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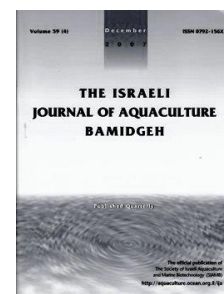
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Cellular Basis of Metallic Iridescence in the Siamese Fighting Fish, *Betta splendens*

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Abstract

The ultrastructural morphology of chromatophores that gives rise to the highly valued metallic iridescence in the Siamese fighting fish (*Betta splendens*) was examined by transmission electron microscopy (TEM). Melanophores, iridophores, xanthophores, erythrophores, and leucophores were observed in the epidermis and dermis of *B. splendens*. Specific combinations of these chromatophores formed the pigmentation patterns of the dark blue, ultra-marine, turquoise-bronze, and golden strains. Under the TEM, large spherical melanosomes appeared which were highly electron dense in the cytoplasm of melanophores. These cells also possessed slightly electron dense organelles which could be partially melanized pre-melanosomes. The wide spectrum of metallic hues on the body of *B. splendens* is attributed to refraction, reflection, and thin-film interferences from reflecting platelets in iridophores that are closely associated with other chromatophore types. The designation "irido-melanophore unit" is proposed for one such close association which comprises an iridophore underlined by a melanophore. Varied inclination angles and thicknesses of reflecting platelets are elucidated to cause the iridescent sheen. Thin platelets oriented at small angles to the long axis of a cell produce purplish-blue hues whilst numerous thick and thin platelets at larger angles generate blue, green, and silvery-golden sheens.

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Introduction

Dark pigmentation in poikilothermic vertebrates is attributed to melanophores which possess brown or black melanin pigments that are synthesized and stored in spherical organelles called melanosomes (Bagnara and Hadley, 1973; Bagnara, 1983; Fujii, 1969, 1993). In addition to brightly colored chromatophores, erythrophores and xanthophores, which are cells with colorless pigments and crystals, are present in the epidermal and dermal layers. These are termed guanophores due to the presence of guanine in granular or crystalline form and are thin light-reflecting platelets (Bagnara and Hadley, 1973; Fujii, 1969, 1993). Guanophores comprise two cell types, namely iridophores and leucophores. Iridophores of teleosts have several flat vesicles, possibly cisternae of smooth endoplasmic reticulum, each of which is embedded with a thin reflective platelet (Fujii, 1969). Leucophores or white pigment cells contain granular guanine-loaded particles (Fujii, 1969, 1993).

The Siamese fighting fish, *Betta splendens* Regan (1910), which belongs to the family Belontiidae, is popular among ornamental fish breeders worldwide due to its convenient size, hardiness, and noticeable physical, physiological, and behavioral traits, and most importantly, for its brilliant and striking color variations (Wallbrunn, 1958; Lucas, 1968; Kirpichnikov, 1981). In South-East Asia, it has been cultured for centuries mainly for the sport of "fish fighting" (Lucas, 1972). Selection for pugnacity, long fins, and bright colors has produced an immense variety of phenotypes, none of which is similar to the short-finned wild form that is widely distributed in the hill streams, forest creeks, sluggish rivers, swamps, and paddy fields of South-East Asia (Regan, 1910; Wallbrunn, 1958; Lucas, 1968; Witte and Schmidt, 1992; Kottelat and Ng, 1994).

In recent years, the major focus of *B. splendens* culture is to improve their brilliant metallic pigmentation, patterns, and color quality for the commercial ornamental fish trade. One of the major issues with *B. splendens* culture is black and red blotching of the scales which disrupts the uniformity of their metallic iridescence and thus reduces their marketability. A similar problem was noted in red tilapia strains with black melanin blotching (Avtalion and Reich, 1989; Rajaei, 2011). A few studies have characterized the pigment patterns of *B. splendens* (Wallbrunn, 1958; Lucas, 1968, 1972; Royal and Lucas, 1972; Khoo, 1995; Khoo, et al., 2012; and Amiri and Shaheen, 2012). The objective of this study was to investigate, at the transmission electron microscopy (TEM) level, the cellular basis of metallic blue, purple, green, and silvery-golden iridescence in *B. splendens*.

