Mechanism of variable structural colour in the neon tetra: quantitative evaluation of the Venetian blind model

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The structural colour of the neon tetra is distinguishable from those of, e.g., butterfly wings and bird feathers, because it can change in response to the light intensity of the surrounding environment. This fact clearly indicates the variability of the colour-producing microstructures. It has been known that an iridophore of the neon tetra contains a few stacks of periodically arranged light-reflecting platelets, which can cause multilayer optical interference phenomena. As a mechanism of the colour variability, the Venetian blind model has been proposed, in which the light-reflecting platelets are assumed to be tilted during colour change, resulting in a variation in the spacing between the platelets. In order to quantitatively evaluate the validity of this model, we have performed a detailed optical study of a single stack of platelets inside an iridophore. In particular, we have prepared a new optical system that can simultaneously measure both the spectrum and direction of the reflected light, which are expected to be closely related to each other in the Venetian blind model. The experimental results and detailed analysis are found to quantitatively verify the model.

Keywords: tunable colour; colour change; iridophore; fish colouration

1. INTRODUCTION

Butterfly wings and bird feathers are well-known examples of structural colour in nature [1]. Some of them possess very high reflectance with saturated colours that are produced by highly sculpted microstructures [2–5]. In these examples, the microstructures mainly consist of chemically stable materials such as dried cuticles in butterfly wings or keratin and melanin granules in bird feathers. Thus, the brilliancy of these structural colours can last for a very long time unless the microstructures are destroyed.

On the other hand, some fish have very different structural colours from those of butterfly wings and bird feathers: the colour can vary depending on the conditions of the surrounding environment. Such colour changes have been reported for the Atlantic killifish (*Fundulus heteroclitus*, [6]), the cardinal tetra (*Paracheirodon axelrodi*, [7]), the blue damselfish (*Chrysiptera cyanea*, [8]), the common surgeonfish (*Paracanthurus hepatus*, [9]), the paradise whiptail (*Pentapodus paradiseus*, [10]) and so on. Physiological colour change is possible because colour-producing microstructures are inside living cells, called iridophores, which are motile.

From the view point of biomimetics, it seems quite interesting to learn how these fish realize the colour tunability, since such a property is expected in various types of tunable optical applications like lasers,

band-pass filters and laser-reflecting mirrors. In fact, an attempt has recently been made to synthesize materials that have submicron periodic structures with the capability of mechanical transformation [11,12]. However, the exact mechanism responsible for the change in structural colours of fish has not been fully understood, even for one of the most striking examples, the neon tetra (*Paracheirodon innesi*).

The lateral stripe of the neon tetra looks brilliant blue in the day time, while the colour changes to deep violet at night [13]. It can even assume a yellow colour when the fish is excited or under stress [14]. The microstructures inside the iridophores forming the lateral stripe have been already revealed by electron microscopy [13,15]. As illustrated in figure 1, it is known that one iridophore contains mainly two, occasionally one or three, stacks of thin platelets of guanine crystals. Since the platelets are observed to be periodically stacked, it is indicated that multilayer interference phenomena are the origin of the structural colour; a multilayer system can selectively reflect light whose wavelength satisfies the interference condition [16,17]. In fact, Lythgoe & Shand [13] experimentally confirmed the correspondence between the spacing of the platelets and the wavelength of the reflected light. Thus, there is now little doubt that the stack produces structural colours through multilayer optical interference phenomena. Consequently, the colour variation is thought to originate from the varying thickness of the cytoplasm part.

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Figure 1. Schematic of the (a) neon tetra and (b) iridophore (reproduced from [15] with permission). The lateral stripe of the neon tetra consists of many iridophores, which are arranged like pavement tiles. Under a higher magnification, it is observed that a single iridophore contains mainly two stacks of the thin light-reflecting platelets (RPs). The nucleus is located in the lower part of the iridophore below the stacks of the platelets.

