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Ontogenetic Changes in Color Vision in the Milkfish (*Chanos chanos* Forsskål, 1775)

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The milkfish (*Chanos chanos* Forsskål, 1775) is a euryhaline fish widely distributed in tropical and subtropical Indo-Pacific waters. It is unique in having in the cephalic region adipose eyelid tissue that begins to develop in the larval stage and is completely formed by the juvenile stage. The formation of the adipose eyelids coincides with the onset of active swimming ability. Larval and juvenile milkfish have different dietary modes and habitats. This study was aimed to investigate ontogenetic changes in color perception ability with the use of microspectrophotometry (MSP). Larval milkfish had rod cells and red, green, blue, and violet cone cells, while juvenile milkfish lost the violet cone cells, and the blue cones shifted to shorter wavelengths. Histological sections showed the presence of cone cells of the single type (but no double or twin types) in the retina, which implies that the milkfish may not have polarized vision.

Key words: adipose eyelid, microspectrophotometry, cone cell, retina, polarized vision

INTRODUCTION

The milkfish (*Chanos chanos*, Gonorhynchiformes) is a pelagic euryhaline fish inhabiting the tropical and subtropical Indo-Pacific Ocean and Red Sea, where temperatures are higher than 20°C (Winans, 1985). This species is regarded as one of the most suitable species for fish aquaculture (Bardach et al., 1972), with hundreds of years of operation (Bagarinao, 1991). It takes 3 to 5 years for this pelagic migratory fish to reach maturity, at which standard length (SL) exceeds 1 m (Bagarinao, 1994). During the breeding season, adult milkfish swim from the pelagic region to spawning sites located offshore near coral reefs or small inlands. Newly hatched larvae move from the spawning grounds to inshore water, which takes about 10 days. The trip inshore is largely confined to the surf zone; while lacking the ability to swim, the larvae are transported by currents (Taki et al., 1987; Morioka et al., 1996). After reaching in inshore region, milkfish larvae are about 10–17 mm in total length (TL). Here, they are called “fry” or “seed” and are caught for aquaculture. During the larval stage, milkfish are planktivorous and their main feeding mode is swallowing (Taki et al., 1987). They capture prey mostly with the aid of vision (Blaxter, 1988), and the eye is likely the major sensory organ to detect food at this stage, because they only take food under lighted conditions (Kawamura and Hara, 1980).

To reach the juvenile stage (TL>20 mm), a milkfish undergoes metamorphosis (Kawamura and Hara, 1980; Kawamura, 1984; Bagarinao, 1994) and becomes a more powerful swimmer. It then migrates to lagoons, mangroves, and estuarine waters where the food is rich and the environment is more sheltered (Bagarinao and Kumagai, 1987). Feeding behavior switches from swallowing to filtering (Hiatt, 1944), and the diet becomes more variable and includes blue-green algae, diatoms, copepods, arthropods, nematodes, and detritus at this stage (Bagarinao and Thayaparan, 1986). Milkfish juveniles longer than 25 mm SL already possess most adult characters. These juveniles leave the nursery waters and move into the ocean. Occasionally, some milkfish live for many years in the large lagoons, atolls, or lakes and have adult body sizes, but do not reach sexual maturity (Bagarinao, 1994).

Light environments in aquatic habitats are quite different from those on the land. Because of the selective absorbance of different light wavelengths by the water, suspended particles, and phytoplankton (Maske and Haardt, 1987; Cleveland and Weidemann, 1993; Sogandares and Fry, 1997; Schubert et al., 2001; Babin et al., 2003) with increasing depth, the light environment is bluer in the clear open ocean, but greener in costal waters (McFarland, 1986). Fishes have to express suitable visual pigments to adapt to the environmental light spectrum and shift their visual spectrum accordingly when they migrate to different habitats (Shand et al., 1988; Wood et al., 1992; Douglas et al., 1998; Shand et al., 2002; Carleton et al., 2005; Pointer et al., 2005). Many examples of fishes shifting the visual spectrum to ultraviolet (UV) (280–400 nm) in different life stages illustrate this adaption. For instance, the salmonids, yellow perch (*Perca flavescens*), and bluegill sunfish (*Lepomis macrochirus*) have UV-sensitive pigments in the juvenile stage but lose it in the adult stage, while many minnows, goldfish (*Carassius auratus* L.), and some damselfishes possess UV photoreceptors only in the adult stage (Losey et

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al., 1999).