Materials and Methods

Dark blue, ultra-marine, turquoise-bronze and golden strains of the long-finned *B. splendens* were obtained from a commercial farm in Singapore (Figs. 1a-c), and maintained (Khoo et al., 2012). Scales from the dorso-lateral regions were detached and mounted in teleost physiological saline for bright-field and epi-illuminated light microscopy at 200-1,000x magnification (Khoo et al., 2012). The ultrastructural morphology of the chromatophores was studied using a transmission electron microscope (TEM) at 7,200-54,000x magnification. Scales from the dorso-lateral region of each strain were processed, sectioned, mounted, and stained for TEM microscopy, and subsequently photomicrographed (Khoo et al., 2012).

Results

The cellular basis of metallic coloration of the dark blue, ultra-marine, turquoise-bronze and golden strains (Figs. 1a-c) was investigated using light microscopy and transmission electron microscopy. Chromatophores of *B. splendens* were observed in the pigment cell region below the basal lamina of a vertically sectioned scale (Khoo et al., 2012). The five main chromatophore types observed in these *B. splendens* strains were melanophores, iridophores, xanthophores, erythrophores and leucophores. These chromatophores could be categorized into the corolla, dendritic, and punctuate shapes. The primary focus of this study was the iridophores and melanophores as these cells gave rise to metallic blue, purple, green, golden and silver iridescence in *B. splendens* (Fig. 3).

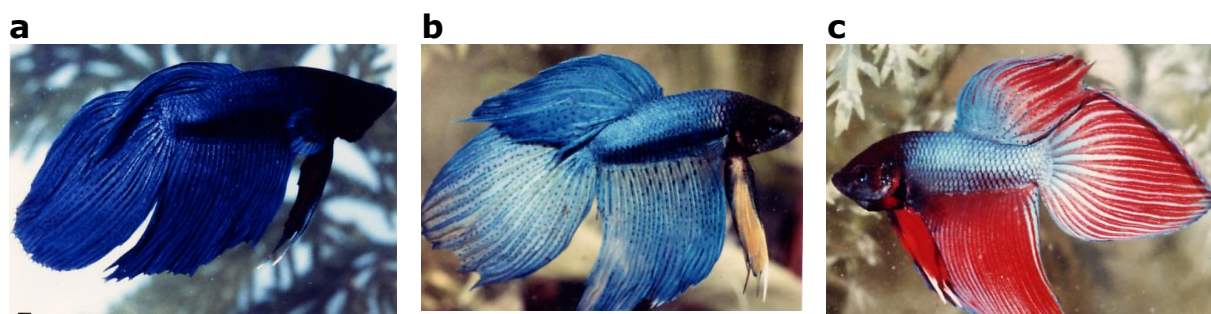


Fig. 1. (a) Dark blue strain covered with deep purplish-blue iridescence. (b) Ultra-marine strain with extensive metallic blue iridescence on body and fins. (c) Turquoise-bronze strain with iridescent bluish-green body and red fins.