As a mechanism of the spacing variation, Lythgoe & Shand [13,18] considered the inflow of water into an iridophore from the physiological experiments of changing osmotic pressure. The inflow of water may cause swelling of the cell and consequently increase the spacing. On the other hand, Nagaishi *et al.* [19] proposed a different model that is called the Venetian blind model, as shown in figure 2. In this model, the tilt angles of the platelets are assumed to be physiologically controlled: some filaments connect the platelets to motor proteins, which move on the microtubules and change the tilt angle, just like a Venetian blind of a window [20]. It is easily understood that the platelet tilt affects the platelet spacing (compare figure 2*a,b*).

The above two mechanisms are different with respect to the relationship between the wavelength and direction of the reflected light: the Venetian blind model expects that the direction of the reflected light varies during the colour change owing to a change in the tilt of the platelets, while the simple swelling of an iridophore affects neither the platelet angle nor the direction of the reflected light. In fact, Nagaishi *et al.* [19] have reported several optical observations that qualitatively indicate a variation in the direction of the reflected light. However, in their observations, the simultaneous illumination of many iridophores made it difficult to directly confirm the change in the tilt angle of the platelets inside a single stack.

Further, it should be pointed out that the above two mechanisms, the swelling and the tilt angle variation, are not mutually exclusive with each other; they can coexist, in principle, in an iridophore to produce colour variability. Therefore, optical investigations into a single stack are necessary to quantitatively evaluate the adequacy of the Venetian blind model. In this study, we have performed detailed optical

Figure 2. Venetian blind model. The longitudinal section of an iridophore is schematically illustrated. There exist many light-reflecting platelets that are tilted at an angle θ with respect to the basal plane of the cell. (a,b) Two different tilt angles. As indicated by a pair of arrows, the spacing between the adjacent platelets is larger in (b) than that in (a). Another difference is noted in the direction of the reflected light. (c) A case when the entire iridophore is tilted by an angle α with respect to the optical axis of the microscope. In fact, our experiments are performed with non-zero α so that the reflected light appears around the centre of the far-field pattern. The inset in (b) shows the definitions of the parameters of the multilayer interference phenomenon. The thicknesses (refraction angle) of the guanin platelet and cytoplasm part are denoted by d_g (ϕ_g) and d_c (ϕ_c), respectively. Further, a constant parameter $D = (d_g + d_c) / \sin \theta$ is introduced to represent the distance between two adjacent platelets located on the bottom (or top) surface of the stack.

investigations on the structural colour of the neon tetra in order to quantitatively evaluate the validity of the Venetian blind model as the mechanism for colour variability. To this end, we have devised an optical set-up that can simultaneously function both as a microspectrophotometer and as a microscatterometer so that we can obtain information on the spectrum and direction of the reflected light, respectively.

2. MATERIAL AND METHODS

2.1. Sample preparation

Neon tetra were purchased from a local commercial source (Suita, Osaka) and kept in an aquarium until

experiments commenced. The sample skin of the stripe part was prepared in a manner similar to previously published methods [21]. Briefly, after a fish was decapitated in Ringer solution, the scales on the outer surface, as well as the internal muscle fibre and the oil droplets, were removed very carefully so as to avoid damaging the iridophores. A colour change was induced in the iridophores by replacing the Ringer solution with one containing a higher concentration of potassium ions, according to a previous study [21].

The skin preparation was placed on the bottom surface of a sample chamber. A few short fragments of a thick fishing line were used to hold the sample: one end of a fragment was firmly attached by adhesive to the chamber's bottom surface and the sample was sandwiched between the fragment and the bottom surface of the chamber so as to be gently held by the elastic force of the fishing line. As shown in figure 3, the sample chamber was placed on a goniometer having two rotational degrees of freedom, by which the skin preparation could be tilted with respect to the optical axis of the microscope.

