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Cuticular Microstructures and Their Relationship to Structural Color in the Shieldbug *Poecilocoris lewisi* Distant

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Abstract. The microstructures of the elytron cuticle of the shieldbug *Poecilocoris lewisi* Distant that exhibits a distinctive structural color are examined by optical reflectance measurement, optical microscopy, and scanning and transmission electron microscopies. The yellowish-green area and the reddish-brown area of the elytron are covered in common with a superficial light-blue color. This color results from the scattering of light by small, transparent cuticular microtubercles with a cone shape. The microtubercles have a light wavelength size small enough to produce the Tyndall or Mie scattering effect. The scattering is more effective for the short wavelengths of incident light than the long wavelengths. Colors ranging from yellow to green are due to the reflectance of the multilayers system and dark pigment backing layer that acts as an absorber of the transmitted light. However, the reddish-brown striped area has a lower number of multilayers and lacks the dark pigment layer. The numerical models accurately predict the experimentally observed coloration.

1. Introduction

The colorful display due to the structural coloration in insects has more recently attracted the attention of biologists than the color resulting from the pigment (FOX, 1976; CHAPMANN, 1978; PARKER, 2000). Structural colors are caused by the interference, diffraction (HINTON, 1976), or scattering of light by arrays of minute, transparent, structure elements, such as multiple thin layers, grating, or particles; whereas pigmentary colors are due to the selective absorption by chemical substances. Accurate, detailed studies of the mechanisms of the structural colors significantly commenced with ANDERSON and RICHARDS (1942) following the introduction of the electron microscope.

The colorful iridescence and superficial light-blue of the dorsal surface has been observed in specimens of the shieldbug *Poecilocoris lewisi* Distant, a distinctive insect in Japan (TOMOKUNI *et al.*, 1993; KOSAKU and MIYAMOTO, 1994). The dorsal surface of *P. lewisi* is made up of a yellowish-green area, a reddish-brown area of stripes, and a light-

blue covering common to both areas. The superficial light-blue color has especially attracted our interest.

MASON (1923) advocated that the main criteria for the recognition of Tyndall colors are: (1) no pronounced change in hue or intensity when viewed from different angles; (2) reflected (scattered) light blue to bluish white; transmitted light reddish brown to yellowish; (3) presence of minute structures with dimensions less of than 0.4 μm, and different index of refraction from that of the surrounding medium; (4) scattered light more or less polarized; vibrations in a plane perpendicular to the direction of the incident beam. The light-blue of *P. lewisi* meet almost these criteria and, although one needs to confirm this effect based on other observations and experiments.

In a few insects, Tyndall blue occurs in the epidermal cells beneath a more or less transparent cuticle, e.g. in several dragonflies (VERON, 1973), and in the grasshopper *Kosiuscola* (FILSHIE *et al*., 1975). Some dragonflies (MASON, 1926) and some other adult insects develop a waxy bloom on the surface of the cuticle after emergence. The Tyndall effect produced by this waxy material is destroyed by washing with a wax solvent (PARKER, 2000). The only recorded Tyndall blue in the cuticle occurring in the larvae of some tent caterpillars (BYERS, 1975), to the best of our knowledge, is the color due to the transparent cuticular filaments.

In this study, we tried to elucidate the mechanism of coloration in *P. lewisi* based on spectrophotometric measurements and microscopic observations of the cuticle structures at several levels of magnification.

2. Materials and Methods

2.1. Specimens

The specimens used in this study were the shieldbug *Poecilocoris lewisi* Distant collected from several sites on the Dokkyo University School of Medicine campus and surrounding areas. No sexual differences in coloration were apparent between either individuals. They were placed in a box on the day of capture where they remained living until examination in the laboratory. The dead individuals were fixed in 5% formalin solution then preserved in 70% ethanol.

