Electronic Supplementary Material

1. Materials and Methods

1.1. Histology and iridophore layer thickness measurements

Lookdowns were captured from coastal waters near Port Aransas (Texas, USA) and the Florida Keys (Florida, USA), euthanized and stored at -20^{o}C until used for analysis. Animals were handled following procedures in accordance with the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC) at University of Texas at Austin under the protocol #AUP-2012-00033. Statistical analysis of polarization reflectance data from freshly euthanized lookdowns [1] with ones thawed from storage showed no significant differences in their optical properties. For tissue analysis or processing, skin tissues (~ 1 cm^2) with the underlying muscle were cut with a sharp razor from the dorsolateral, mid-lateral and ventrolateral flanks of the fish. The tissues were fixed in 2% glutaraldehyde and 2% formaldehyde prepared in phosphate-buffered saline (PBS) overnight and then submerged in 30% sucrose for 24 hours. The tissues were subsequently frozen in a tissue freezing medium (TBS Triangle Biomedical Sciences). 14-µm thick cross sections were cut on a cryostat (Microm HM 550 Microtome cryostat) and placed on microscope slides. Each section was immediately covered with 50% glycerol in PBS followed by cover glass for imaging by a Nikon Eclipse 80i microscope under epi-illumination and transmitted illumination. The thickness of the iridophore layer (stratum argenteum) in the skin was determined by the measurement tool in Nikon's NIS-Element software.

Denton and Nicol [2] effectively used optical methods to estimate the orientation of the reflecting surface of guanine platelets with respect to the fish surface, especially those associated with the fish scales. They also used histological methods to determine the orientation of the guanine platelets in the stratum argenteum. However, caution should be used in interpretation of their histological data, some of which appeared to be distorted by artefacts of tissue processing and drying. For example, the drying artefact greatly exaggerated the angles between the fish surface and the reflecting platelets in some of the fish they examined (Plate 5A-B, Plate 6A-B &D, Plate 7A-B &D, Plate 9C, and Plate 10B in [3]). We also observed similar artefacts in our tissue sections after the drying process. When cryosections were kept moist immediately after sectioning, the artefacts (vacuoles, gaps and curved guanine platelets) were never seen in the tissue sections by light microscopy.

For statistical analysis of iridophore layer thicknesses, two pieces of skin tissues (one from each side of the fish) were collected from each of three regions of interest (dorsolateral, mid-lateral and ventrolateral flanks) from each fish. Two to three cryo-sections from each piece of tissue were analysed by light microscopy. Up to ten images were collected from each section and three thickness measurements were made in each image. The measurements were repeated for four individual fish.

1.2. Measurements of guanine platelet dimensions

Skin tissues were dissected from dorsolateral, mid-lateral, or ventrolateral flanks from each fish and submerged in 0.5% crude type IA collagenase (Sigma Aldrich product # C9891; dissolved in 50 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$, pH 7.4) at room temperature for 15 min. Scales and the collagen layer (stratum compactum) were carefully removed with fine forceps under a dissecting microscope. The iridophore layer (stratum argenteum) was peeled off from the muscle with the forceps and gently torn apart to facilitate the release of guanine platelets. 100 µL of the solution containing dissociated guanine platelets was pipetted onto a microscope slide and covered with cover glass for image acquisition. This process was repeated up to ten times for each piece of tissue, i.e. up to a total of 1000 µL of the solution with guanine platelets was analysed. A total of five individual fish were used for this analysis. After completion of image acquisition, the Nikon's NIS-Element software was used to measure the length and width of individual guanine platelets.

To evaluate the dimensions of the fish scales, the scales were removed the mid-lateral flank from two individual fish with fine forceps under a dissecting microscope and placed on the microscope slide for image acquisition. The dimensions of the long and short axes of these oval-shaped scales were measured by the Nikon's NIS-Element software.

1.3. SEM analysis of dissociated guanine platelets

Dissociated guanine platelets were prepared as described for the measurements of the lengths and widths of guanine platelets in a previous section. The solution containing dissociated guanine platelets was spotted on Whatman filter paper and rinsed with water followed by 50% and then 100% ethanol. The filter paper was air-dried and cut into a small piece and attached to the SEM mount. It was coated with palladium/platinum using a sputter coater and then analysed by SEM. Thicknesses of individual guanine platelets were measured in high magnification (>100K) SEM images using NIH Image J software and calculated based on the scale bars in the same images.