2.2. Microspectrophotometer

Since the stack of the platelets inside the iridophore was typically $15\text{ }\mu\text{m}$ in width and $40\text{ }\mu\text{m}$ in length, a microspectrophotometer was necessary to obtain spectra for such a small region. We used an optical microscope (Olympus BX51) equipped with a Xe lamp and a fibre-optic spectrometer (Ocean Optics Inc., USB2000) as described previously [22]. A water-immersion objective (Olympus, LUMPlanFLN $40\times$ W, NA 0.80) was mainly used for the observation and measurement. The spectrum of the reflected light was obtained for a circular region of $5\text{ }\mu\text{m}$ diameter, which was calculated using the diameter of the fibre ($200\text{ }\mu\text{m}$) and the magnification of the objective ($40\times$). As the reflectance standard, we employed a broad-band dielectric multi-layer reflector covering a wavelength range from 300 to 2000 nm (Sigma Koki, TFMS-30C05-3/20). Since the reflection from an iridophore was observed to be nearly specular, it was in principle possible to determine the quantitative reflectance. However, in practice, the reflected light intensity was found to be very sensitive to adjustments of the height of the mirror surface on the focal plane of the objective. Repeated measurements of spectral intensity with readjustments revealed an experimental error of 0.1. The images of samples were photographed using a CCD camera (Olympus, DP25).

2.3. Microscatterometer

The direction of the reflected light can be evaluated by observing the intensity pattern on the back focal plane of the objective, since this plane corresponds to the far-field pattern of reflection [23]. In the case of transmission illumination, a Bertrand lens is commonly used for observations in this plane. However, under epi-illumination, several additional optical components are necessary, as shown in figure 3. This type of optical apparatus is now called a microscatterometer [24]. The details of our setup are described below.

Figure 3. Optical system of a microscope that functions both as a microspectrophotometer and as a microscatterometer. Light from a Xe lamp is focused on a $100\text{ }\mu\text{m}$ diameter pinhole placed in the plane of the aperture stop (AS). The pinhole is imaged on the back focal plane of the objective lens so that the illuminating light becomes nearly collimated at the sample position. The real image of the sample is observed with a CCD camera (DP25). Using an optical fibre, the reflected light from a small part is guided into a spectrometer. Another CCD camera (PL-B954HU) observes the intensity pattern of the back focal plane of the objective lens through a beam splitter inside the dual-port unit (U-DP) and a macro zoom lens (model 54363). A pinhole of a $200\text{ }\mu\text{m}$ diameter is placed in the plane of field stop (FS) to restrict the illuminated area during the measurements. The lower right illustration shows that the sample chamber is placed on the goniometer attached to translation stages.

The white light from a Xe lamp was first focused on the plane of the aperture stop (AS), where a small pinhole with a $100\text{ }\mu\text{m}$ diameter was placed. The image of this pinhole was focused on the back focal plane of the objective so that the illuminating light became nearly

collimated at the sample position. When the sample had a specular surface, the reflected light was focused by the objective lens again on the back focal plane as a small spot. The intensity pattern of this plane was observed by a CCD camera (PixeLINK, PL-B954HU) through a beam splitter, which was placed inside a dual-port unit (Olympus, U-DP), and a macro zoom lens (Edmund, model 54 363).

To evaluate the assembled microscatterometer, we first used a piece of a flat glass plate as a sample, which was placed on a goniometer for tilting. It was verified that the reflection spot in the far-field pattern moved as expected from a change in the tilt angle. From the diameter of the observed spot, the aperture of the illuminating beam was estimated to be 2° in angle. On the other hand, when a diffuse white material was used as a sample, a large white circle appeared in the far-field pattern, since the light was scattered in every direction. The edge of this circle corresponded to the maximum angle $\theta_{\max}$ of the detectable range of the far-field pattern, which was determined by the numerical aperture (NA) of the objective. The angle $\theta_{\max}$ was calculated to be 35.7° by using the relation $n \sin \theta_{\max} = \text{NA} = 0.8$ with the refractive index $n = 1.37$ for cytoplasm. This circle was always used to estimate the reflected light direction from the position of the spot in the far-field pattern according to the sine condition for the objective lens.

To restrict the illuminated area to within a small part of a single stack, a $200\text{ }\mu\text{m}$ diameter pinhole was placed in the plane of the field stop, which approximately corresponded to a diameter of $20\text{ }\mu\text{m}$ at the sample position when the objective of $40\times$ was used. If this pinhole was too small, the illuminating beam became less collimated owing to optical diffraction. We confirmed that this effect was negligible for this pinhole diameter.