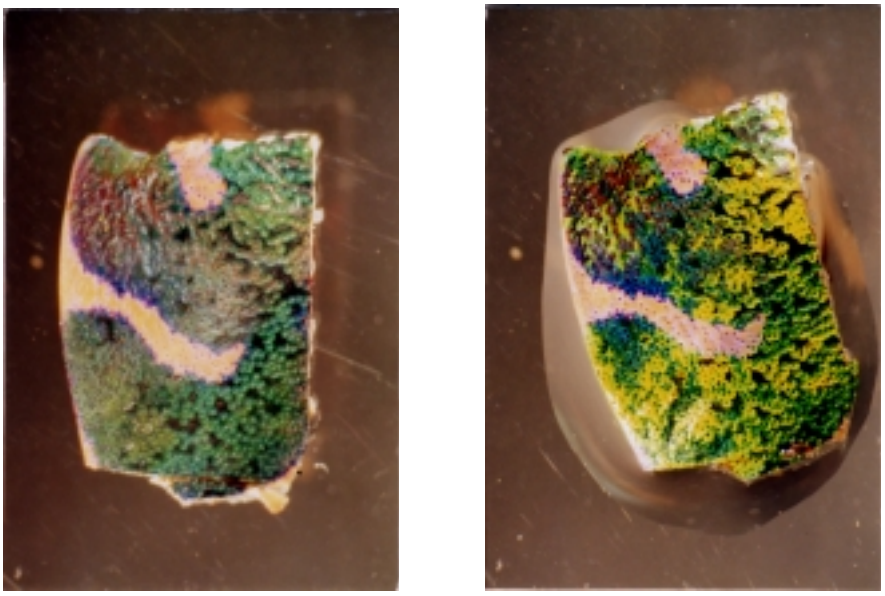
2.2. Coloration

The coloration of the dorsal surface of *P. lewisi* (adults) consists of a yellowish-green area including gold metallic brilliance on the main parts and reddish-brown stripe area placed mainly on the border of the elytra and pronotum makeup (Fig. 1). Both areas were slightly covered by a light-blue color. The color of the yellowish-green area changes from green to yellow or to gold with the angle of viewing, while the color of the reddish-brown area and the light-blue did not change when viewed from different angles. Unlike the reddish-brown and light-blue, the yellowish-green colors were destroyed when the specimen was dried but returned again when it was wetted (Fig. 2).

2.3. Optical spectrometry and microscopy

Pieces of the cuticle (about 5×5 mm^2) extending from the yellowish-green area to the reddish-brown area were removed from the elytron and cleaned of all adhering tissues.

Fig. 1. Dorsal surface view of shieldbug *Poecilocoris lewisi* Distant, photographed when the surface is wet. The individual is 15 mm long.



(a) (b)

Fig. 2. A part of cuticle removed from elytron. (a) dried state. (b) soaked state. Surface light-blue is observed both the states and both coloration areas.

Reflectance measurements of the piece in the visible and near ultraviolet spectral range were made using a Shimazu RF-5000 spectrofluorophotometer equipped with a recorder. The measurements were carried out in the five states of cuticle conditions from immediately after the cuticle soaked with water, 2 min after the soaking, 5 min after the soaking, 10 min after the soaking, and fully dried.

For examination by light microscopy, some pieces of the cuticle were dehydrated and cleared. Semi-thin (0.5–1.5 μm) sections were obtained from the pieces of cuticle embedded in resin. The sections were attached and dried on glass slides, stained with alkaline methylene blue (1% in 1% aqueous borax).

2.4. Scanning electron microscopy

The pieces were washed in ethanol, air dried, and mounted on specimen stubs. The specimens were gold coated (approximately 10 nm thick) in a direct current sputtering device. Micrographs of the cuticle surface were taken using a scanning electron microscope (JSM-25S, JEOL, Tokyo, Japan).

2.5. Transmission electron microscopy

Small pieces (1.0 to 5.0 mm^2) extending from the yellowish-green area to the reddish-brown area were removed from the elytra, then immediately immersed in a fixative mixture consisting of: (a) 10% paraformaldehyde solution, (b) 2.5% glutaraldehyde solution, and (c) phosphate buffer at pH 6.7–7.0. After 2 hours in this fixative, the pieces were washed in phosphate buffer and postfixed in 2% OsO_4 solution in the same buffer. Dehydration was carried out as usual in a water-ethanol series. The pieces were then finally embedded in resin (Epon 812). The sections were cut perpendicular to the dorsal surface with an LKB Ultrotome and mounted on the grids. The tissue was double stained with uranyl acetate and lead citrate. The fine cuticle structure was observed using a transmission electron microscope (JEX-100, JEOL, Tokyo, Japan).

3. Results

The structural colors in the yellowish-green area are iridescent or brilliant, and vary in appearance based on the viewing angle. The intensity is significantly reduced and the colors change to a dull brown when the surface of the cuticle is dried or the individual is died. If the dried state is, in turn, restored to a wetted state using water, the colors immediately regain their original intensity. The color of the reddish-brown stripe area seems to originate in the pigment of the cuticle, because the color does not change by the viewing directions and irrespective of its wetness. The light-blue covered commonly reddish-brown stripe area as well as the yellowish-green area are flat, i.e. not shiny or iridescent, and do not change in hue when viewed from different angles. The intensity of the light-blue remains even if the cuticle is dried. For the transmitted light passing through the cuticle, which has a thickness of about 110 μm, the color from the reddish-brown area is reddish brown, while the color from the yellowish-green area is dark reddish purple, the complementary color.