1.4. Reflectance spectrum from the lookdown skin

The reflectance spectrum from the ventral flank of the lookdown was measured using the BLACK-Comet spectrometer with a fibre optic probe (StellarNet, Inc.). A white Lambertian reflectance standard (Model RS50 from StellarNet, Inc.) was used as the reference. Reflectance spectra were collected from three different spots in the ventral flank of the fish. Three spectra were collected from each spot. All the data were averaged to produce the reflectance spectrum for the ventrolateral flank. The reflectance spectrum was normalized to its peak.

1.5. Birefringence of fish surface and skin sections

Birefringence of the fish surface was evaluated by cross polarization microscopy, i.e. illuminating the fish with polarized light and acquiring image through a polarization filter orthogonal to the polarization plane of the illuminating light. The image of the fish surface would be completely dark in the absence of birefringence or depolarization effects. The birefringence effect is indicated by the differences of intensity for different fish orientations. Because an adult lookdown was too large for this type of analysis, juvenile lookdowns (standard length ≈ 9-10 cm) were used. Juvenile lookdowns exhibited similar patterns of birefringence as those of the adult fish. The fish was submerged in a tray containing PBS and placed under a Leica stereomicroscope (Leica M205 C). Polarized light was produced by the illuminator covered with a polarization filter (the polarizer). Light reflected from the fish passed through another polarization filter (the analyser) orthogonal to the polarizer before entering the objective of the microscope. An image of the fish without the polarizers was first acquired when the fish was at its natural swimming position, i.e. the line between the eye and the centre of the caudal peduncle was horizontal. Subsequently, the image of the fish was obtained in the presence of both polarization filters. The next image was acquired after the fish was rotated by 22.5^{o} clockwise on a circular rotating stage. The procedure was repeated until the fish had been rotated by 90^{o} from its initial horizontal position.

1.6. Visualization of reflectance distribution

To evaluate the reflectance distribution from the lookdown skin, a piece of skin with the underlying muscle (~ 1 cm^2) was excised from the mid-lateral flank of the fish. The skin preparation was taped along two parallel edges on to a microscope slide and submerged in a plastic box containing PBS. The microscope slide was placed against one side of the plastic box (Fig. S6*a*). The plastic box was prepared by cutting a rectangular opening on one side of its walls and a microscope slide was glued to cover the opening with silicone sealant. 1% nonfat dry milk dissolved in PBS was added to the plastic box in a volume ratio of 1:100 and mixed so that the milk was evenly distributed. Laser beam from a green laser pointer (523 nm, Class III 5 mW constant wave) was directed to pass through this glass window and targeted on to the skin sample (Fig. S6*a*). The incident angle of the laser beam was approximately 30^{o} (the angle between the ray of light and the normal to the surface). The plastic box was placed under the Leica stereomicroscope and the laser beam in the solution was photographed. For comparison, a piece of aluminium foil (the dull side) and a silver screen reflection standard (polystyrene board with S1 Screen Paint Silver from Paint On Screen, Inc.) were also placed in the box and photographed. All the images were acquired with the same exposure time. The intensity profile of the reflectance at a particular plane was generated by Nikon's NIS-Element software.

2. Results

2.1. Thicknesses of iridophore layers

In images of cross sections of the lookdown skin acquired from light microscopy with transmitted illumination (Fig. S1*a-f*), the iridophore layers appeared mostly dark-brownish, suggesting that light was scattered and dissipated by the densely packed guanine platelets. The iridophore layer in the dorsolateral skin was much thinner than those in mid-lateral and ventrolateral flanks. Each iridophore layer consisted of two distinct sub-layers (labelled as 1 and 2 in Fig. S1*d* and as 3 and 4 in Fig. S1*e,f*). The average total thicknesses of the iridophore layer in the dorsolateral, mid-lateral and ventrolateral flanks are shown in Fig. S1*h* and listed in Table 1. One-way ANOVA shows that these layers were significantly different in thickness ($F=2043$, $p < 0.001$). Dukey-Kramer post hoc tests (with error rate $\alpha = 0.01$) indicate that the iridophore layer in the dorsolateral flank is significantly thinner than those in the mid-lateral or ventrolateral regions, and that the iridophore layer in the mid-lateral flank is also significantly thinner, but to a lesser degree, than that in the ventrolateral skin (Table 2). These results suggest that the thickness of the total iridophore layer increases progressively from the dorsolateral to the ventrolateral flanks.

In the dorsolateral skin (Fig. S1*d*), the guanine platelets with large surface area (Type 1) formed a layer about twice as thick as the platelets with small surface areas (Type 2). In contrast, in the mid-lateral or ventrolateral skin (Fig. S1*e,f*), the guanine platelets with large surface areas (Type 3) formed a layer that was approximately one quarter of the thickness of the platelets with small surface areas (Type 4). It is also noted that the long axes of nearly all guanine platelets except for some of Type 2 platelets were aligned parallel with the skin surface (Fig. S1*a-f*).