2.4. Measurements of platelet dimensions

The size and thickness of a single light-reflecting platelet were measured by using a commercial interference microscope (Ryoka Systems Inc., VERTSCAN 2.0). This microscope was able to quantitatively measure the surface profile with very high accuracy: the vertical resolution was less than 1 nm . In addition, an atomic force microscope (SII Nanotechnology Inc., SPA-400) was employed since it had a much better spatial resolution in the lateral direction. In these observations, the iridophores were dissected first, and a droplet of Ringer solution including many platelets was aspirated into a glass pipette and put on a glass slide. After the water evaporated, the surface profile of the platelet was observed using the above-mentioned two types of microscopes. It was proved that at least the lateral sizes of the platelets were independent of being dry or in watery condition.

3. RESULTS

3.1. Surface profile of a single light-reflecting platelet

First, we examine the surface profile of the light-reflecting platelets by using the interference microscope. As

Figure 4. The profile of a single light-reflecting platelet. (a) A colour image created by the height data obtained by using an interference microscope. The inset shows the same platelet observed by a normal optical microscope under epi-illumination (scale bar, $10\text{ }\mu\text{m}$). The height profiles are shown in (b,c), which are obtained along the longitudinal and cross sections, respectively, as indicated by the corresponding letters in (a).

shown in figure 4, one platelet has an elongated hexagonal shape. We have observed many platelets and found that the dimensions are rather distributed, but the platelets are typically $10\text{ }\mu\text{m}$ in length and $3\text{ }\mu\text{m}$ in width. Further, the thickness is not observed to be perfectly uniform within one platelet, but in cross section the platelet is tapered, as has been reported in the case of different fish [25,26]. Along the longitudinal direction, the sectional profile has a flat-top shape (figure 4b), while a round profile is observed in cross section (figure 4c). The thickest part of the 10 platelets examined has a thickness of $60 \pm 5\text{ nm}$ with two exceptions of 70 and 80 nm. These dimensions have been confirmed by measurements performed using the atomic force microscope.

3.2. Specular reflection from the iridophore

Next, we investigate the optical properties. Figure 5a–c shows microphotographs of several iridophores observed under epi-illumination. Each blue part corresponds to a stack of light-reflecting platelets, which are illustrated in figure 1. Figure 5d–f shows the corresponding observed far-field patterns. When a small part of a single stack is illuminated, which is indicated by a

Figure 5. Tilt angle dependence of the real image and the far-field pattern of the iridophores. By using the goniometer, the sample chamber is tilted through angles of 0° (*a,d*), 12° (*b,e*), and 22° (*c,f*). Photographs (*a–c*) show the real image of the same part, in which several iridophores containing many blue stacks of the platelets are shown (scale bar, $20\ \mu\text{m}$). The white circle shows the illuminated area when the far-field patterns are observed. The images (*d–f*) are the observed far-field patterns corresponding to (*a–c*). As the sample is tilted, the spot laterally moves as shown in (*e*) and goes out of the observable angular range in (*f*). The two inner dotted circles indicate the angles of 15° and 30° with respect to the optical axis of the microscope (the centre of the far-field pattern), and the solid circle indicates the maximum angle 35.7° of the detectable angular range. The plot (*g*) shows the relation between the tilt angle of the goniometer and the change in the angle of reflection. The line is drawn according to the general relation in specular reflection: the reflection angle is twice the tilt angle.

white circle in figure 5*a*, a small blue spot appears in the far-field pattern, as shown in figure 5*d*. The appearance of this small spot implies that the reflection is almost specular, but not perfectly. From the observed spot size, the angular range of reflection is estimated to be 4° , which is larger than the aperture of the illumination, namely, 2° . We have examined many stacks of different iridophores and sometimes observed an elongated spot shape, which indicates a poorer specular characteristic. However, the angular range is still less than about 10° in the elongated direction.