UV vision is more common in fishes (Jacobs, 1992; Honkavaara et al., 2002) than in other vertebrates and aids in visual communication, camouflage, and color vision (Cott, 1940; Neumeyer, 1992; Losey et al., 1999). UV vision may also protect the eye against overexposure to UV light, which can be harmful (Salo et al., 1998a; Salo et al., 1998b; Losey et al., 1999; Salo et al., 2000; Jokinen et al., 2001).

The presence of UV-protective pigments in zooplankton is thought to reduce UV transparency and to subsequently enhance contrast under UV light (Johnsen and Widder, 2001); therefore, many fish larvae and planktivorous fishes have UV vision (Loew et al., 1993; Britt et al., 2001; Flamarique, 2005), and UV contrast assists them in detecting planktonic prey (Tovée, 1995; Britt et al., 2001; Siebeck and Marshall, 2001).

The ontogeny of milkfish from the larval to the juvenile stage is accompanied by significant changes in environments and feeding habits. Therefore, it is of scientific merit to investigate how the visual spectrum of milkfish adapts to various visual environments. The first part of this study addressed: (1) How many kinds of photoreceptor cells do milkfish have? (2) Since milkfish larvae are planktivorous, do they use UV vision to find their prey? (3) What are the visual spectral ranges of larval and juvenile milkfish? (4) Does a shift in the visual spectrum occur during the ontogeny of milkfish, reflecting the change in feeding habits and diet during growth?

The milkfish is unique in having adipose eyelids, which extend from the post-snout to the opercular region of the head (Stewart, 1962). Chang et al. (2009) investigated the composition and transmission spectrum of these eyelids and found that they can filter out part of UV light, which offers protection by preventing harmful UV light from reaching the retina. The second part of this study addressed the following two questions relevant to the adipose eyelids: (1) What is their pattern of ontogeny? (2) Do the UV-filtering adipose eyelids interfere with the visual spectrum?

MATERIALS AND METHODS

Fish

Juvenile milkfish with an average standard length of 13.41±1.07 cm were bought from a tackle shop in Keelung, Taiwan. Larval milkfish 3 weeks old, and averaging 6.54±0.7 mm in total length (by convention, the total length of larval fish is measured), were bought from a fish hatchery in Pingtung, Taiwan. The juvenile milkfish were kept in seawater (ca. 32‰ salinity) or freshwater in either 2- or 4-ton FRP tanks at 25–30°C under a photoperiod of 12L:12D (hours) for at least two weeks prior to use. They were fed ad libitum with artificial milkfish feed twice a day. The larval milkfish were kept in seawater in 2-ton FRP tanks at 25–30°C under ambient photoperiod. They were fed green algae, egg yolk, artificial rotifers, and fish feed three times each day.

Microspectrophotometry (MSP)

For microspectrophotometry, the larval and juvenile milkfish were both kept in seawater (ca. 32‰ salinity) at 25–30°C under a 12L:12D photoperiod for at least two weeks prior to use. Microspectrophotometry (MSP) was used to measure the color reception of the retinal cells. Loew (1982) described the principles and methods of MSP in detail. To protect the photoreceptor cells from light bleaching, the following procedures were carried out inside a dark room, where only a dim red light (620 nm) source was used to provide the needed illumination. After at least 6 hours of dark adaptation, the eyes of either larval or juvenile milkfish were enucleated and then dipped into an ice-cold 6% sucrose PBS solution (Sigma P4417, USA). The eye was then dissected under an SMZ1000 dissecting microscope (Nikon, Japan) viewed through with a 26–1020 night-vision scope (Bushnell, USA) to separate the retina. Preliminary data showed that the cone and rod cells maintained activity for up to 24 hours when kept in an ice-cold 6% sucrose PBS solution (Sigma P4417, USA), because within 24 hours after retinal preparation, the cells still responded to a typical 120-sec exposure of 350–750 nm bright light, the so-called "bleaching reaction."