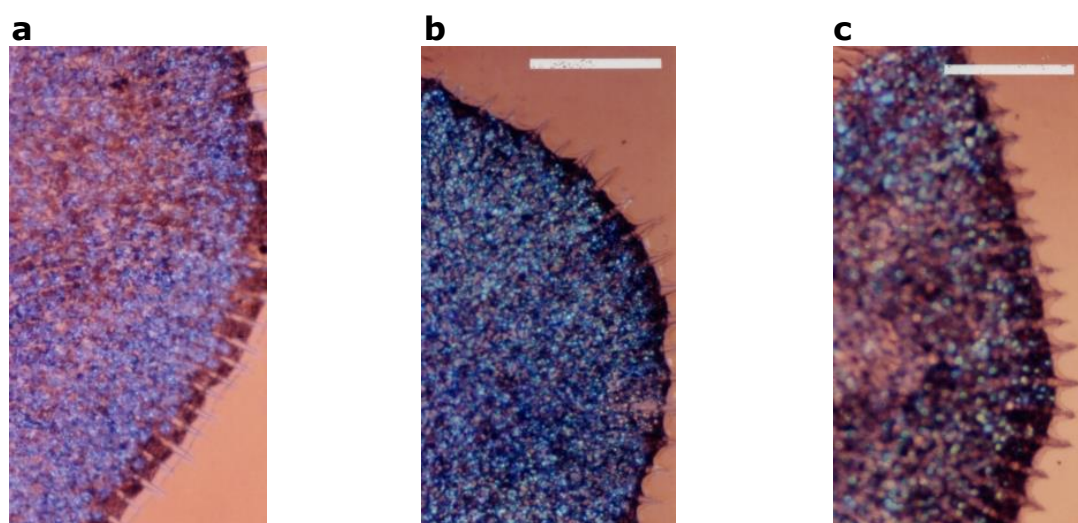


Fig. 2. Under epi-illumination of a light microscope, the dorsal scale of the (a) dark blue strain showed dark purplish-blue iridescence, (b) ultra-marine strain gave metallic blue iridescence, and (c) turquoise-bronze strain had less uniform bluish-green, golden and silver metallic iridescence. Bar = 300 μ m.

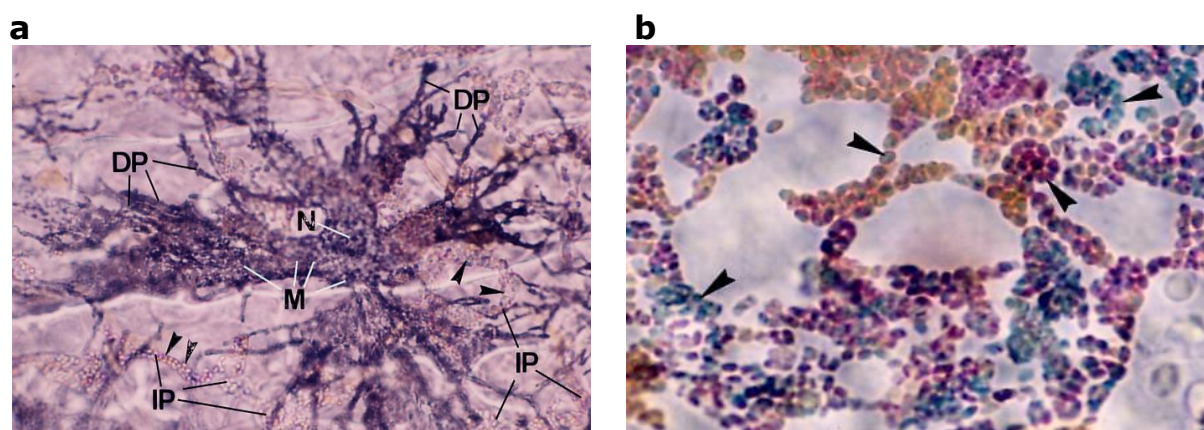


Fig. 3. (a) Melanophore of dark blue strain showing melanosomes (M), nucleus (N) and long dendritic processes (DP), with multicored iridophore platelets (arrowheads) in long interlinking processes (IP) surrounding the melanophore (1,000 \times). (b) Multicored platelets (arrowheads) in iridophores which were interlinked to form a mesh-like network (1,000 \times).

Black and brown pigmentation in *B. splendens* was attributed to melanophores (Fig. 3a) which contained a dark, dense melanin pigment, bound within melanosomes. The heavily melanized mature melanosomes in melanophores appeared to be highly electron dense under the TEM (Fig. 4). These membrane-bound melanosomes were primarily spherical organelles in the cytoplasm and seemed larger in size than the pterinosomes in erythrophores (Khoo, 1995; Khoo *et al.*, 2012). Sectioned melanophores showed the

presence of large broad nuclei in these cells (Fig. 4). Small slightly electron dense ellipsoidal organelles, presumably partially melanized pre-melanosomes, were noted in the melanophores of some adult fishes.