The results of the spectrophotometric analysis of light reflected and scattered from the yellowish-green area including the reddish-brown stripe area in *P. lewisi* elytron are shown

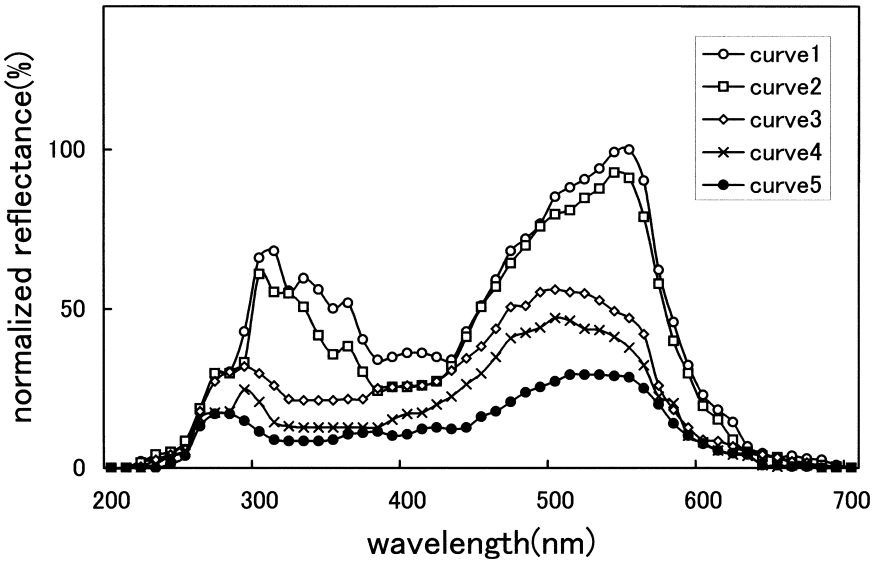


Fig. 3. Reflectance spectra of *P. lewisi* elytra cuticle: curve 1, immediately after soaked by water; curve 2, 2 min after the soaking; curve 3, 5 min after the soaking; curve 4, 10 min after the soaking; curve 5, fully dried.

in Fig. 3. The measured values of the reflectance of the specimen in the water-containing condition (Fig. 3, curve 1) are the highest then the values gradually decrease (Fig. 3, curve 2 to 4) and reach a fully dried condition (Fig. 3, curve 5) that is the lowest. The apparent reflection of the specimen in the wetted condition extends to the regions from about 450 nm (blue) to 580 nm (orange) with the maximum 550 nm (yellow-green). Another reflectance peak of 310 nm is found in the ultraviolet region extending from 280 nm to 380 nm. These reflectance peaks decrease to about 29% and 13%, respectively, when the specimen is completely dried. There are small peaks or flat parts between two major peaks of the spectra in Fig. 3. These are likely to be detected the light-blue color in the dorsal surface.

An optical micrograph of the semi-thin section of the elytron cuticle including both the yellowish-green area and reddish-brown area is shown in Fig. 4. The border of two areas is around the middle of this image. The epicuticle including the exocuticle (the surface layer of the cuticle) is about 5.9 μm thick on both areas. The cuticle of the reddish-brown area (Fig. 4, left of the middle) is devoid of a pigment layer in the exocuticle, whereas the cuticle of the yellowish-green area (Fig. 4, right of the middle) has about a 4.5 μm thick dark pigment layer on the bottom of the exocuticle. It is possible that the pigment is made up of closely spaced, irregularly shaped, and dark brown granules (BYERS, 1975).

The elytra has an uneven surface which consists of convex shape with 130–250 μm wide and with vertical interval of 12–18 μm. The angle of elevation of the surface from horizontal plane lies in the range between 0 to about 27 degrees.