To conduct MSP, a drop of 6% sucrose PBS solution and a small piece of retinal tissue were placed onto a cover slip, and a dissecting blade was then used to tease apart a small piece of retinal tissue, in order to dissociate the photoreceptor cells. After mounting these retinal cells between cover slips, we performed the MSP measurement at room temperature (20–23°C). The monochromator of the microspectrophotometer allowed the scanning of light wavelengths from 350 nm to 750 nm. The beam scanning was first performed on a clear background site near the visual cell to obtain background readings. The light beam was then moved to the outer segment of either the cone or rod cell, and a second scan was performed, and the values were recorded. The computer software then subtracted the background values from the recorded values and calculated the absorption spectrum of that particular retinal cell. To prove the viability of the retinal cell examined, right after the recording a strong bright light was shone on the retinal cell for 2-min to bleach it. Another MSP reading was then made, with no response from the bleached cell indicating that the previous reading was valid. The visual spectra of rod cells and different kinds of cone cells were compared between larval and juvenile milkfish by using Minitab software (one-way ANOVA, Tukey's pairwise comparisons).

Ontogeny of the adipose eyelids

Larval milkfish were sampled once every 3 days for observation of the ontogenetic development of the adipose eyelids. Under an SMZ1000 dissecting microscope (Nikon, Japan), milkfish with adipose eyelids were separated from those without them. These two classes of milkfish were both fixed and preserved in 10% formaldehyde solution in aqueous phosphate buffer (Malinckrodt Lot H121 B47754, USA). After fixation, the milkfish were measured (TL) and their developmental stage was determined. The definitions of the larval and juvenile stages followed that of FishBase (http://www.fishbase.org/search.php): a larva is a young fish which at birth or hatching is fundamentally unlike its parent and must pass through metamorphosis before assuming adult characters; a juvenile is a young fish, mostly similar in form to an adult but not yet sexually mature.

Histological sectioning and staining

For paraffin sections, retinas sampled from juvenile milkfish and whole heads from larval and juvenile milkfish were fixed in a 10% formalin solution in aqueous phosphate buffer (Malinckrodt LOT H121 B47754, USA) for 24 hours. The fixed heads were then bathed in decalcification solution (Lot 930803, Shimakyu's Pure Chemicals, Japan) for 3 days, with the solution changed every day. The fixed retinas and decalcified heads were then dehydrated in a graded ethanol series (50%, 50%, 70%, 80%, 95%, 100%, 100%), for 1 hour in each step. The dehydrated samples were transferred twice into xylene, for 1 hour each time, and then into liquid paraffin in an oven at 60°C, for two days. The samples were then embedded and sectioned (Leica RM2245, Germany) at a thickness of 5–7 μm. These sections were stained with hematoxylin-eosin (H&E). The stained sections were observed under an E600 microscope (Nikon, Japan), and photomicrographs were taken with a Coolpix 4500 digital camera (Nikon, Japan).

