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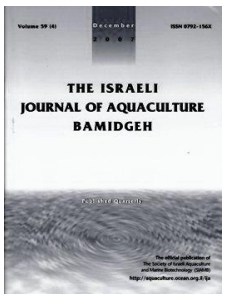

Ultrastructure of Erythrophores and Xanthophores of the Siamese Fighting Fish, *Betta splendens*

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Abstract

The ultrastructural morphology of brightly colored pigment cells (chromatophores) of Siamese fighting fish, *Betta splendens*, was investigated by transmission electron microscopy. The major pigment cells in the epidermis and dermis of the red and golden strains were erythrophores and xanthophores, respectively. Specific combinations of these chromatophores formed the basis of pigmentation patterns in both strains. The ultrastructure of the erythrophores was characterized by ellipsoidal electron-lucent vesicles that had limiting membranes and inner lamellae. The latter appeared whorl-like due to a concentric arrangement of parallel membranes. The xanthophores contained small and large cytoplasmic vesicles that appeared hollow and electron-lucent, with some vesicles displaying slightly electron dense particles. Sections of some large vesicles also revealed a very thin membrane enveloping these droplet-like vesicles.

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Introduction

Most teleosts possess five basic types of pigment cells (chromatophores): melanophores, erythrophores, xanthophores, iridophores, and leucophores (Bagnara and Hadley, 1973; Bagnara, 1983; Fujii, 1969, 1993). *Xiphophorus* spp. have a unique chromatophore, called the xantho-erythrophore, which has a yellow center and a red periphery. Chromatophores can be separated into three main shapes: a corolla with petal-like processes radiating from a disc-like center, dendritic with irregular processes branching from a small cell body, and small round punctate cells with short stumpy processes.

Erythrophores contribute to red pigmentation in many fishes (Fujii, 1969, 1993). In goldfish, erythrophores contain a mixture of drosopterin compounds enclosed within spherical cytoplasmic organelles called pterinosomes (Matsumoto, 1965; Matsumoto and Obika, 1968). Besides pterinosomes, erythrophores also contain carotenoid pigments, specific components for storage (Fujii, 1969).

Yellow or xanthic (gold) coloration in fishes is attributed to xanthophores, which contain carotenoids (Fujii, 1969, 1993). The lipid-soluble carotenoid pigments are dietary in origin and stored among the pterinosomes in vesicles of different sizes. These vesicles possess an extremely thin membrane extending from or in direct contact with the endoplasmic reticulum (Obika, 1993). Zeaxanthin and lutein are the carotenoids responsible for yellow coloration (Goodrich et al., 1941). Besides carotenoids, xanthophores also have yellow and colorless pteridines enclosed within pterinosomes. The xantho-erythrophore, found only in Xiphophorine fishes, is a single cell with both pteridines and carotenoids (Valenti, 1973). Its yellow center contains carotenoids while pteridines are found in the red periphery.

Fishes of the family Belontiidae are popular with aquarists primarily because of their varied array of pigmentation patterns. Previous investigations concentrated on the genetic basis of fancy color strains and morphological mutants of the Siamese fighting fish, *Betta splendens* Regan (Regan, 1910; Wallbrunn, 1958; Lucas, 1968, 1972). This study endeavors to clarify the cellular basis of coloration of the long-finned *B. splendens* using bright-field light and transmission electron microscopy (TEM) techniques.

Materials and Methods

Red and golden strains of long-finned *B. splendens* (Fig. 1) were obtained from a commercial farm in Lim Chu Kang, Singapore. The fish were maintained in 1.5-l mineral water bottles, each of which contained one male *B. splendens* (Khoo, 1995). The fish were fed freeze-dried bloodworms daily and fresh tubifex weekly. Water was changed weekly to maintain water quality.

Scales from the dorso-lateral regions were detached and mounted individually in teleost physiological saline (TPS: 6.5 g NaCl, 0.4 g KCl, 0.15 g $CaCl_2.2H_2O$, 0.15 g $MgSO_4.7H_2O$ in 1 l deionized-distilled H_2O, pH 7.3). An Olympus BHS-2 binocular light microscope (Tokyo, Japan) was used at 200-1,000× magnifications to observe the chromatophores.

a b

Fig. 1. *Betta splendens*: (a) red strain with dull blood-red body and fins, (b) golden strain with xanthic-colored body, i.e., metallic silvery-golden spots and yellow fins.