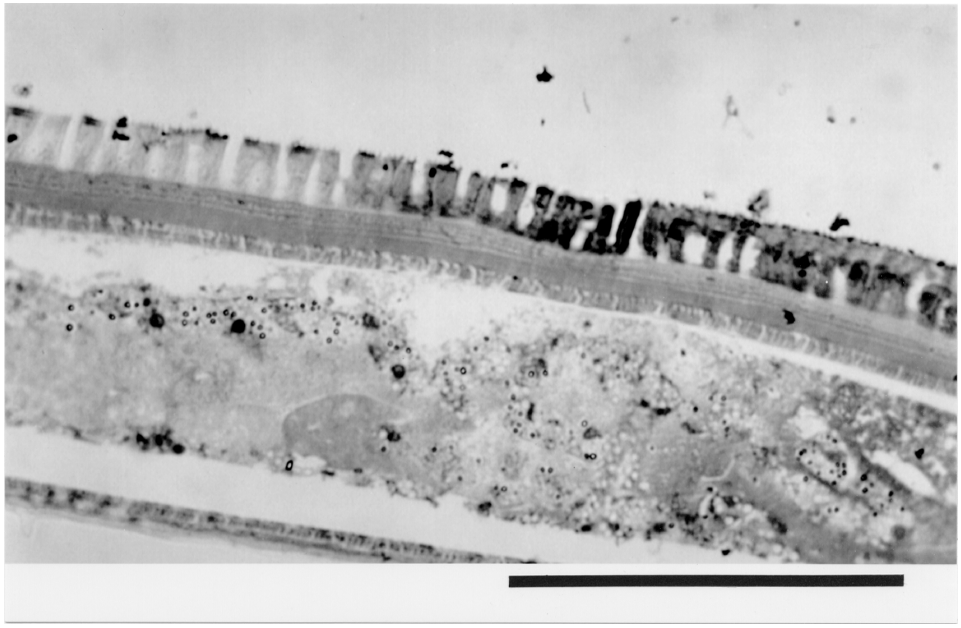


Fig. 4. Optical micrograph of semi-thin section of cuticle from elytron of *P. lewisi*. The border of reddish-brown area and yellowish-green area is in the middle. Pigment layer is seen at the bottom of exocuticle of yellowish-green area (right side of the middle) as a dense distribution, but it is not seen in reddish-brown area (left side of the middle). Stained with alkaline methylene blue. Scale bar represents 40 μm. (An artifact formed during the section preparation is seen as the longitudinal cracks in the epicuticle and the exocuticle.)

The scanning electron microscope image shows that the entire surface is covered with microtubercles with conical shapes having dull points, about 900 nm in height, 224 nm in diameter at the tip, 500 nm in diameter at the end, and 362 nm in average diameter (Fig. 5). The microtubercles are distributed at about 1 μm intervals (or 4.4×10^6 mm^2) of the surface of both the yellowish-green area and reddish-brown areas of the epicuticle. The microtubercles, the thickness of which is comparable to the wavelength (400 nm to 700 nm) of light and distributed irregularly on the cuticle surface, will scatter a light of particular wavelength and cause the cuticle surface to be superficially colored. The numerical relationship between the microtubercle sizes and scattered light wavelength will be discussed below.

The transmission electron micrographs of the ultrathin perpendicular section of the cuticle show the multilayer reflectance system of the yellowish-green area (Fig. 6(a)) and reddish-brown area (Fig. 6(b)). The former multilayer reflector consists of about 20 thin layers; a single thin layer approximately 110 nm thick and separated by 80 nm body fluid filled gaps. In contrast to the former refrector, the latter reflector consists of about 5 thin layers; the thickness of the single thin layer is 75 nm in thickness and has a gap of 75 nm.

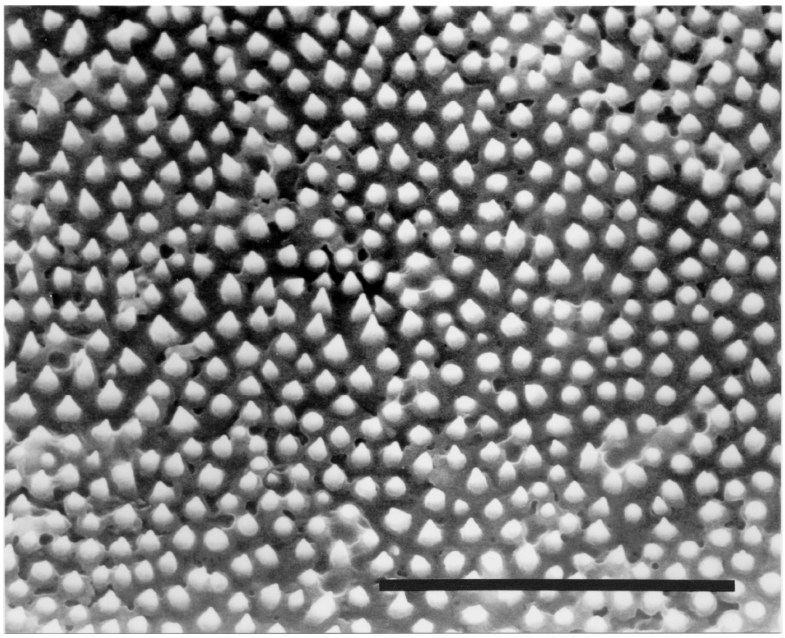


Fig. 5. Scanning electron micrograph of the cuticle surface of *P. lewisi*. The cuticle bears conical microtubercles with dull point existing commonly both of cuticle areas. Scale bar represents 10 μm.