RESULTS

Visual spectra of the larval and the juvenile milkfish

MSP measurements indicated that the larval milkfish had four types of single cone cells (red, green, blue, and violet) and one type of rod cell in the retina. The maximal absorbance wavelength (λ_{max}) in larval milkfish was 578.61±10.39 nm (mean±SE, N=28) for the red cone cells, 506.56±10.24 nm (N=44) for the green cone cells, 468.45±9.78 nm (N=64) for the blue cone cells, 423.16±9.12 nm (N=19) for the violet cone cells, and 507.37±5.30 nm (N=271) for the rod cells (Fig. 1A). The λ_{max} in juvenile milkfish was 580.27±6.05 nm (N=75) for the red cone cells, 502.28±8.48 nm (N=65) for the green cone cells, 441.97±7.70 nm (N=30) for the blue cone cells, and 497.05±7.81 nm (N=37) for the rod cells (Fig. 1B). No violet cone cells were found in the juveniles, and when the milkfish passed from the larval into the juvenile stage, the blue cone cells showed a statistically significant spectral shift to a wavelength shorter by 30 nm ($P<0.05$). A typical relative absorbance spectrum for each type of visual cell is shown in Fig. 2A–F.

Ontogeny of the adipose eyelid

Both histological sections and dissection microscopic observations showed that the adipose eyelid was absent in larvae, but present in juveniles (Fig. 3A–D). The adipose eyelid began to develop at the periphery of the eye socket, and then gradually expanded to the center of the eye. The extending adipose eyelid finally coalesced and formed a chamber between the eyelid and eye. H&E stained sections showed that the medial connective tissue did not comprise the greatest part of the adipose eyelid as the latter began to form, but continued to grow so that the thickening of eyelid was due mainly to this tissue. The youngest milkfish with an adipose eyelid was 10.5 mm in TL, and the oldest without an adipose eyelid was 18.7 mm in TL (Fig. 4). These results indicate that formation of adipose eyelid was complete once the milkfish had reached the juvenile stage.

Histology of the retina

The retina of both larval and juvenile milkfish comprised six layers beneath the pigment epithelium (PE) layer (Fig. 5A): (1), the rod and cone layer, containing the outer segments of the rod and cone cells; (2) the outer nuclear layer (ONL), which contained the nuclei of the rods and cones, with the positions of the cone nuclei usually higher than those of the rods; (3) the outer plexiform layer (OPL), which contained the synapses of the dendrites of the photoreceptor cells and the axons of the bipolar cells and horizontal cells; (4) the inner nuclear layer (INL), which contained the nuclei of the bipolar, horizontal, amacrine, and neuroglial Müller's cells; (5) the inner plexiform layer (IPL), which contained the axons of the bipolar cell synapses and the dendrites of the amacrine and ganglion cells; and (6) the ganglion cell layer (GCL), in which

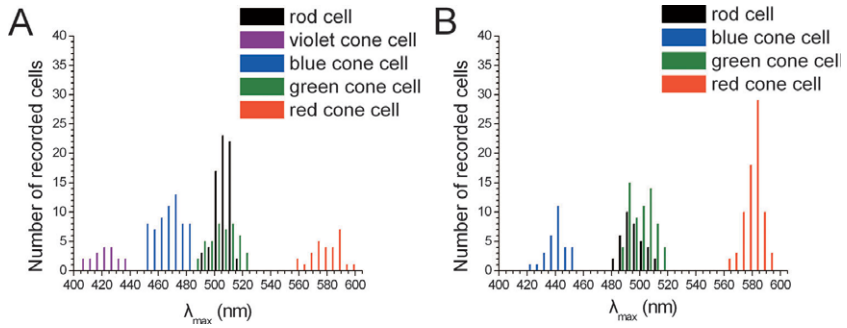

Fig. 1. **(A)** MSP data for larval milkfish. **(B)** MSP data for juvenile milkfish. In both panels, the y-axis shows the number of recorded rod cells (black), red cone cells (red), green cone cells (green), blue cone cells (blue), and violet cone cells (violet) and their maximum adsorption wavelength (λ_{max}) values.