The ultrastructural morphology of the pigment cells was studied using a Jeol JEM-100CX II transmission electron microscope (Tokyo, Japan) at 7,200-54,000× magnifications. Scales were detached from the dorso-lateral region of each strain and pre-fixed for 60 min at 20-23°C in a 2.5% glutaraldehyde-2% paraformaldehyde mixture (TAAB Laboratories Equipment Ltd., UK) prepared in Sorensen's phosphate buffer (11.88 g $Na_2HPO_4.2H_2O$ and 9.08 g $KH_2PO_4.H_2O$ in 1 l deionized-distilled H_2O respectively, pH 7.2. Osmolarity was adjusted with sucrose to 224 mOsm using a Wescor 5500 Vapor Pressure Osmometer). The scales were immersed in the TPS, then repeatedly washed with Sorensen's buffer for 20 min. Scales were post-fixed for 60 min at 20-23°C with 1% OsO_4 (TAAB Laboratories Equipment Ltd., UK), washed thoroughly in buffer for 20 min, and dehydrated through an ascending ethanol (Merck, Germany) series (30-100%). This was followed by infiltration with a 1:1 propylene oxide (TAAB Laboratories Equipment Ltd., UK) and 100% ethanol mixture, and a 100% propylene oxide. A 1:1 mixture of propylene oxide and Spurr's low viscosity embedding resin (EM Sciences, USA) was used to enhance resin infiltration before the scales were finally embedded in Spurr's resin at 80°C in a Memmert oven (Schmidt Scientific, Germany) for 48-72 h.

Ultrathin sections (80-120 nm) of the scales were cut on an LKB Ultrotome Nova (Bromma, Sweden) with newly prepared glass knives and mounted on formvar-coated SPI 100-150 mesh size copper grids (USA). The sections were dried in a desiccator, stained with uranyl acetate (TAAB Laboratories Equipment Ltd., UK) for 15 min, washed thoroughly with five changes of distilled water to remove excess stain, and double-stained with lead citrate (TAAB Laboratories Equipment Ltd., UK) for a further 15 min before a final rinsing with distilled water. Mounted, stained longitudinal sections of the scales were dried overnight in a desiccator and observed at an accelerating voltage of 80-100 kV. Photomicrographs were taken at different magnifications using Kodak 4489 ESTAR film.

Results

The colors and pigment patterns of red and golden *B. splendens* were due to the presence of different types of pigment cells. Analysis of the cellular basis of coloration using light microscopy and transmission electron microscopy shows pigment cells in the pigment cell region below the basal lamina of a vertically sectioned scale (Fig. 2). The five main chromatophore types were noted: erythrophores, xanthophores, melanophores, iridophores, and leucophores (Khoo, 1995). The present study focuses on the first two as they were brightly pigmented and provided background coloration in both strains, usually in combination with the other types.

Under high-magnification light microscopy, red spherical pterinosome granules seemed uniformly distributed in the erythrophores while xanthophores had large vesicles

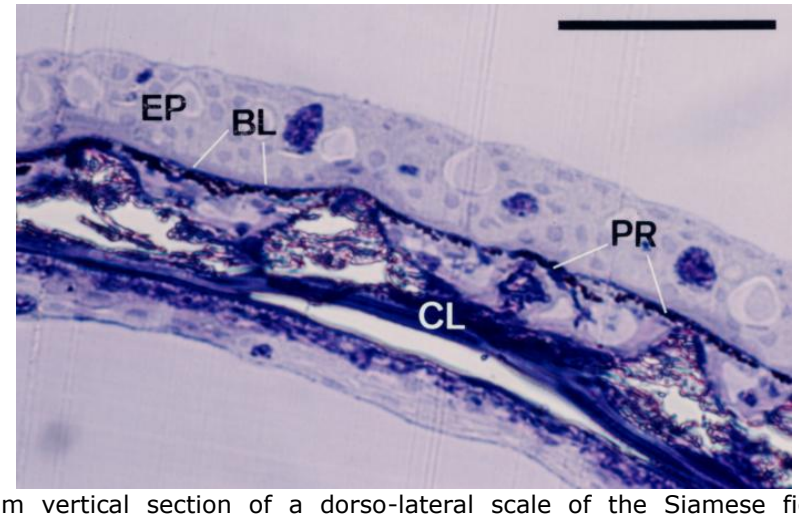

Fig. 2. One µm vertical section of a dorso-lateral scale of the Siamese fighting fish, *Betta splendens*, showing epidermal layer (EP), basal lamina (BL), pigment cell region (PR), and collagen layer (CL). Bar = 50 µm.

containing yellow carotenoids and smaller pterinosomes (Fig. 3). The erythrophores were distinctly corolla-shaped with only a few having long dendritic processes when viewed under the light microscope. The xanthophores were small and dendritic-shaped.