4. Discussion

Scattering of light occurs when a beam of light passes through a transparent medium or a vacuum containing randomly dispersed particles of different refractive indices. When the particle sizes exceed about 1,000 nm, the scattered light is white rather than blue. However, when the particle sizes are in the range between 100 nm to 1,000 nm, blues and violets of high intensity are produced.

In a few insects, a blue color due to this scattering effect were found although widely known to occur in some dragonflies (FOX and VEVERS, 1960), tent caterpillars (BYERS, 1975), and grasshoppers (FILSHIE *et al.*, 1975). This effect used to be called "Tyndall blue" by many investigators of the structural color of insects. However, to examine the relationship between particle sizes and the spectrum of scattering light, it is more general to adopt the Mie scattering theory when the particles have dimensions about equal to or somewhat less than the wavelength of blue light, that is, about 400 nm.

The scattering theory for spherical particles has been described in textbooks (such as VAN DE HULST'S, 1957; BOHREN and HUFFMAN, 1998). However, the final results are listed below to explain the terminology used herein. The total efficiency factor of Mie scattering for an incident plane wave is defined as

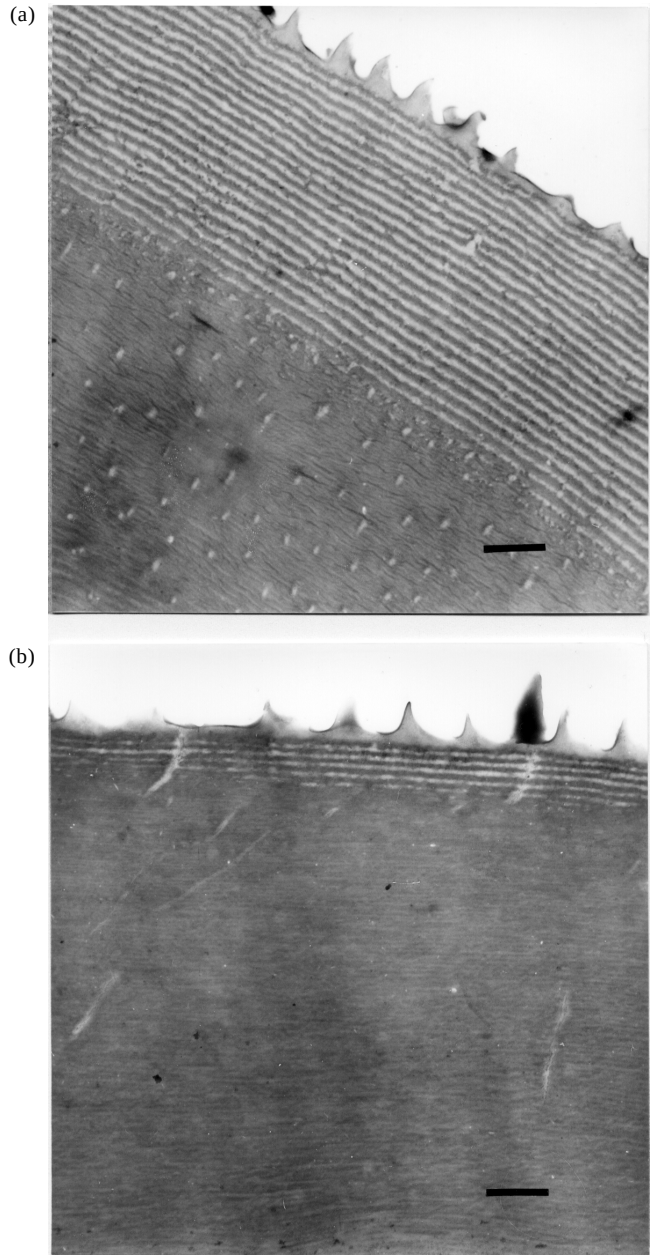


Fig. 6. Transmission electron micrographs of ultra-thin section of cuticle. (a) Multilayer system of yellowish-green area cuticle consists of about 20 thin layers and 110 nm thick of a thin layer, and 80 nm space gap. (b) Multilayer system of reddish-brown area cuticle consists of about 5 thin layers; 75 nm thick of thin layer, and 75 nm space gap. Scale bars represent 1 μm.