Silvery-golden metallic iridescence on the body and fins of light-colored *B. splendens* strains, such as golden, were contributed mainly by iridophores (Fig. 3b) that were present as individual cells or closely associated with xanthophores and leucophores (Khoo, 1995). Under bright-field light microscopy, iridophores could be observed as large multi-coloured granules or oval-shaped structures having a range of blue, purple, green, pink, yellow, and white hues (Fig. 3b). Leucophores are not presented here because these transparent hyaline cells were difficult to locate and photograph among the overlapping pigmented cells (Khoo, 1995).

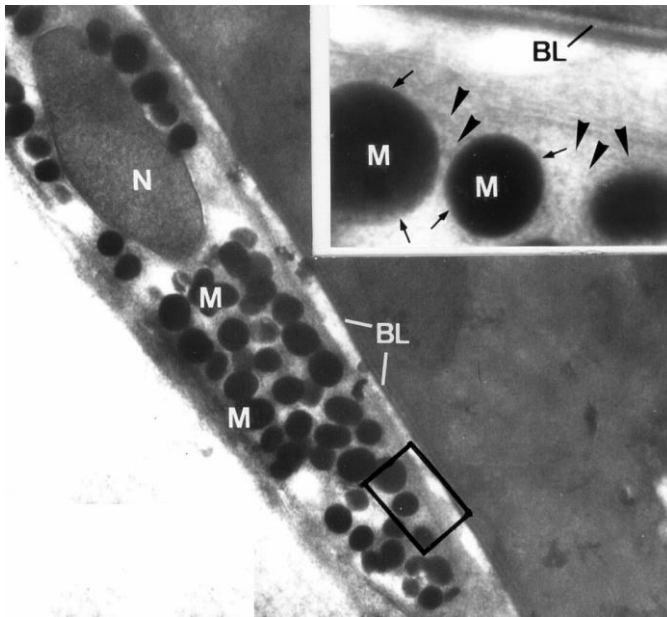


Fig. 4. Melanophore with highly electron dense melanosomes (M) and a large nucleus (7,200 \times). Inset: Outlined region of the melanophore highly magnified to show membrane-bound (small arrows) melanosomes (M) (28,500 \times). (BL: basal lamina, N: nucleus)

In contrast to melanophores, iridophores were observed to possess stacks of membrane-bound platelets arranged horizontally or inclined at various oblique, but constant, angles to the basal lamina and an underlying melanophore layer (Fig. 5). These platelets were stacked at an equal distance from each other in parallel arrays. Fig. 5 depicts two types of iridophore platelets, namely, thin reflecting platelets and thicker ones. At high magnifications, these platelets appeared to be membrane-bound. Actual reflecting platelets were generally not observed in ultrathin sections. These vertically sectioned platelets were represented by membrane-limited empty spaces (sacs) since they became detached during processing, microtoming and staining of sections (Fig. 5).

Some reflecting platelet sacs might appear distorted or bulging due to deformation during specimen processing or exposure to the electron beam during TEM observations. It was possible that some platelets might be preserved completely (Figs. 5c & 5d). Each platelet (visible as a thin line), was surrounded by a membrane in the center of the sacs. In all iridophore sections, the nuclei were large and broad while some were polymorphic in shape (Figs. 5c-e).