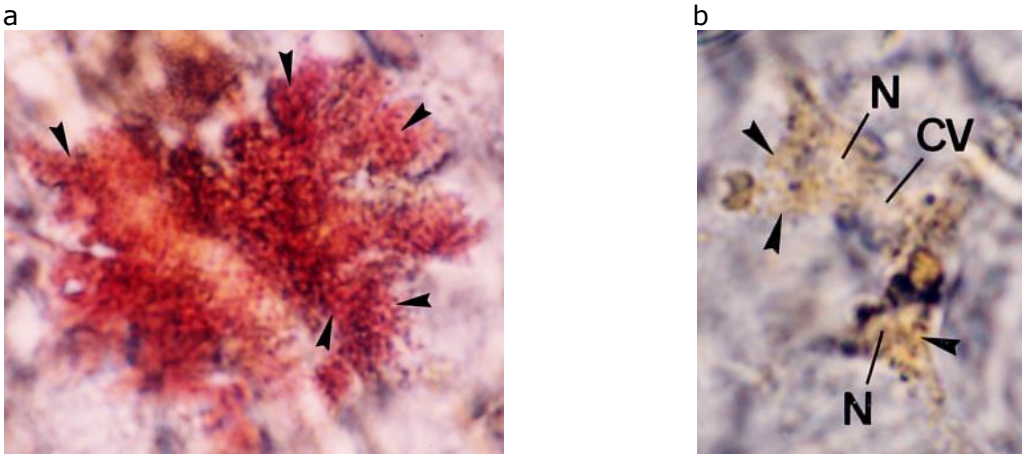

Fig. 3. (a) Corolla-shaped erythrophore of the red strain of *Betta splendens* with red pterinosomes (arrowheads) uniformly distributed in the cytoplasm, 1,000×, and (b) dendritic xanthophores of the golden strain of *Betta splendens* with yellow pterinosomes (arrowheads), carotenoid vesicles (CV), and colorless nucleus (N), 1,000×.

Under TEM, vertically sectioned erythrophores displayed large spherical cytoplasmic organelles that possessed a limiting membrane and inner lamellae (Fig. 4a). Small electron-lucent vesicles were also present in the cytoplasm, together with a large nucleus. The internal lamellae of the larger organelles contained whorl-like rings of concentrically arranged parallel membranes, ostensibly of a dense fibrous material (Fig. 4b). Slightly electron dense particles and vesicular inclusions seemed to be present in some of these organelles.

The xanthophores displayed two main types of cytoplasmic vesicles (vacuoles), i.e., small and large, which appeared hollow and electron-lucent under TEM (Fig. 5). The smaller vesicles were more numerous than the larger ones. Some appeared to enclose slightly electron dense particles. Sections of some large droplet-like vacuoles revealed a