When the sample skin is tilted by 12° by using the goniometer for the sample chamber, the spot appears

in a different position in the far-field pattern, as shown in figure 5*e*, while the corresponding real image (figure 5*b*) does not exhibit any large differences from figure 5*a*. Further, when the sample is tilted by 22° , no light spot is observed in the far-field pattern (figure 5*f*). That is because the reflected light goes out of the aperture of the objective. Consequently, the real image of the stack looks almost completely dark, as shown in figure 5*c*. Figure 5*g* shows the experimentally observed relation between the tilt angle of the goniometer and the change in the reflected light direction. As generally expected in specular reflection, the reflection angle changes by twice the tilt angle.

Figure 6. Illuminated area dependence of the far-field pattern. (a–c) The same photograph of the real image of several iridophores. The white circle shows the illuminated area when the corresponding far-field patterns (d–f) are observed. (d–f) The white circle indicates the maximum angle of 35.7° of the detectable range. The experimental artefact of a white spot is noted in (e, f), which originates from the back surface of the objective. Scale bar, $20\ \mu\text{m}$ in (a–c).

3.3. Variation in the facing direction in different stacks

It is quite interesting to note that in figure 5c, some stacks look almost completely dark, while others are observed as bright as before the sample is tilted. This fact suggests that the platelet surfaces of different stacks do not face the same direction. We have confirmed this conjecture by observing the far-field patterns with different illuminated areas. As shown in figure 6, more reflection spots are observed as the illuminated area becomes larger. We have verified that these patterns do not result from disturbance of the skin surface, which might be caused during sample preparation, by observing similar far-field patterns with distributed spots in intact fish. The distributed spots clearly indicate that the platelets of different stacks are differently oriented, even though the stacks are located next to each other. The observed light spots are scattered in almost all the detectable angular range. This implies that the distribution in angle exceeds $\pm 15^\circ$ and that the orientational distribution widens the angular range of visibility of the brilliant blue stripes.

3.4. Optical properties during colour change

Next, we investigate the optical properties of an iridophore during colour change. In order to induce a colour change, the Ringer solution in the sample chamber is replaced with a solution having a potassium ion concentration 50 times higher than normal. As shown in figure 7a, the colour of the iridophore gradually changes from blue to yellow. Completion of this process requires about 10 min. However, the speed of colour change largely depends on the sample, since the penetration of the chemicals can be different depending on the

composition of the skin preparation, e.g. it may contain muscle fibres or oil droplets. With regard to the colour change, the reflectance spectra shifts into the longer wavelength region, as shown in figure 7c, roughly maintaining the spectral line shape. The spectra consist of a broad rectangle-like component accompanied by a few side peaks on the shorter wavelength side. The peak reflectance is observed to be as high as 0.7–0.8. We have examined many iridophores in different skin samples and obtained similarly high reflectance values.

In addition, the reflected light spot in the far-field pattern moves by about 10° during a colour change, as shown in figure 7b. This fact clearly demonstrates the change in the platelet angle. The spot looks small when the colour is blue, while it has an elongated shape when the stack colour turns into yellow. The red and green colours are also noted in the spot of the far-field pattern. These facts may indicate that the tilt angles of the platelets are distributed within a single stack, broadening the reflected light direction. In fact, the colour of the real image is not perfectly uniform within the stack when the colour is yellow.

From the spot position in the far-field pattern, it is possible to quantitatively determine the tilt angle θ of the platelets, which is defined as the angle between the platelets and the bottom (and also top) surface of the stack, as depicted in figure 2. In fact, we have first adjusted the angles of the goniometer such that the real image of the entire top surface in a single stack is clearly focused using a high-power objective (Olympus, LUMPlanFl 100 $\times$ W, NA = 1.0). Since this objective has a very shallow depth of field, the top surface of the stack is oriented perpendicular to the optical axis of the microscope. Then, the goniometer is tilted by an angle α , as depicted in figure 2c, so that the

Figure 7. Optical properties of the iridophore during colour change from blue to yellow. (a) Real image of the iridophore during the colour change. The red circle indicates the illuminated area for the measurements shown in (b,c) (scale bar, 20 μm). (b) The far-field patterns obtained corresponding to the real images shown in (a). The two inner dotted circles indicate the angles of 15° and 30° with respect to the optical axis, and the solid circle indicates the maximum angle 35.7° of the detectable angular range. (c) The reflectance spectra obtained corresponding to the images in (a,b).

reflection spot appears near the centre of the far-field pattern. From the angle α and the spot position in the far-field pattern, we can estimate the tilt angle θ .