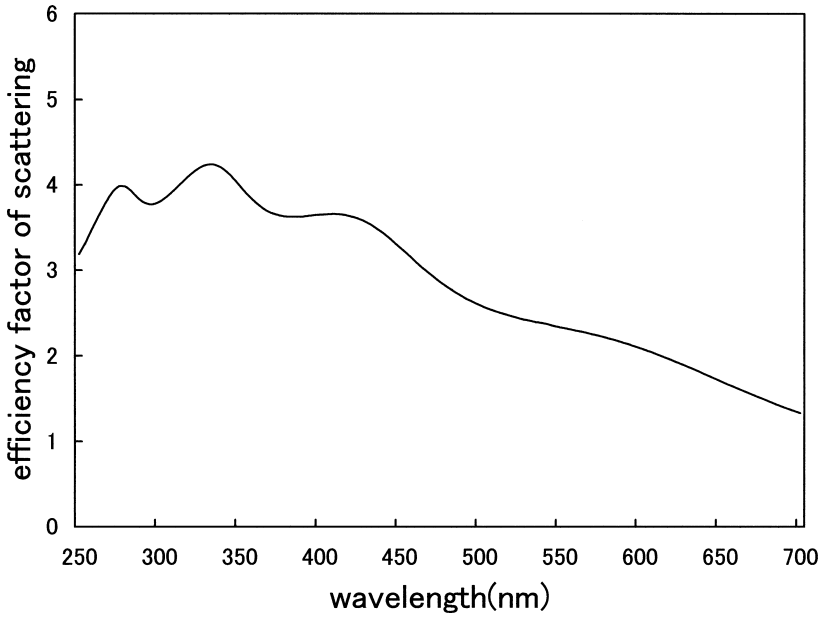


Fig. 7. Efficiency factor of scattering vs. incident light wavelength. The calculations are carried out on the conditions that the particle diameter, 362 nm, complex refractive index of the particle, 1.58 + 0.01*i* (chitin), and that of the surrounding medium (air), 1.00.

$$K^{(s)} = \frac{2}{\alpha^2} \sum_{m=1}^{\infty} (2m+1)\left[|a_m|^2 + |b_m|^2\right], \tag{1}$$

where $\alpha = \pi d/\lambda$ is the size parameter of a particle, d is the diameter of a particle, λ is the wavelength, and a_m and b_m are defined as the Mie coefficients, and m is an integer. The efficiency factor of scattering is defined as the ratio of the scattering cross section to the geometrical cross section of the particle, $\pi d^2/4$. The factor is in the range between 0 and about 4. The calculation of $K^{(s)}$ can be accomplished by introducing the series expansions of spherical Bessel and of the spherical Neumann functions into the Mie a_m and b_m coefficients (PENNDORF, 1962).

A computer program has been provided in a web site (BERNHARD, 2000) to calculate the coefficients or spectra associated with Mie scattering theory. The program is based on the condition that the optical scattering from a single homogeneous spherical particle illuminated by a plane incident light wave. In this program we used the real refractive index of 1.58 with a 0.01 value for the imaginary part of the spherical particle and the refractive index of 1.0 for the surrounding air. The microtubercles of the epicuticle surface are made of cuticle-containing chitin; the real index of refraction of 1.58 in the visible range is for the chitin. The spherical particle diameter is set at 362 nm as an average of the diameter of

the microtubercles with a conical shape. Figure 7 shows that the $K^{(s)}$ values calculated from Eq. (1) for the single-scattering properties of a spherical particle in air as a functions of the incident wavelength, λ. The scattering curve in the visible light region increases as the wavelength becomes shorter, which results in the blue coloring, as indicated by the blue coloring of the *P. lewisi* dorsal surface (Fig. 7). The peak of the scattering occurs at approximately 335 nm, which is in the ultraviolet region. This is in good agreement with the experimental result in the range of ultraviolet to the shorter wavelength of visible light.

The explanation why the dorsal surface is commonly colored light blue to yellowish-green or reddish-brown although both have a Mie-scattering surface, can be tested either directly by reflectance spectrophotometry or theoretically.

Light may be strongly reflected by constructive interference between reflections from the different interfaces of a stack of thin layers with alternately high and low refractive indices. For this to occur, reflections from successive interfaces must emerge with the same phase. In a multilayer consisting of a large number of layers, the refractive index of the medium (n_a) with the thickness (d_a) and surrounding medium of refractive index (n_b) with the thickness (d_b), the central wavelength (λ) due to constructive interference of the reflected waves at normal incidence is expressed by the equation:

$$\lambda = 2(d_a n_a + d_b n_b). \tag{2}$$

The multilayer system of *P. lewisi* elytra for the case of 20 layers is about 3.8 μm thick. Substituting the refractive index, $n_a = 1.58$, the thickness of a single layer, $d_a = 110$ nm, the refractive index of water $n_b = 1.33$ and $d_b = 80$ nm into Eq. (2), we obtain the central wavelength $\lambda = 560$ nm when viewed from the angle of 0 degree. The colors of these wavelengths correspond to yellow or orange.