Table 1. Thicknesses of iridophore layers

Region of interest	Thickness (μm) (Mean ± SD)	No. of Fish	No. of Tissues	No. of Measurements
Dorsolateral	16.5 ± 3.6	4	8	n = 322
Mid-lateral	78.7 ± 18.3	4	8	n = 306
Ventrolateral	89.6 ± 18.1	4	8	n = 159

Table 2 Tukey-Kramer post hoc test for layer thicknesses (Error rate $\alpha = 0.01$)

	Dorsal vs. Middle	Dorsal vs. Ventral	Middle vs. Ventral
Absolute difference	62.24	73.12	10.89
Critical range	3.19	4.19	4.02
Different?	yes	yes	yes

2.2. Guanine platelet types and their dimensions

SEM analysis of dissociated guanine platelets from the lookdown skin showed crystals with distinct morphologies (Fig. S2*a-d*). In the dorsolateral skin, the two primary types of guanine platelets, Type 1 and Type 2, exhibited a dramatic difference in size. Stacks of Type 1 platelets were frequently seen in preparations (Fig. S2*b*). In the mid-lateral skin, the other two types of guanine platelets, Type 3 and Type 4, were both elongated with Type 3 being much wider than Type 4 (Fig. S2*c*). Morphologies of guanine platelets in the ventrolateral skin are similar to those in the mid-lateral flank. Often observed in stacks of Type 4 guanine platelets were gaps between individual platelets (arrows in Fig. S2*d*). This indicates that one reflecting plane can consist of multiple smaller platelets instead of a continuous surface (i.e. a single platelet) within one stack of guanine platelets. The presence of these gaps is expected to contribute to scattering events and colour variations.

Lengths and widths of individual platelets were analysed by light microscopy and plotted in Fig. S2*e* and their averages are shown in Fig. S2*e* and in Table 3. Type 2 guanine platelets were too small to be reliably measured by light microscopy. Their dimensions were measured from SEM images (Table 3).

Analysis of SEM images (see the inlet in Fig. S2*d*) showed that thicknesses of individual Type 1 and Type 3 guanine platelets are significant smaller than those of Type 2 and Type 4 (Fig. S2*f* and Table 3). One-way ANOVA indicates significant differences among the thicknesses of different types of platelets ($F=177.6$, $p < 0.001$). Dukey-Kramer post hoc tests show that each type of the platelets is different from another one in thickness (with error rate $\alpha = 0.01$). A reason that the measured average thicknesses of the guanine platelets with large surface areas were smaller is that many of them were very thin at their edges, and therefore, the average measurements became smaller. Broken edges were frequently seen in SEM images indicating their fragility.

Table 3 Dimensions of guanine platelets

Type of guanine platelets	Type 1	Type 2	Type 3	Type 4
Length (μm) (mean ± SD)	18.3 ± 3.4	4.60 ± 0.89	28.2 ± 9.7	14.2 ± 7.1
Width (μm) (mean ± SD)	8.4 ± 1.6	2.54 ± 0.45	4.6 ± 1.3	1.0 ± 0.19
Length/width ratio	2.20 ± 0.28	1.86 ± 0.46	6.43 ± 2.78	14.72 ± 8.72
Number of fish	5	3	5	5
Number of tissues	15	6	15	15
Number of measurements	n = 394	n = 244	n = 168	n = 433
Thickness (nm) (mean ± SD)	53.5 ± 16.9	73.0 ± 16.8	40.1 ± 13.9	90.1 ± 40.1
Number of fish	3	3	3	3
Number of tissues	6	6	6	5
Number of measurements	n = 148	n = 120	n = 74	n = 98

2.3. Birefringence of the lookdown skin surface

Cross-polarization microscopy was used to analyse birefringence of different surface areas of juvenile lookdowns. These areas include the dorsolateral (Fig. S3*a1-a7*), mid-lateral (Fig. S3*b1-b7,* also shown in Fig. 7 in the main text), and ventrolateral (Fig. S3*c1-c7*) flanks, the caudal penducle (Fig. S3*d1-d7*) and operculum (Fig. S3*e1-e7*). Most of the areas exhibited the maximum birefringence effect when the fish was rotated by 22.5° or 45°. This is likely due to variation in the pitch angles of guanine platelets skin or different contributions from the collagen layer in different regions of the lookdown.

