JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
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Structural Basis for the Brilliant Colors of the Sapphirinid Copepods

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Supporting Information

ABSTRACT: Males of sapphirinid copepods use regularly alternating layers of hexagonal-shaped guanine crystals and cytoplasm to produce spectacular structural colors. In order to understand the mechanism by which the different colors are produced, we measured the reflectance of live individuals and then characterized the organization of the crystals and the cytoplasm layers in the same individuals using cryo-SEM. On the basis of these measurements, we calculated the expected reflectance spectra and found that they are strikingly similar to the measured ones. We show that variations in the cytoplasm layer thickness are mainly responsible for the different reflected colors and also that the copepod color strongly depends on the angular orientation relative to the incident light, which can account for its appearance and disappearance during spiral swimming in the natural habitat.

Some of the most spectacular colors produced by organisms are derived from periodic layered structures on the length scale of the wavelengths of visible light.[1] Such photonic structures consist of regularly alternating layers of two transparent materials with different refractive indices, such that light reflected from the different layers undergoes constructive interference for some wavelengths and destructive interference for others.[1f,2a−c] A multilayer stack can act as a spectrally selective reflector when the optical thickness nd (the product of the physical thickness d and the refractive index n) of the layers falls within the wavelength range of visible light, resulting in the observation of distinct colors.[1f,2c−f] The most efficient reflector arrangement is the quarter-wave stack, where the optical thickness of both layers is equal to the one-fourth of the wavelength of the reflected light.[2c,f,g]

One of the most striking examples of such photonic structures are the male sapphirinid copepods, small marine crustaceans that produce a variety of different colors, but only when the incident light is at specific angles to the animal's dorsal surface. Thus, the copepods "flash" light of a specific color, but as they move they become transparent and suddenly seem to almost completely disappear (a movie showing this behavior is available at http://www.liquidguru.com/octopod-copepod/). The goal of this study is to understand the structural basis for both the variability of the colors and the strong angular dependence of the reflected light.

Members of the copepod family Sapphirinidae are found between the ocean surface and a depth of 300 m.[3a,b] Their reflectivity and color are thought to play a role in interspecies communication and mate recognition in the open ocean.[3a−d] The iridescent colors of the males of each species are closely related to their distribution in the epipelagic zone and are thought to provide increased visibility against the ambient background.[3c,d] Species with warm colors (i.e., with longer wavelengths) are usually found in shallow waters, whereas species with blue colors are usually found in deeper waters, where the spectrum of the filtered solar light is primarily in the blue-green range.[3b,e] Even within the Sapphirinidae family, the *Sapphirina metallina* males, the main focus of this study, are exceptional in the variety and brilliance of their colors.

The multilayer reflectors responsible for the colors in *S. metallina* are composed of stacks of anhydrous guanine crystals[3a,4] separated by cytoplasm, similar to those found in iridescent fish scales, silver spiders, and chameleons.[1f,2a,5] In contrast to the crystals found in chameleons, the guanine crystals in the sapphirinids, as well as those in fish and spiders, are thin {102} plates. The exceptionally high refractive index along the axis normal to the biogenic plate crystals (n = 1.83) provides high index contrast relative to the cytoplasm (n = 1.33).[6] Unlike the guanine crystals in fish and spiders, the sapphirinid crystals are perfectly regular hexagons in an extremely ordered arrangement (Figure 1). Earlier studies reported that the measured thickness of the crystals did not match the expected reflectance (or reflectivity) calculated assuming an ideal multilayer system.[3a,d] Furthermore, no connection was found between the thickness of the crystals and the copepod colors.

The crystal layer is located just below the chitin dorsal cuticle, forming a continuous mosaic surface of closely packed hexagonal crystals over the whole area of the dorsal integument. The crystals form inside specialized cells and are arranged in arrays of 10−14 alternating layers of guanine crystals and cytoplasm in *S. metallina* and 5−8 layers in *Copilia mirabilis* (Figures 1 and 2; see the Supporting Information (SI) for crystal characterization).