Figure 8 shows the relation between the tilt angle θ of the platelets and the wavelength λ_c of the reflectance spectrum. The results of two measurements for different iridophores are shown. Since the spectrum has a broad rectangular component, the centre wavelength λ_c is used to characterize the reflectance spectrum, which is defined as the average of the two wavelengths where reflectance is at half maximum. On the other hand, the tilt angle θ is estimated from the centre of the elongated spot shape in the far-field pattern. The plot points in figure 8 clearly show that the wavelength increases with the tilt angle.

4. ANALYSIS

4.1. Relation between wavelength and tilt angle

The above experiments show that the direction of reflected light varies with the colour change. Here, we

investigate whether the Venetian blind model quantitatively describes this variation. We consider a tilted multilayer system that consists of guanine and cytoplasm layers, as shown in the inset of figure 2b, whose thicknesses (refractive indices) are denoted by d_g (n_g) and d_c (n_c), respectively. The wavelength λ_p that satisfies the constructive interference condition is expressed by the following relation [17]:

$$m\lambda_p = 2(n_g d_g \cos \phi_g + n_c d_c \cos \phi_c),$$

where m denotes an integer; and ϕ_g (ϕ_c), the refraction angle inside the guanine (cytoplasm) layer. When the iridophore is tilted by an angle α , as illustrated in figure 2c, the relation $\phi_c = \theta - \alpha$ holds under the assumption that the illumination is along the optical axis of the microscope (the vertical direction in figure 2). In addition, since the layer spacing d_c varies with θ , a constant parameter D is introduced with the definition $D \sin \theta = d_g + d_c$, which is the distance between the platelets along the bottom surface of the stack. Using the above relation and Snell's law, n_g

Figure 8. Experimentally obtained relation between the centre wavelength λ_c of the rectangular component in the spectrum and the tilt angle θ of the platelets. The results of two measurements for two different iridophores are shown by triangles and circles. The curves are drawn according to relation (1) for the parameters $D = 500, 570, 670, 800$, and 1100 nm from the top to bottom. The parameter value $\alpha = 11.3^\circ$ is used, except for the curve with $D = 570$ nm where α is 12.0° . See text for the other parameters. When the shorter wavelength edge of the rectangular component cannot be observed owing to the limitation on the spectral range, λ_c is determined from the edge of the longer wavelength side and the approximate half width of the rectangular component that is estimated from other spectra.

$\sin \phi_g = n_c \sin \phi_c$, we finally obtain the relation between θ and λ_p as

$$m\lambda_p = 2 \left(d_g \sqrt{n_g^2 - n_c^2 \sin^2(\theta - \alpha)} + n_c(D \sin \theta - d_g) \cos(\theta - \alpha) \right). \quad (4.1)$$

We have drawn this relation in figure 8 for several values of D with the parameters $m = 1$, $n_g = 1.83$, $n_c = 1.37$ and $d_g = 60$ nm. The values of the refractive indices are taken from previous studies [26], and the thickness d_g has been determined by the surface profile measurement. The tilt angle α is known from readings of the goniometer to be 11.3° or 12° , depending on the experiments.

The curve-fitting analysis using the above equation reveals that $D = 570$ and 670 nm give reasonable agreement with the two different measurements, as shown in figure 8. Here, we emphasize that D is the only parameter in the fitting procedure. From this reasonable agreement, we can say that the Venetian blind model quantitatively explains the variation in the angle and the wavelength of the reflected light. We have repeated similar measurements and analysis five times for different iridophores and have confirmed that the Venetian blind model always reproduces the experimental results. However, the determined parameter D ranges from 570 to 740 nm, indicating inhomogeneous characteristics of the iridophores.