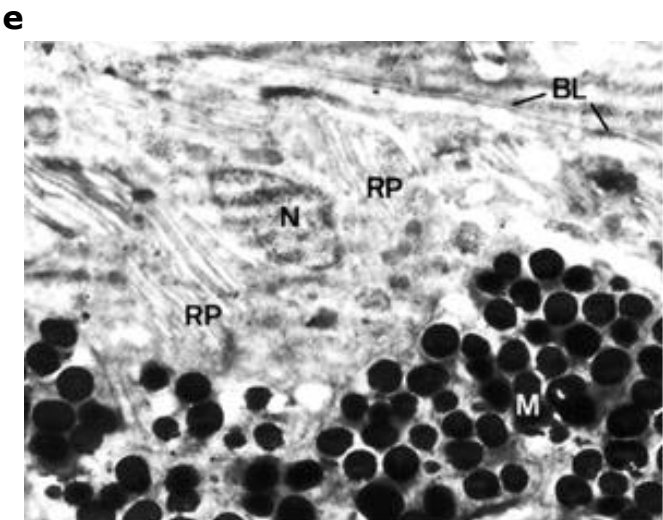
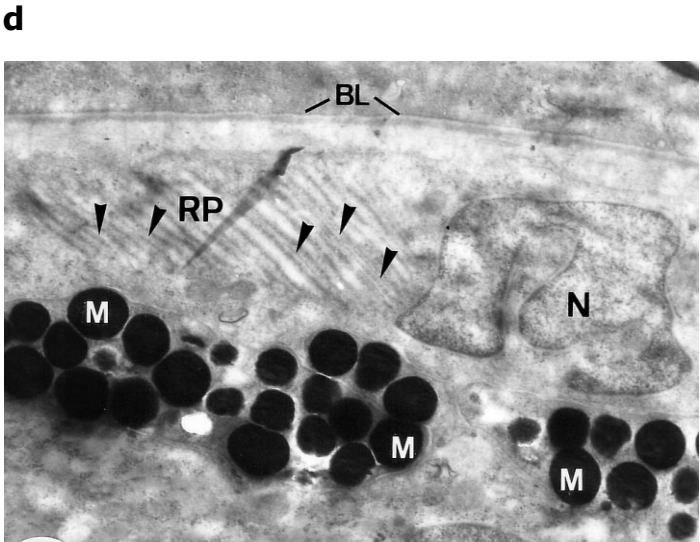
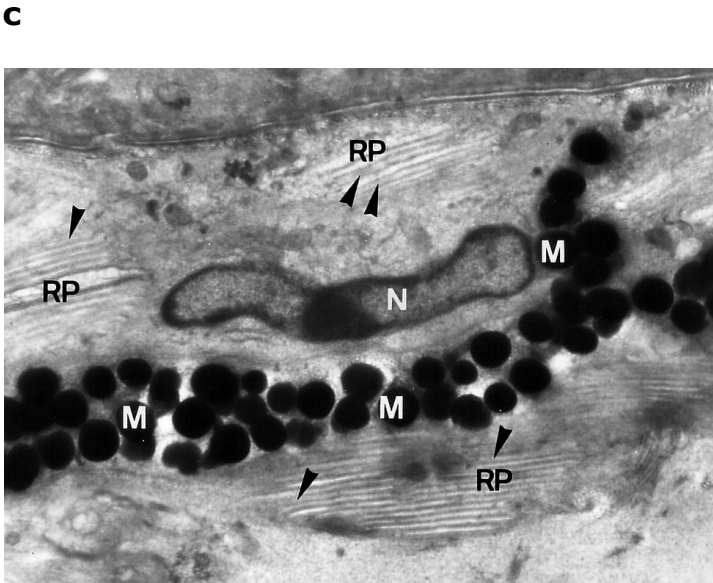
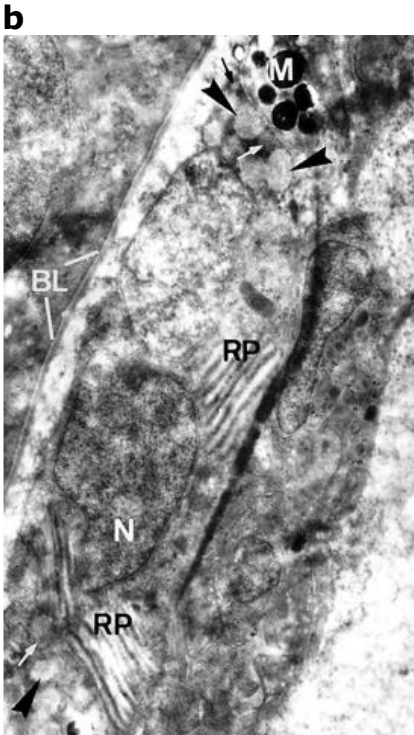