In order to definitively establish the relation between specific structural parameters and the observed reflectance, we designed a correlative experiment in which the reflectance of individual copepods was first measured and then the thicknesses of the crystal and cytoplasm layers were measured using cryogenic scanning electron microscopy (cryo-SEM) on the same individuals. The study involved the following stages: (i) live individuals of violet-magenta, blue-green, yellow, and red *S. metallina* copepods as well as indigo-violet *C. mirabilis* were

Received: May 27, 2015
Published: June 22, 2015

DOI: 10.1021/jacs.5b05289
J. Am. Chem. Soc. 2015, 137, 8408−8411

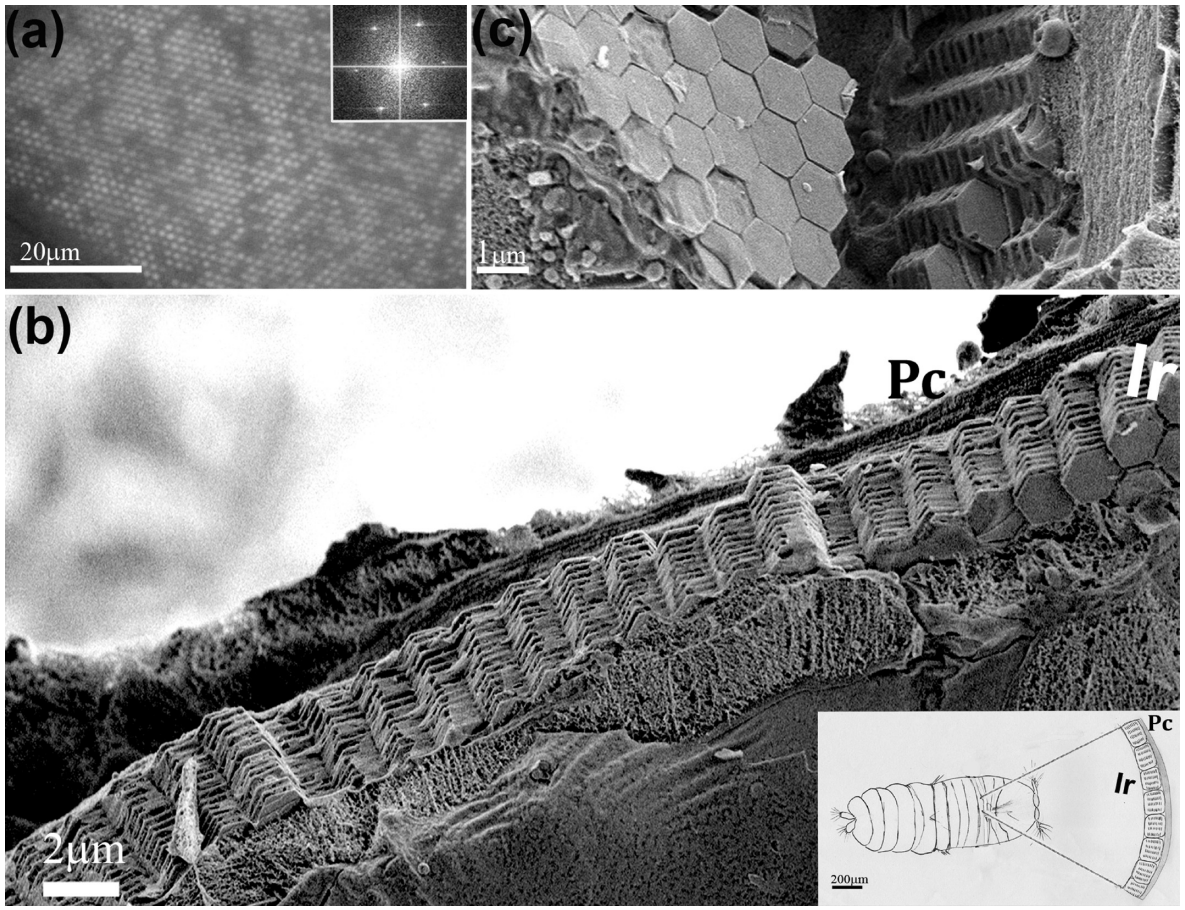

Figure 1. Ultrastructure of *Sapphirina metallina*. (a) Light microscope image viewed through the dorsal surface showing an array of tightly packed hexagonal crystals. Inset: Fourier transform of the image demonstrating the regular packing. (b, c) Cryo-SEM micrographs of a high-pressure-frozen, freeze-fractured *S. metallina* copepod: (b) Transverse view showing the guanine crystals aligned perpendicular to the dorsal surface of the animal. The iridophores (Ir) are located just beneath the animal chitin procuticle (Pc). Inset: Schematic representation of the position of the crystal-containing cells (Ir) relative to the copepod anatomy. The copepod is presented in dorsal view. The right-hand side is a schematic representation of the cross section of the region indicated, which corresponds to the cryo-SEM micrograph. (c) Dorsal view showing the tightly packed perfectly hexagonal crystals and the alternating layers of guanine crystals and cytoplasm beneath the procuticle.