2.4. Birefringence of the guanine platelet layers

Three different skin sections from the mid-flank were analysed so that the birefringence of the guanine platelets was examined from three directions that are orthogonal to one another. In the cross section (Figs. S4*a1-a6, 5a*), the long axes of the guanine platelets were nearly parallel to the section while their short axes and reflecting surfaces were nearly perpendicular to the section. Minimum intensity was observed when the long axis of the platelets was perpendicular (Fig. S4*a2*) or parallel (Fig. S4*a6*) to the polarizer. The maximum birefringence effect occurred when the long axis the platelets was at 45° away from the polarization plane (Fig. 4*a4,a7*). This indicates that the optical axes of the guanine platelets (Types 3 and 4) were either along their long geometric axis and/or perpendicular to the reflecting surface (Fig. S5a).

Similar effect of birefringence was observed for both Types 3 and 4 platelets in the parasagittal section (Fig. S4*c1-c6;* also shown in Fig. 7*b1-b6* in the main text) in which both the long and short axes and the reflecting surfaces of these platelets were mostly parallel to the section. This indicates that the optical axis of the guanine platelets was either aligned with their long or short geometric axis (Fig. S5*c*).

In the horizontal section (Fig. S4*b1-b6*), the long axes and reflecting surfaces of the guanine platelets were nearly perpendicular to the section while their short axes were nearly parallel to the section (Fig. S5*b*). The birefringence effect of Type 3 guanine platelets (marked by arrows in Fig. S4*b1-b6*) was similar to those observed in the cross and parasagittal sections, i.e. nearly invisible when their short axes were perpendicular (Fig. S4*b2*) or parallel (Fig. S4*b6*) to the polarization plane of illuminating light and reaching maximum intensity when the section was rotated by 45° (Fig. S4*b4*). This demonstrates that the optical axis of Type 3 platelets were along their short geometric axis or perpendicular to the reflecting surface (Fig. S5*b*).

An interesting observation was that Type 4 guanine platelets (other than those marked by arrows in Fig. S4*b1-b6*) exhibited very little change in intensity as the tissue section was rotated from 0° to 90°. Although the average intensity reached its maximum at 45°, the overall change was much less than those in cross and parasagittal sections. This can be explained by the fact that the short axes of Type 4 guanine platelets exhibited random yaw orientations (Fig.

4 in the main text). Therefore, as the tissue section was rotated, different subsets of the guanine platelets always had different angles with respect to the polarization plane of the illuminating light. Higher magnification images revealed subtle changes in intensity in localized areas as the tissue section was rotated. These observations also indicate that the optical axes of Type 4 platelets were aligned with their short geometric axis or perpendicular to the reflecting surface (Fig. S5*b*).

Considering the birefringence effects of the Types 3 and 4 guanine platelets in all three directions and assuming that these guanine platelets have one optical axis (uniaxial), it could be concluded that there were at least two populations of guanine platelets with optical axes orthogonal to each other (Fig. S5). If all the platelets had the same optical axis, we would not be able to observe all the birefringence effects at all three directions shown in Fig. S6. Any combination of two out of the three possibilities could explain all the effects observed in our experiment. However, these data were insufficient to determine which combination of the two different optical axes would exist, whether all three different populations of platelets were present. A more likely possibility is that these platelets have two optical axes (biaxial) [4]. A combination of any two of three possible orientations of the optical axes can explain the cross-polarization microscopy data.

2.5. Reflectance distribution of the lookdown skin

To visualize the reflectance distribution of the lookdown skin, the skin preparation from the mid-flank region was submerged in a plastic container with a low concentration of nonfat dry milk (0.01%) dissolved in PBS to scatter light. A laser beam was directed onto the skin through the glass window on one side of the container (Fig. S6*a1*). When the skin sample was placed in an orientation consistent with the fish's natural swimming position (i.e. anterior-posterior axis of the fish being parallel to the water surface), the laser beam was scattered diffusely (Fig. S6*a1*). At this orientation, the long axes of the guanine platelets were nearly perpendicular to the water surface. The very thin guanine platelets were expected to function as an irregular grating and caused significant diffraction. The random yaw angles of the Type 4 guanine platelets and scattering from edges of the guanine platelets contributed to the diffusion of light. When the same piece of the fish skin was turned 90°, a strong component of specular reflection was observed (Fig. S6*a2*). When a silver screen reflection standard (Fig. S6*a3*) was used to replace the fish skin, it primarily caused scattering as expected while a piece of aluminium foil (Fig. S6*a4*) produced prominent specular reflection. The intensity profiles along the red lines in Fig. S6*a1-a4* are shown below each image (Fig. S6*b1-b4*).

References

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