The elytron has a fine uneven surface of each unit with convex shape and with an elevation angle of 0–27 degrees to the entire surface. For the oblique angle of incidence, which is same as the angle elevation, the wavelength of light that constructively interferes will be shorter than that for light at normal incidence. Therefore, as the angle of elevation changes, the observed color also changes from 560 nm to 500 nm. Assuming that a convex surface approximates a hemisphere, the area at which a light is incident of oblique angle θ made by the normal line of the surface with the line normal to the zenith is proportional to sin θ. Therefore, the intensity from the surface of an oblique angle will be greater than one at normal incidence, and the shorter wavelength (500 nm, green) predominates in the mixed color from the entire elytron of yellowish-green. It is considered that the underlying dark pigment layer sustains to make this color due to the interference conspicuous.

These estimations are almost consistent with our observations and the spectrum of the curve 1 in Fig. 3. A difference in the thickness of the layer provides a change in the color observed from unidirectional polychromatic light. However, in the multilayer system with 20 high index layers, reflection efficiencies can reach almost 100% and (LAND, 1972) highly metallic and very purified colors are expected to appear.

At the reddish-brown area of the elytra, the multilayer system with a 750 nm thickness consists of five layers. Substituting a single layer of about $d_a = 75$ nm in thickness with the refractive index, $n_a = 1.58$, and the space between the layers, $d_b = 75$ nm, with the refractive index, $n_b = 1.33$, into Eq. (2), we obtain the central wavelength of 436 nm at normal

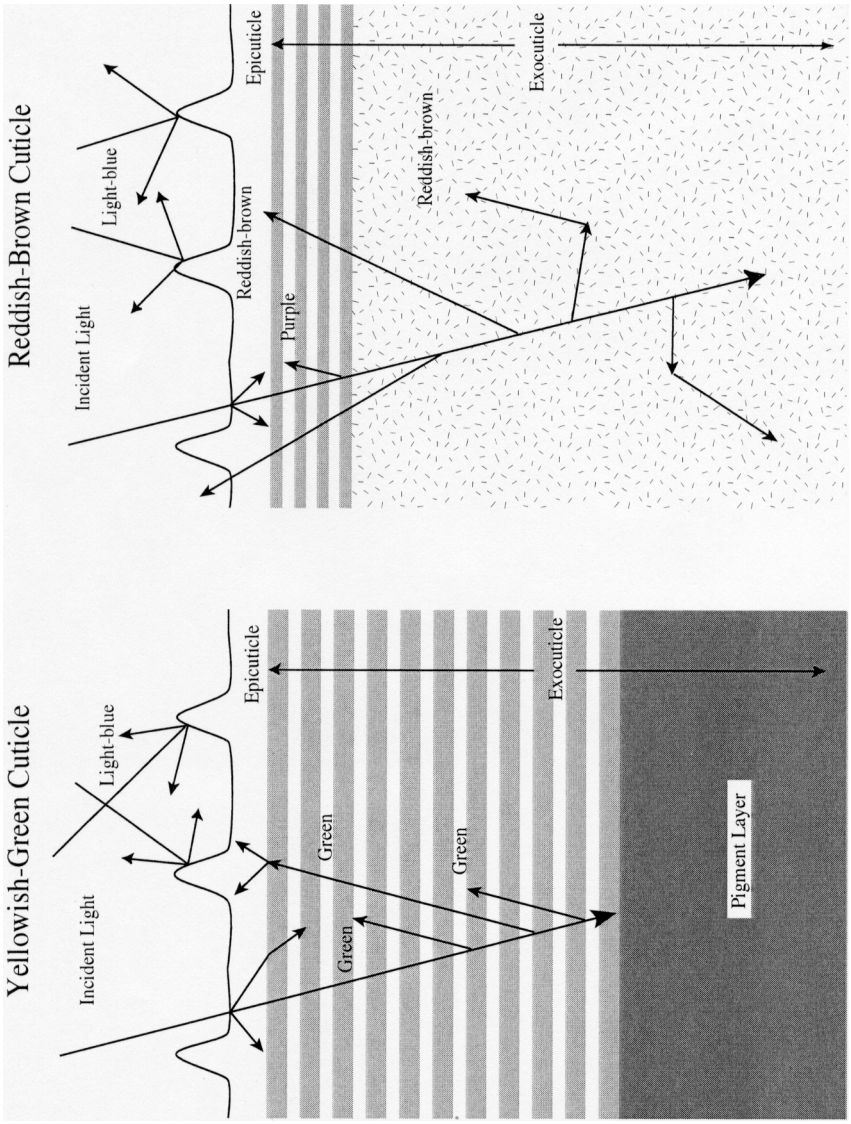


Fig. 8. Diagram depicting the scattering and reflectance of light from (a) cuticle of yellowish-green area and (b) cuticle of reddish-brown area.