selected; (ii) individual copepods were photographed, and their reflectance spectra were measured using a tailor-made microscope (see the SI); (iii) the same individuals were high-pressure-frozen, freeze-fractured, and imaged by cryo-SEM to avoid dehydration artifacts that would change the thickness of the hydrated cytoplasm layers. The spacings of the crystal/cytoplasm layers were measured from micrographs viewed more or less orthogonal to the layers and statistically evaluated on the basis of 50−150 measurements per specimen; (iv) the derived structural parameters were used to simulate the reflectance spectra using a Monte Carlo transfer matrix calculation; (v) the simulated and measured reflectance spectra were compared.

The results are shown in Figure 2, and the agreement between each of the simulated and measured reflectance spectra is striking. This demonstrates that the crystal/cytoplasm spacings indeed control the reflected colors. In individual copepods, the standard deviation of the measurements over the whole cross section is around ±10%, showing the uniformity of the thicknesses of both guanine crystals and cytoplasm layers (Figure 2). Note that the crystal thicknesses are similar among all the differently colored copepods, ∼70 nm, but the thicknesses of the cytoplasm layers vary considerably, ranging between 50 and 200 nm (Figure 2). This indicates that practically all of the observed variations in the reflected light are due to changes in the thickness of the cytoplasm layers and not of the crystals. Despite the fact that the reflectors can deviate significantly from being quarter-wave stacks, the observed peak reflectivities are close to unity. This is due to the relatively large number of repeats of low/high index layers, which is higher than the ∼6 repeats needed to achieve near-unity reflection in quarter-wave stacks (considering the refractive index contrast of anhydrous guanine and water). This also explains why the previous work that considered only the crystal's thickness while calculating the predicted reflectance could not account for the variety of different colors.[3a,d]

For a dielectric reflector in which the main peak is in the near-infrared, a second-order peak can be observed within the shorter wavelengths of visible light, i.e., violet or deep blue. This is the case of the violet-magenta copepod, which has the largest cytoplasm spacings (Figure 2a): the first-order peak is at ∼800 nm, and the second-order peak is at ∼420 nm. The combination of the two peaks results in the observed magenta color.

One of the most surprising features of copepod reflectance is the sudden disappearance of any reflectance in swimming copepods, which makes them virtually invisible (see the movie mentioned above). The wavelength of the reflected light should therefore vary with the angle of incidence of the incoming light, with shorter wavelengths being observed at higher incidence angles. Moreover, because of the birefringence of guanine,[6b,7] the