Figure 9. (a) Example of an experimentally obtained reflectance spectrum from a small part of a yellow-coloured stack. (b) Theoretical reflectance spectrum calculated with the parameters $d_g = 60$ nm and $d_c = 155$ nm. These thicknesses are selected so as to satisfy the constructive interference condition at a wavelength of 625 nm with refractive indices $n_g = 1.83$ and $n_c = 1.37$ and an incidence angle of 15° . The number of guanine platelets is assumed to be 12 . Since the incidence angle is not normal, reflectance depends on the light polarization. The reflectance spectra calculated for two perpendicular polarizations are averaged, since the incident light is assumed to be unpolarized.

In the above analysis, we have paid attention to the fact that the wavelengths λ_p and λ_c are theoretically different from each other in a strict sense, since they are differently determined: λ_p is determined from the interference condition; λ_c from the centre wavelength of the spectral component. However, the theoretical calculations of the reflectance spectrum show that the analysis using the wavelength λ_c instead of λ_p gives only a small correction in D , of less than 10 nm.

4.2. Reflectance spectrum

The experimentally determined spectra have several characteristics that are generally observed in a thin-film multilayer system: the main peak has a broad rectangular shape with high reflectance, accompanied by several side peaks. Although the platelets have finite dimensions, we compare the experimental spectrum with the theoretical one for a multilayer system that assumes flat and infinitely wide layers.

Figure 9 shows the comparison when the colour of the stack is yellow. The theoretical reflectance is

calculated for two polarizations of light, and the average of the two reflectance spectra is shown in figure 9b. The following parameters are used for the calculation: incidence angle 15° , $d_g = 60$ nm, $d_c = 155$ nm, and the refractive indices have the values stated above. The layer thickness d_c is determined such that the interference condition is satisfied at a wavelength 625 nm. The number of guanine layers is assumed to be 12, which is determined as the number of platelets inside a circular region of 5 μm diameter, with the parameters $D = 670$ nm, $\theta = 15^\circ$, and a length of 3 μm of the platelets being along the longitudinal direction of the stack. It is found that the theory and experiments are in reasonable agreement in several aspects: high reflectance, width of the main component, a few side bands in the shorter wavelength side. On the other hand, one discrepancy is noted on the longer wavelength side of the main peak: a few side bands expected from the theory are not observed in the experiment. The reason for this discrepancy is unclear at present. However, the finite size of the platelets and the tilt angle distribution, which is experimentally observed as the elongated shape of the spot in figure 7b, are probably related to it.

5. DISCUSSION

We have shown that the relation between the wavelength and the tilt angle during colour change is well explained by the Venetian blind model. If the swelling of the iridophore is the origin of the colour change, it is expected that the wavelength just shifts without a change in tilt angle. Namely, the plot points in figure 8 should appear on a nearly horizontal line. If both of these mechanisms, the tilt of the platelets and swelling, are involved in the colour variation, the plot points should appear with an intermediate slope between the horizontal line and the curves drawn according to equation (4.1). Thus, the quantitative agreement in figure 8 safely leads us to conclude that the contribution of swelling is negligible as the origin of the colour change.

There is another theoretical possibility regarding the tilting of the platelets: the entire stack rotates inside an iridophore. This possibility can also be excluded easily, since the slope of the plot points is expected to have the opposite sign from that of the experimental results shown in figure 8. This is because the interference condition is satisfied at a shorter wavelength with an increase in the incidence angle.

Previous studies conducted using electron microscopy have suggested that the thickness of a guanine platelet is very small, only 5–10 nm [13,15]. However, the assumption of such a thin platelet leads to a very weak reflectance, which is only 9 per cent at the maximum and looks totally different from the experimental results. In fact, our quantitative measurements of the surface profile have revealed that the platelets are much thicker, measuring up to 60 nm in thickness, as shown in figure 4. Since the platelets are tapered, the most plausible explanation for the discrepancy with previous electron microscopic observations

is that sections of thinner parts were accidentally observed in previous studies.

It has been found that the examined stacks have different values with respect to the parameter D , which is the spacing between the platelets. Nevertheless, the stripe part of the neon tetra appears to have the same blue colour when it is observed in an aquarium. Thus, it is indicated that there exists some feedback mechanism inside an iridophore to regulate the tilt angle θ of the platelets to reflect light with the same wavelength. A more fundamental question also remains with regard to the molecular basis of the motility: what actually exerts the force to move the platelets? Previous investigations on the influence of various inhibitor chemicals have suggested that motor proteins on the microtubules and the actin filament play an essential role in determining the motility [20,21]. However, further studies are necessary for better understanding of the molecular basis for the motility.