Fig. 5. (previous page) Electron micrographs showing: (a) An iridophore of the golden strain with stacks of thick and thin reflecting platelets oriented at different angles to the basal lamina (10,800 \times). (b) Three pigment cell types, i.e., an iridophore with stacks of thin reflecting platelets, a xanthophore process with carotenoid vesicles (arrowheads) and a melanophore process with highly electron dense melanosomes (7,200 \times). (c) Cell process of a melanophore of the dark blue strain containing electron dense melanosomes located in between iridophores with stacks of thin horizontally oriented reflecting platelets (7,200 \times). (d) Iridophore of the ultra-marine strain with a polymorphic nucleus and reflecting platelets inclined at large angles to an underlying melanophore with electron dense melanosomes (10,800 \times). (e) Melanophore process of the turquoise-bronze strain with electron dense melanosomes closely associated with an iridophore having stacks of obliquely oriented reflecting platelets below the basal lamina (7,200 \times). Reflecting platelets could be observed as thin lines (arrowheads) in (c) and (d). (BL: basal lamina, N: nucleus, M: melanosomes, RP: reflecting platelets).

Fig. 5b is a common representation of the metallic colored *B. splendens* strains in which long melanophore processes containing highly electron dense melanosomes were closely associated with the cell processes of xanthophores that enclosed large carotenoid vesicles (Khoo, 1995; Khoo *et al.*, 2012). These cell processes overlapped and interdigitated with those of iridophores that bore multiple stacks of parallel-arrayed reflecting platelets to form densely interlinked mesh-like networks usually seen under the light microscope (compare Fig. 3a with Fig. 5b). Blue, purple, green, pink, lavender, yellow and white iridophore platelets typically observed in three of the metallic colored strains had been established using the TEM as being due to iridophores which formed a screen above the melanophores. Each iridophore appeared to be associated with an underlying melanophore. Figs. 5c & 5d portray these iridophores as having reflecting platelets inclined at specific angles which might be characteristic of each metallic colored strain. The iridophores of the golden and cambodia *B. splendens* strains did not occur in combination with melanophores and erythrophores (Fig. 5a) (Khoo, 1995).

In the dark blue strain, stacks of thin reflecting platelets were oriented horizontally or inclined at small angles relative to the basal lamina and underlying melanophores (Fig. 5c). Larger stacks of iridophore platelets were inclined at wider angles to melanophores and their processes in the ultra-marine strain (Fig. 5d) compared to the dark blue strain. These platelets, in parallel arrays, were also more numerous and smaller in each stack. In comparison to the dark blue and ultra-marine strains, the turquoise-bronze strain had reflecting platelets inclined at very large angles relative to the basal lamina and underlying melanophores (Fig. 5e). The inclination angles of reflecting platelets in the ultra-marine and turquoise-bronze strains appeared to be generally similar. However, the distribution of iridophores was less uniform in the turquoise-bronze strain in contrast to the dark blue and ultra-marine strains (compare Fig. 2c with Figs. 2a & 2b). This was due to patches or aggregations of melanophores and erythrophores (unassociated with iridophores) which caused blotching of the scales and epidermis of this strain.

Discussion

Dusky pigmentation in *B. splendens* is attributed to dark, dense melanin pigment, bound within melanosomes in the cytoplasm (Fig. 3a). Under the TEM, vertically sectioned melanophores of *B. splendens* reveal large spherical membrane-bound melanosomes in the cytoplasm (Fig. 4). Mature melanosomes are highly electron dense due to their heavy melanization. They are larger than pterinosomes but appear similar in size to carotenoid vesicles (Nakajima and Obika, 1986; Blanchard *et al.*, 1991; Khoo, 1995; Khoo *et al.*, 2012). These large flat melanophores usually have long dendritic cell processes and possess a large centrally-located membrane-bound non-polymorphic nucleus (Fig. 4). Numerous small and slightly electron dense ellipsoidal vesicles in some melanophore sections of *B. splendens* might possibly be pre-melanosomes that develop into mature melanosomes following melanin biosynthesis (Nakajima and Obika, 1986; Blanchard *et al.*, 1991; Khoo, 1995; Khoo *et al.*, 2012).