DOI: 10.1021/jacs.5b05289
J. Am. Chem. Soc. 2015, 137, 8408−8411

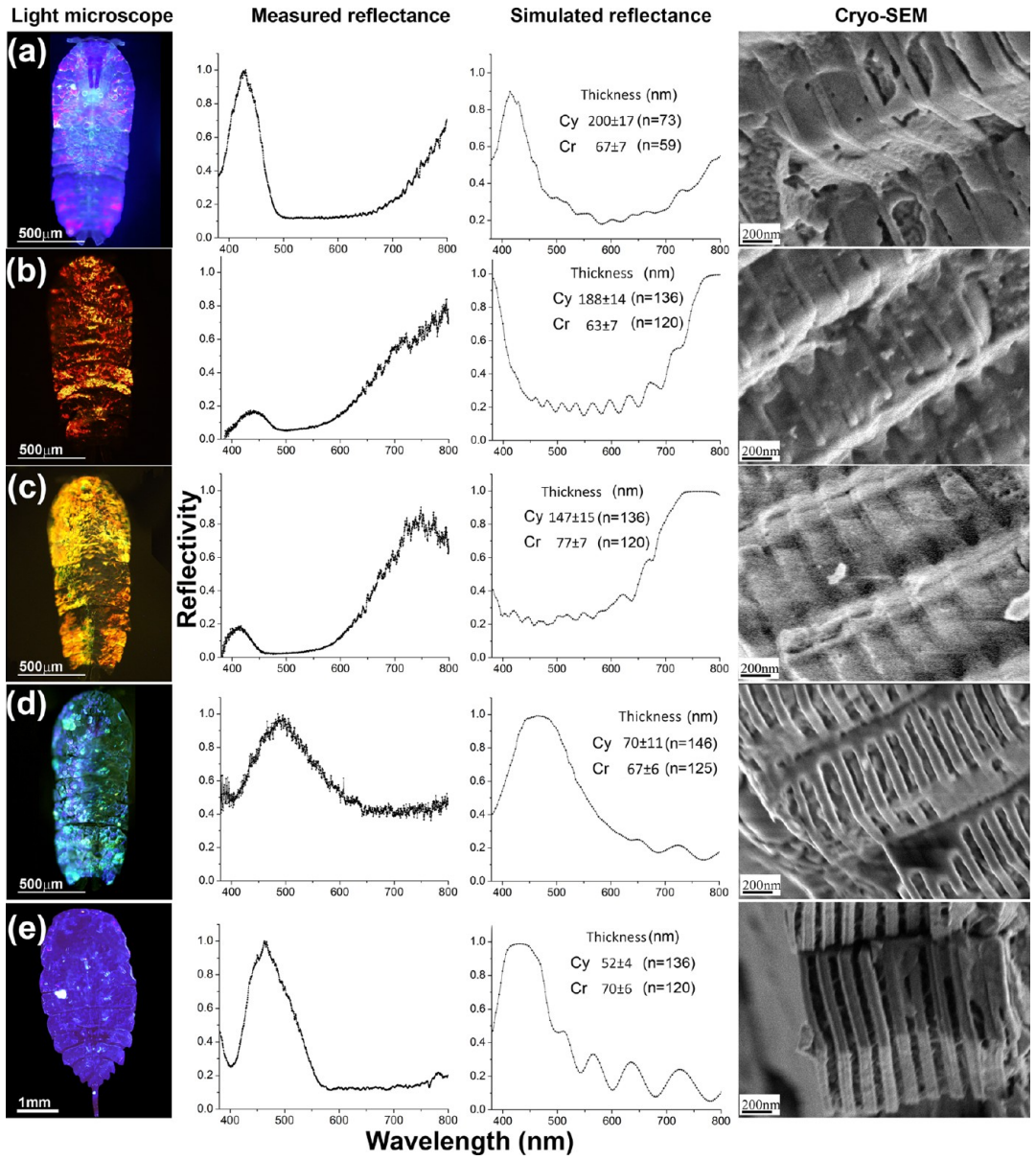

Figure 2. Reflectance and structural properties of individual copepods: (a–d) *Sapphirina metallina*; (e) *Copilia mirabilis*. Column 1 (left) shows light microscope images of representative sapphirinid male specimens and column 2 their measured reflectances. Column 4 shows representative cryo-SEM images of the crystal–cytoplasm layer arrays and column 3 the simulated reflectance spectra. The simulated reflectance was calculated on the basis of the cytoplasm (Cy) and crystal (Cr) thicknesses measured in cryo-SEM images from many differently colored specimens. The results of these measurements are shown in column 3 for each color. The measured reflectance in each spectrum is normalized to a silver mirror; the measured reflectance of (d) was also normalized to the crystal coverage area because of the lack of uniformity of the specimen. The seeming disagreement between the measured and calculated reflectance spectra at short wavelengths in (C) and (D) is due to the short-wavelength edge of the light source (see the SI).

refractive index contrast is reduced for one of the polarization axes, further reducing the reflection intensity and spectrally broadening it. On the basis of the measured structural parameters, we simulated the spectra of the reflected light at different tilt angles and compared them to images taken at the same tilt angles in the violet *C. mirabilis* (Figure 3a) and the blue-green *S. metallina* (Figure 3b). In *C. mirabilis*, when the incident light is normal to the surface of the animal, the observed color is violet with the reflectance peak maximum at 420 nm. When the incident light is tilted to 15°, only a minor variation in the reflected light is observed, while the maximum of the simulated spectrum shifts to 410 nm. At 30° tilt, the reflected light is deep violet, and the peak reflectance shifts to 395 nm. At 45° tilt, the reflectance peak shifts further into the UV, and the copepod becomes practically invisible (Figure 3a). A similar trend was observed for the blue-green copepod, in which the reflectance peak shifted from blue-green (495 nm) at normal incidence to indigo (410 nm) at 45° (Figure 3b). In the magenta *S. metallina* copepods (Figure 2a), where two reflectance peaks are present within the visible-light range, tilting causes a shift of the second-order peak into the UV, whereas the first-order peak shifts into the yellow range, resulting in a yellowish color (not shown). *C. mirabilis* "overcomes" this potential problem by making the thickness of the cytoplasm spacings one-fourth of those of the magenta *Sapphirina.* This locates the first-order peak in the magenta-indigo regime and the second-order peak deep in the UV. Tilting in this case also shifts the first-order peak into the UV, making it invisible to many marine organisms.