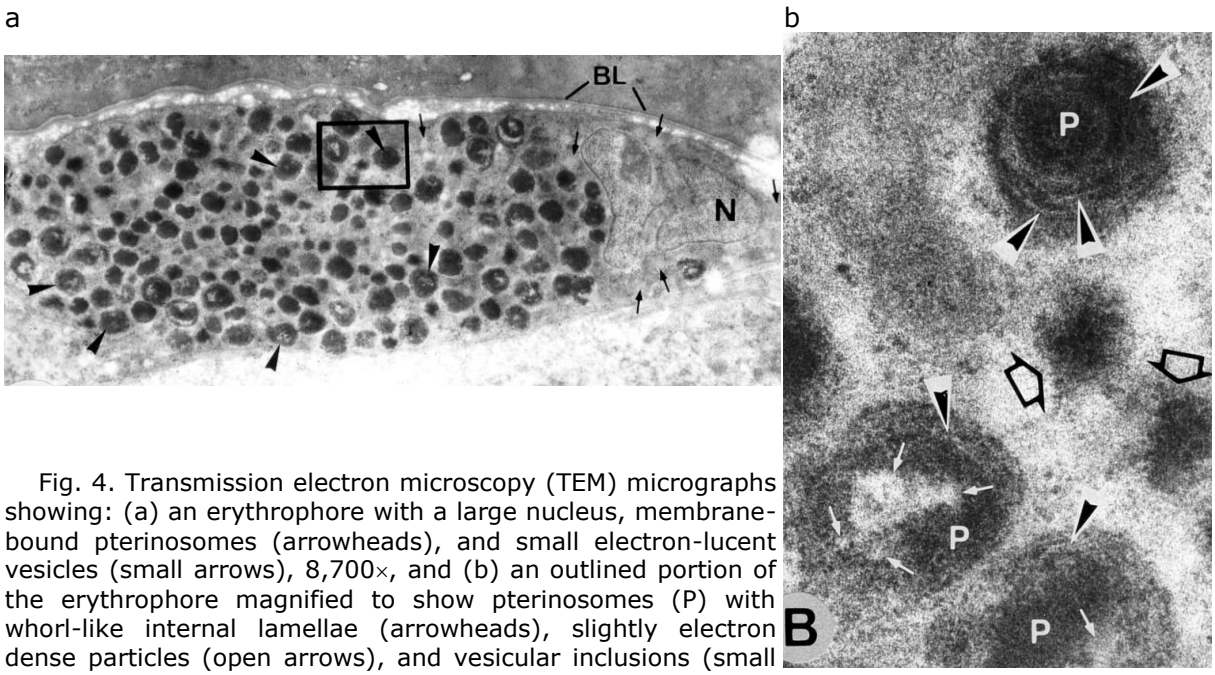

Fig. 4. Transmission electron microscopy (TEM) micrographs showing: (a) an erythrophore with a large nucleus, membrane-bound pterinosomes (arrowheads), and small electron-lucent vesicles (small arrows), 8,700×, and (b) an outlined portion of the erythrophore magnified to show pterinosomes (P) with whorl-like internal lamellae (arrowheads), slightly electron dense particles (open arrows), and vesicular inclusions (small arrows), 54,000×.

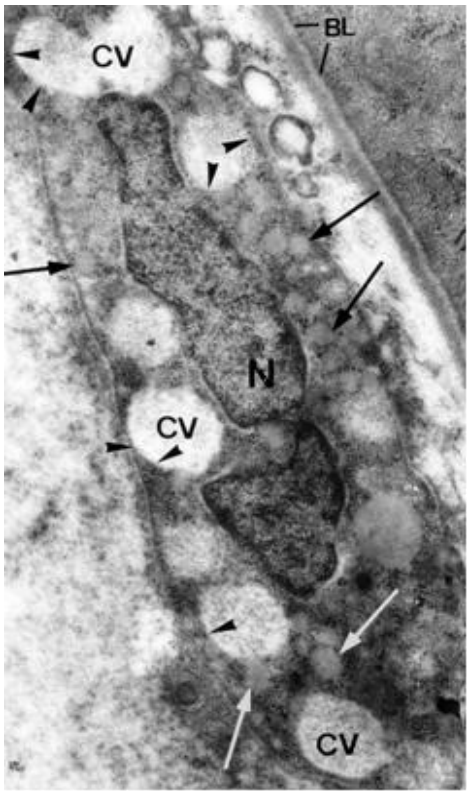

Fig. 5. Xanthophore with large hollow carotenoid vesicles (CV) and smaller ones with slightly electron dense particles (arrows) and a membrane-bound polymorphic nucleus below the basal lamina. A thin membrane (arrowheads) seems to envelop the large vesicles, 21,000×.

very thin membrane enveloping these vesicles. Most of the xanthophores had a large membrane-bound polymorphic nucleus in the cytoplasm while no cytoplasmic organelles with whorl-like internal lamellae were observed in any of the xanthophores.

Discussion

The Siamese fighting fish, *Betta splendens* Regan (1910), is popular among ornamental fish breeders due to its convenient size, hardiness, noticeable physical, physiological, and behavioral traits, and, most importantly, its brilliant and striking color variations (Wallbrunn, 1958; Lucas, 1968; Kirpichnikov, 1981). It has been cultured in southeast Asia for centuries, mainly for the sport of fish fighting (Lucas, 1972). Selection for pugnacity, long fins, and bright color has produced a number of phenotypes, none of which is similar to the short-finned wild form that is widely distributed in hill streams, forest creeks, sluggish rivers, swamps, and paddies of this region (Regan, 1910; Wallbrunn, 1958; Lucas, 1968; Witte and Schmidt, 1992; Kottelat and Ng, 1994).

The genetic basis of body and fin color variations in *B. splendens* and its hybrids, which possess a wide range of morphological variation in finnage patterns, have been well reported by Wallbrunn (1958) and Lucas (1968, 1972). In contrast, the pigment patterns of *B. splendens* have so far been investigated using only conventional bright-field light microscopy. This study incorporated the use of transmission electron microscopy to elucidate the cellular basis of red and yellow pigmentation in *B. splendens*.