incidence and 389 nm at the oblique angle of 27 degrees incidence. The major color of this constructive interference corresponds to purple. But this color may be inconspicuous, because it is under the cover of the reddish-brown of cuticular pigment color and there is no dark pigment layer beneath the epicuticle. Furthermore, the wavelength of purple is close to that of light-blue due to Mie scattering by the surface microtubercles so that two colors are hard to distinguish each other.

The significant differences between the yellowish-green and reddish-brown areas are the number of multiple thin layers, the thickness of the layers, the distances between the layers, and the presence of a dark pigment layer beneath the multilayer system in the exocuticle. This pigment absorbs the light transmitted through the epicuticle with microtubercles and the multilayer system and thereby provides a dark background against which the scattered light and reflected light are conspicuously viewed (Fig. 8(a)). The transmitted light through this cuticle is a complimentary color, dark reddish purple that is the color removed the yellowish-green color of the cuticle from white light. In the area of the elytron cuticle which has a lower number of multiple thin layers and lacks a background dark pigment layer, the scattered light due to surface microtubercles also appears light-blue. However, the light of the constructive interferences due to a mere five multilayer system has a low intensity of purple in color. The visual color is the mixture of the pigment reddish-brown color, the superficial light-blue, and the purple (Fig. 8(b)). The latter two colors have near wavelengths and low intensities. Therefore, the transmitted light through the reddish-brown cuticle mainly appears the same color of the cuticle.

Visual observations (Fig. 2) and the reflectance spectrophotometric measurements (Fig. 3) on the yellowish-green area in *P. lewisi* show that the elytron cuticle changes its color to dull brown when dried. In many insects, such as scarab beetles and butterflies, with structural color arising from multiple layer interference, the original colors are usually preserved after they died and the cuticles dried. The fact that iridescent cuticles of some scarab beetles have been used to decorate ancient oriental crafts suggests that these insects possess permanent colored and tough structured cuticles. The cuticle surface of the shieldbug is very fragile such that the exocuticle together with the epicuticle is easily scraped with a knife and the surface of the endocuticle is bare. The color of the scraped cuticle is not yellowish-green, but reddish-brown which is the pigmentary color of the cuticle. In butterflies, layers are often supported by vertical vanes of the wing scales. Air fills in the spaces and provides the alternate layers of the interference system (ANDERSON and RICHARDS, 1942). Under illumination by white light at normal incidence, the iridescent blue color of the butterfly *Arhopala micale* turns to iridescent green when the air is replaced by acetone. The replacement of the air space with a medium causes a color change due to the alteration of the interference condition, but it is not connected to the dullness of the color. It will be appreciated that the color change of the *P. lewisi* cuticle, unlike the cuticles of the scarab beetles, is attributed to the loss of the body fluid and the shrinkage of spaces in the multilayer until adjacent layers contact. The iridescence color changes to a dull brown or mixture of various colors. The reason why the dried cuticle restores the iridescent color when soaked in water is explained in terms that the water permeates into and fills the spaces of the multilayer of dried cuticle, and forces the spaces to spread until the original intervals are regained.

5. Conclusion

The diverse coloration of the shieldbug dorsal surface is due to the multiple thin layer structures in the exocuticle, tremendous number of microtubercles on the surface of the epicuticle, and dark pigment layer underlying the exocuticle. In sunlight, the combination of the multilayer system and the dark pigment layer produces a bright iridescence of yellowish-green. On the other hand, the stripe area, which has a lower number of multilayer systems and lacks the dark pigment layer appears reddish-brown, the cuticle color itself, and the emergent light is scattered in the cuticle. The microtubercles overlaying both areas causes the scattering of the wavelength at the blue end of the spectrum (Tyndall blue or Mie scattering), then a subtle hue is added to the iridescent structural color as well as the pigment color. The shieldbug possessing the Tyndall color is an unusual insect in a sense that the scattering structure responsible for the Tyndall effect is the true cuticular that processes neither aggregations of secreted substances nor epidermal cells containing colorless granules.

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