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J. Am. Chem. Soc. 2015, 137, 8408–8411

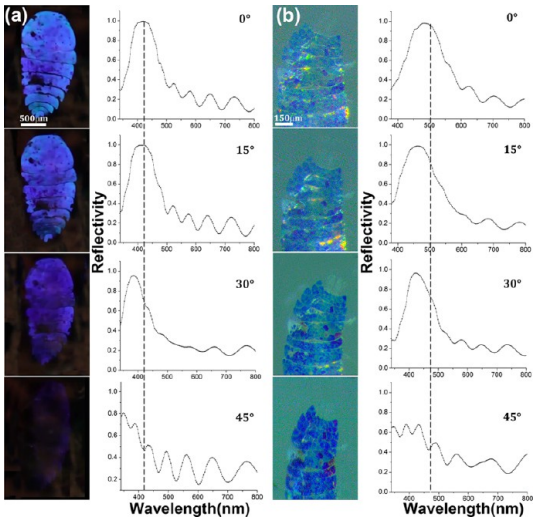

Figure 3. Images of the copepod color changes at different tilt angles and simulated reflected spectra at the same tilt angles: (a) *C. mirabilis*; (b) *S. metallina*. The green background is due to the sample holder.

The spiral swimming behavior of the sapphirinid males is thought to play an important functional role in interspecies communication. When stimulated with light, the males exhibit significantly higher speed and rotating frequency than the females.[8] At the same time spiral swimming changes the tilt of the animal relative to the incident light. This makes them invisible at high tilt angles, possibly providing a defense system against predators. The fact that the crystal thicknesses in copepods from different species are similar implies that different colors may be due to a common mechanism of crystal formation. A similar phenomenon was observed in the silver-colored koi fish, in which the broad-wavelength iridescence is obtained only by fluctuations in thickness of the cytoplasm spacings, whereas the crystal thicknesses remain relatively constant.[5a]

One observation that arises from this study concerns the notion of "ideality". We often consider the quarter-wave reflector as "ideal", as it requires the least number of repeats to achieve high reflectivity. Indeed, when a dielectric reflector is manufactured layer-by-layer in a serial process, this is a significant consideration. For the self-assembled reflectors observed in copepods, the fact that one of the building blocks (the guanine crystals) follows a fixed template likely simplifies its formation significantly, whereas an increase in the number of repeats apparently compensates for the deviation from quarter-wave thickness. The observed 70 nm thickness of the guanine crystals corresponds to a quarter of a wave in the green ($4nd = 4 \times 1.83 \times 70$ nm = 512 nm). The optical thickness of the cytoplasm layer is seen to range from a quarter wave in the near-UV (for the 52 nm spacing) all the way to a quarter wave in the near-IR (for the 200 nm spacing). In total, while the formed stack can only be an "ideal" quarter-wave stack in the green, this design allows for both high reflectivity and excellent spectral tuning of the reflection color across the entire near-UV to near-IR range. Variations in cytoplasm thickness may be metabolically controlled, raising the intriguing possibility that individual copepods are able to "tune" their colors.

The sophisticated strategy evolved by copepods to produce and manipulate their brilliant structural colors may serve as an inspiration for the construction of artificial photonic crystal structures with controlled characteristics.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and additional results. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b05289.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Eden Rosenfeld-Gur for her help in preparing the figures and schemes and the Israel Science Foundation (Grant 2012\224330*), the Crown Center of Photonics and the ICORE: The Israeli Excellence Center "Circle of Light", and the Interuniversity Institute for Marine Sciences for support. L.A. and S.W. are the incumbents of the Dorothy and Patrick Gorman Professorial Chair of Biological Ultrastructure and the Dr. Trude Burchardt Professorial Chair of Structural Biology, respectively.

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DOI: 10.1021/jacs.5b05289
J. Am. Chem. Soc. 2015, 137, 8408−8411