Light microscopy and TEM reveal that brightly colored pigment cells of *B. splendens* display morphological and ultrastructural features specific to each cell type. Erythrophores of *B. splendens* appear to possess discrete spherical red pterinosomes (Fig. 3a; Matsumoto, 1965; Matsumoto and Obika, 1968; Fujii, 1969, 1993) that can be separated into different drosopterin compounds (Matsumoto, 1965; Matsumoto and Obika, 1968; Valenti, 1973). Conversely, the reddish-brown pigments in *B. splendens* erythrophores might be of non-pteridine origin (Khoo, 1995), e.g., phaeomelanins (Fujii, 1993) or an intermediate product of a blocked melanin synthesis pathway (Goodrich et al., 1941; Royal and Lucas, 1972).

Photomicrographs of pterinosomes in erythrophores of *B. splendens* show large discrete oval-shaped cytoplasmic organelles with a trilaminar limiting membrane and whorl-like concentric internal lamellae of parallel membranes (Fig. 4; Khoo, 1995). The ultrastructural morphology of these organelles is identical to that of the pterinosomes in swordtail, *Xiphophorus helleri* (Matsumoto, 1965), sailfin molly, *Poecilia latipinna* (Blanchard et al., 1991), and medaka, *Oryzias latipes* (Obika, 1993). Some appear similar to the pterinosomes of goldfish (*Carassius auratus*) erythrophores (Matsumoto and Obika, 1968) and *O. latipes* xanthophores (Obika, 1993) in that they have slightly electron dense particles and vesicular inclusions (Fig. 4b). The large cytoplasmic organelles may be associated with pteridine-based pigments while carotenoids are possibly enclosed within the smaller electron-lucent vesicles (Bagnara and Hadley, 1973; Bagnara, 1983). These intracellular structures might represent different maturity stages of organelles that possess well developed inner lamellae (Obika, 1993) and are

comparable to pre-melanosome vesicles derived from the Golgi complex during melanogenesis (Bagnara, 1983; Blanchard et al., 1991).

Xanthic or golden pigmentation in *B. splendens* is dependent on yellow carotenoid pigments. Carotenoid pigments are present in xanthophores in terrestrial vertebrates (Bagnara and Hadley, 1973; Bagnara, 1983) and fish species (Fujii, 1969; Valenti, 1973). Due to the dense yellow pigment, xanthophores appear diffused and do not show discrete margins under light microscopy (Fig. 3b). Ultrastructural morphology of xanthophores shows two types of cytoplasmic vesicles (vacuoles), i.e., those with large seemingly hollow electron-lucent vesicles, presumably containing carotenoids, and smaller ones that have slightly electron dense particles (Fig. 5). The large droplet-like vesicles seem enveloped within a very thin limiting membrane, and are apparently attached to endoplasmic reticular-like tubulo-vesicular structures (Khoo, 1995). These vesicles appear empty, possibly due to the dissolution of lipid-soluble carotenoids during the fixation and dehydration process in preparation for electron microscopy (Obika, 1993). Small electron-lucent vesicles interspersed among the large vesicles may contain yellow and colorless pteridines (Khoo, 1995). The carotenoid vesicles of *B. splendens* seem identical to the vesicles in xanthophores of *C. auratus* and *O. latipes* (Matsumoto, 1965; Matsumoto and Obika, 1968; Obika, 1993). Under TEM, xanthophores of *B. splendens* often display polymorphic nuclei (Fig. 5).

This study enables aquaculturists to understand the importance of red (pteridine-based) and yellow (carotenoid-based) pigmentation in sexual selection, mate choice, and signal evolution in fishes. Carotenoid-based sexual coloration is a well-known signal of mate quality (Bagnara and Hadley, 1973; Bagnara, 1983; Grether et al., 2001). However, animals are unable to synthesize carotenoids and must ingest them from dietary sources such as plants and microalgae. The presence of carotenoid-containing xanthophores may be a direct indicator of foraging ability and, thus, an indirect indicator of health and vigor in male fishes. Carotenoids are not the only pigments in *B. splendens* (Khoo, 1995). Pteridines are also present but, unlike carotenoids, they can be synthesized by animals. Erythrophores and xanthophores of *B. splendens* contain both red pteridines (drosopterins) and carotenoids (Khoo, 1995), similar to the guppy (*Poecilia reticulata*) in which there is a positive correlation between drosopterin use and higher carotenoid availability in the environment (Grether et al., 2001). Developing a method to improve male health and vigor is especially important to fish breeders because *B. splendens* males have high commercial value in the ornamental fish industry.

In conclusion, this study provides an in-depth description of the ultrastructural basis for red and yellow coloration in the perennially popular long-finned *B. splendens*. Further investigation into the dietary use of carotenoids and pteridines in aquaculture feeds is needed to enhance the red and yellow pigmentation of this commercially important species.

Acknowledgements

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