During melanin synthesis in melanocytes and immature melanophores, tyrosine is oxidized to 3,4-dihydroxyphenyl-alanine (Dopa) and then to Dopa quinone with tyrosinase as the crucial catalyst. Dopa quinone subsequently forms melanin through polymerization and is stored within Golgi- or endoplasmic reticulum-derived pre-

melanosomes and pre-formed melanosomes which fuse, grow and develop into mature melanosomes (Nakajima and Obika, 1986; Blanchard *et al.*, 1991). It is possible that these presumptive pre-melanosomes of different sizes in *B. splendens* are undergoing various stages of premelanization and development at the ultrastructural level to form mature melanosomes in young melanophores (Khoo, 1995; Khoo *et al.*, 2012).

The occurrence of reddish-brown transformation products between tyrosine and melanin have led to a hypothesis that some red pigments in melanophores might belong to the melanin group, but these findings have been contradicted in Xiphophorine fish (Goodrich, *et al.* 1941). These pigments could, nevertheless, be an intermediate product from a blocked melanin synthesis pathway (Goodrich *et al.*, 1941; Royal and Lucas, 1972), or phaeomelanins (Fujii, 1993). These reddish-brown pigment cells and melanophores that occur in scattered patches and large aggregations result in blotching patterns on the scales and epidermis of the turquoise-bronze *B. splendens* (Khoo, 1995). *B. splendens* strains with blotching do not fetch as high commercial value in the ornamental fish trade as strains with uniform metallic iridescence. A similar reduction in value was also reported in red tilapia strains with blotched patterns (Avtalion and Reich, 1989; and Rajae, 2011).

Glitter, iridescence, silvery, or metallic tones such as blue, purple, turquoise, and green colors are caused by refraction, reflection, diffraction, thin-film interference and Tyndall scattering of light from reflecting platelets in iridophores (Bagnara and Hadley, 1973; Menter *et al.*, 1979; Fujii, 1969, 1993; Zarnescu, 2007; Amiri and Shaheen, 2012). These reflecting platelets have been termed "refractosomes" (Menter, *et al.*, 1979). Each guanine platelet in an iridophore is regularly arranged in parallel stacks, thus forming an acute angle of inclination with respect to the surface of scales and skin along the lateral plane of the fish (Nagaishi *et al.*, 1990; Nagaishi and Oshima, 1992; Fujii, 1993). These platelets have been found to contain hypoxanthine and xanthine besides guanine and a mixture of purines which might possibly form isomorphic crystals as observed in the iridophores of the blue damselfish, *Chrysiptera cyanea* (Kasukawa *et al.*, 1987), larval amphibians, *Rana pipiens* and *Pachymedusa dactinolor* (Bagnara *et al.*, 1988), common carp, *Cyprinus carpio*, Nile tilapia, *Oreochromis niloticus* (Fujii *et al.*, 1988), blue-green damselfish, *Chromis viridis* (Fujii *et al.*, 1988, 1989), freshwater goby, *Odontobutis obscura* (Matsuno and Iga, 1989) and neon tetra, *Paracheirodon innesi* (Nagaishi *et al.*, 1990; Nagaishi and Oshima, 1992; Fujii, 1993).

Silvery or metallic iridescence on the body and fins of *B. splendens* is contributed mainly by iridophores that are present as individual cells or closely associated with an underlying layer of melanophores and other chromatophores (Khoo, 1995; Amiri and Shaheen, 2012). Under bright-field light microscopy, iridophores were observed as large multicolored granules or oval-shaped platelets having a range of purple, pink, yellow, white, blue, and green hues (Fig. 3b). The platelets of *B. splendens* were observed to lack well-defined corners and had seemingly wavy or rippled alternating light and dark bands (Lucas, 1968). Light microscopy observations on iridophores of the neon tetra also revealed that these plates of guanine crystals are hexagonal in shape (Nagaishi *et al.*, 1990).

