their body or relocate to a different habitat when displaying a high-contrasting signal, whereas cephalopods have the ability to modify their skin to produce such displays at any time. There are over 700 species of cephalopods in the world's oceans today but what we know about their color change system comes from only a handful of mainly near-shore species. While cephalopods seem to share a basic framework of skin structures that create body patterns for signaling and camouflage (chromatophores and light reflectors), it is nevertheless amazing how diverse the mechanisms of color change are within this invertebrate group.

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