Next, we will compare the structural colour of the neon tetra with other systems of colour variation found in different animals. Since there are generally two ways of colouration, pigmentary and structural colours, colour variation can be attributed to one of them or both. Among wide varieties of animals that can physiologically change their colours (for a recent review, see [27]), we here restrict our discussion to several cases in which structural colouration has been reported to play a major role.

It has been reported that some species of beetles, e.g. the Hercules beetle (*Dynastes hercules*, [28,29]) and the Panamanian tortoise beetle (*Charidotella egregia*, [30]), physiologically control the amount of water in porous three-dimensional structures in their elytra. The infiltration or expulsion of water causes colour variation, since the refractive index of the pores is changed and, consequently, the amounts of light scattered, reflected and absorbed are altered. As compared with the change in the colour of the neon tetra, these beetles exhibit very broad-band reflection, which results in a less-saturated colour, and the spectral change is almost characterized by a variation in the magnitude of reflectance.

Some cephalopod species (squid, cuttlefish and octopus) exhibit a remarkable colour change: the body pattern can be almost instantaneously changed for camouflage and signalling [31,32]. In particular, a unique structural variation has been observed inside a dermal iridophore of a squid, *Loliguncula brevis*: a multilayer reflector is gradually formed using proteinaceous material during the appearance of iridescence [33,34]. Although detailed optical properties of the constructed multilayer system have not been exactly clarified, the colour-varying mechanism seems quite different from that of the neon tetra, in which the movement of rigid crystalline platelets is the main origin of colour variation.

Fish is another group of animals that show physiological colour changes. As it is observed in the neon tetra, the fish iridophore generally contains a stack of crystalline platelets of guanine. Although the direct correspondence between the platelet spacing and the

wavelength of reflected light has not been experimentally confirmed, except for the neon tetra [13], it is generally considered that the stack of platelets produces structural colours through multilayer interference phenomena. However, the stack arrangement is not always the same: for instance, in the blue damselfish, one iridophore contains many stacks that are radially arranged around the nucleus [14,35]. Therefore, reflection is omnidirectional even for a single iridophore. That is in contrast with the iridophore of the neon tetra that shows specular reflection. Further, the platelets in one stack are stacked not in a tilting manner as in the neon tetra, but in a regularly aligned way. Thus, colour variation of the damsel fish is simply thought to result from a change in the platelet spacing without a change in the reflected light direction.

Kasukawa *et al.* [8] reported a wavelength shift of 155 nm for the blue damsel fish, while for the neon tetra, a larger shift was observed up to 250 nm (data not shown). Although more investigations are necessary to arrive at a conclusion, the observed difference in the wavelength shift may indicate that a tilted stack has some advantage in producing a larger spectral shift. Assuming a separation distance of $D = 700$ nm, angular change of only 7.5° is sufficient to produce a wavelength shift of 250 nm according to equation (4.1). When a larger D is assumed, the required angular change becomes smaller, as shown in figure 9. This fact inspires us to consider one design of artificial colour-changing reflectors: a stack of tilted rigid platelets that is embedded in soft elastic matter. This composite material may exhibit large colour variation when small shear stress is applied.

On the other hand, the variability or flexibility of the microstructure implies that it is inevitably accompanied by temporal fluctuations [36]. The dynamical measurement of the reflected light intensity may reveal such fluctuation in the variable multilayer system, which is thought to include random motion of a single platelet owing to the Brownian motion of water molecules and also the collective motion of the several platelets within a stack. Thus, a new question arises as to how such temporal fluctuations modify reflectance spectra. Detailed optical investigations on the fish iridophores or other colour-variable systems may expand the research field of the structural colours into a new dimension that includes the tunability and dynamics of optical characteristics.

This study is supported by a Grant-in-Aid for Scientific Research (NOS. 20740242 and 22340121) from the Ministry of Education, Culture, Sports, Science and Technology.

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