Metallic colors of dark blue, ultra-marine and turquoise-bronze *B. splendens* are a result of reflection from a melanophore screen beneath the iridophores (Fig. 3a), with combinations of melanophores and xanthophores imparting a wide range of blue and green hues as in the blue-green lateral stripes of the neon tetra (Nagaishi *et al.*, 1990). Besides reflection, white light is also refracted by iridophores and Tyndall-scattered by white and colorless guanine particles (leucosomes) in leucophores (Fujii, 1993). The Tyndall effect produced by leucophores of the golden *B. splendens* together with refraction from iridophores that interdigitate with yellow xanthophores might generate silvery-golden iridescence (Khoo, 1995). Lucas (1968) suggested that the "spread iridocyte color" of metallic blue is a result of Tyndall blue when viewed against a dark screen of melanophores. Hence, yellow colored xanthophores interspersed among melanophores and densely packed refractive iridophores might cause Tyndall blue to appear green in the turquoise-bronze *B. splendens* (Fujii, 1993; Khoo, 1995).

Electron microscopy of iridophores yielded interesting results for the metallic colored strains. Light-colored metallic strains, such as the golden, have two types of membrane-bound reflecting platelets in iridophores, i.e., large thick platelets and small thin ones (Fig. 5a; Khoo, 1995). The stacked platelets appear to be regularly oriented in parallel arrays at various oblique angles. The actual platelets are usually not observed but are represented by empty membrane-enclosed spaces as they might have become detached during sectioning and staining. In this study, some platelets were well preserved and could be observed as thin lines enveloped within fine membranes (Fig. 3). These transparent platelets, inclined at varying angles, form a multilayered thin-film interference system in the liquid cytoplasm, thus rendering the cells very refractive and reflective (Nagaishi et al., 1990; Fujii, 1993; Khoo, 1995; Amiri and Shaheen, 2012).

Iridescent blues and greens, for which *B. splendens* is well-known, could be traced to two chromosomal loci whereby one locus affects the density of overlying iridophores, and the other the thickness of guanine platelets and the refraction of a particular color (Wallbrunn, 1958). The dark blue, ultra-marine and turquoise-bronze strains possess iridophores that are situated above melanophores with cell processes that interdigitate and overlap amongst the cells (Figs. 5b-e; Khoo, 1995). It has been proposed that the designation "irido-melanophore unit" be used to define the close association represented by these two pigment cell types of *B. splendens* (Khoo, 1995). This study also reveals that the irido-melanophore unit of *B. splendens* consists of these two cell types which form interdigitations with xanthophore processes (Fig. 5b). The irido-melanophore unit of *B. splendens* parallels the melanophore-backed iridophores of the neon tetra (Nagaishi et al., 1990; Fujii, 1993) and the dermal chromatic unit of Bagnara and Hadley (1973) and Amiri and Shaheen (2012).

Reflecting platelets in the irido-melanophore units are arranged in parallel arrays and enclosed within multiple stacks in the cytoplasm (Fig. 5; Khoo, 1995). Variable inclination angles and thicknesses of the iridophore reflecting platelets primarily generate the metallic iridescent hues of *B. splendens*. Inclination angles of reflecting platelets in the irido-melanophore unit appear to increase in the following ascending order: dark blue < ultra-marine < turquoise-bronze (Figs. 5c-e; Khoo, 1995). Calculated angles of platelet orientation in iridophores of the neon tetra reveal that dark violet hues are generated by very small angles while red iridescence is attributed to the large angles (Nagaishi et al., 1990; Fujii, 1993). In essence, light of shorter wavelengths such as blue and violet are reflected by small angles but larger angles reflect light of longer wavelengths, e.g., yellow and red (Fujii, 1993; Amiri and Shaheen, 2012). This supports the observations for *B. splendens* in which platelets that are almost horizontally oriented generate the deep purplish-blue sheen of the dark blue strain while larger angles produce bluish-green and silvery-golden iridescence of the turquoise-bronze and golden strains, respectively (Figs. 1, 2 & 5).

In conclusion, this study has provided an in-depth description of the cellular basis for the brilliant color patterns of the perennially popular long-finned Siamese fighting fish, and has clearly illustrated the specific combinations of pigment cells that are responsible for the tremendous range of color variations of this teleost species. Additionally, reflection, refraction, thin-film interference, and Tyndall effect, coupled with varying inclination angles and thicknesses of guanine reflecting platelets in iridophores that are closely associated with other chromatophores, are inferred to produce a wide spectrum of metallic iridescent colors ranging from dark blue to silvery-golden in *B. splendens*.

Acknowledgements

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