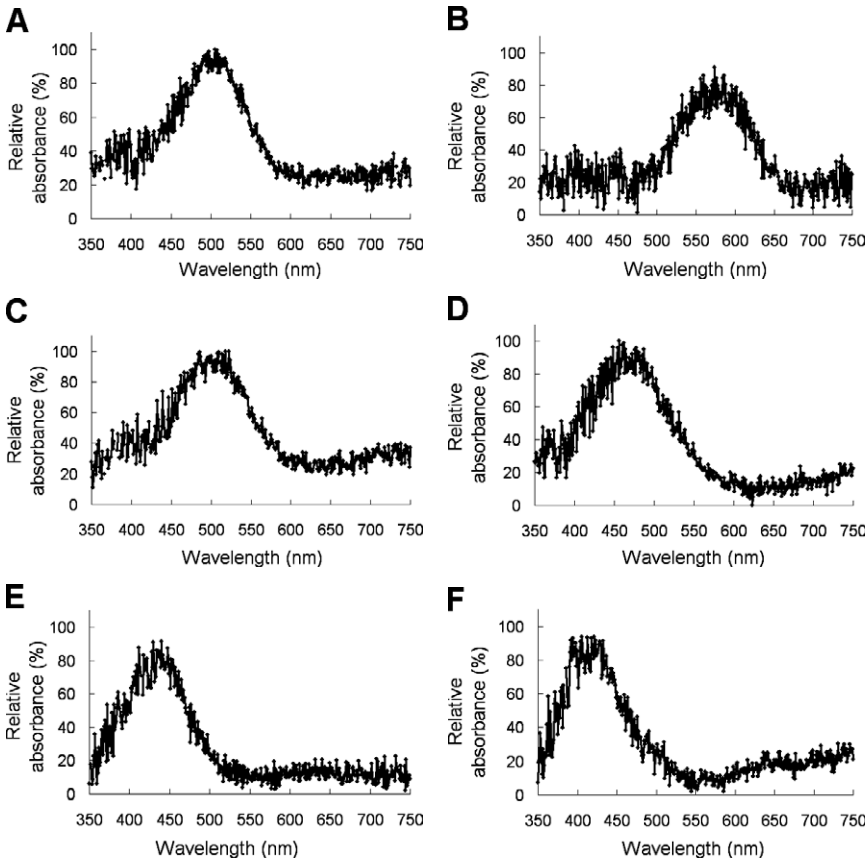

Fig. 2. Examples of typical relative absorbance spectra of visual cells. **(A)** Rod cell. (509 nm) **(B)** Red cone cell. (570 nm) **(C)** Green cone cell. (500 nm) **(D)** Blue cone cell (470 nm). **(E)** Blue cone cell (440 nm). **(F)** Violet cone cell. (425 nm).

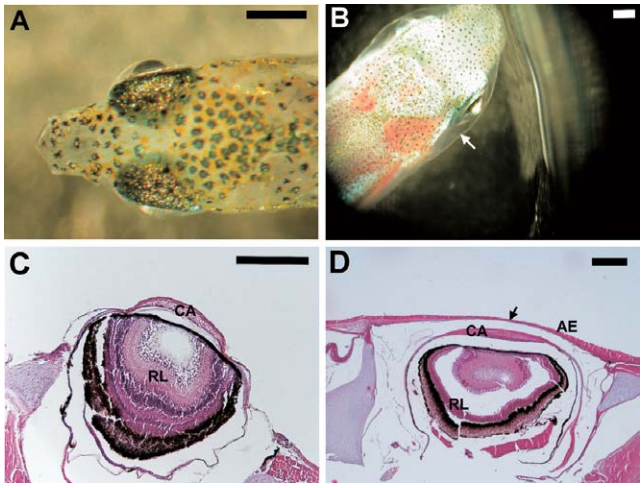

Fig. 3. **(A, B)** The head region of **(A)** larval and **(B)** juvenile milkfish photographed under a dissection microscope. The adipose eyelid is absent in the larval stage but present in the juvenile, as indicated by the arrow in (B). **(C, D)** Sagittal sections of the **(C)** larval and **(D)** juvenile milkfish head; arrow in (D) indicates the adipose eyelid. AE, adipose eyelid; CA, cornea; RL, retinal layer. See Fig. 5 for details of the RL. H&E staining. Scale bars, 0.5 mm (A, B), 250 μm (C, D).

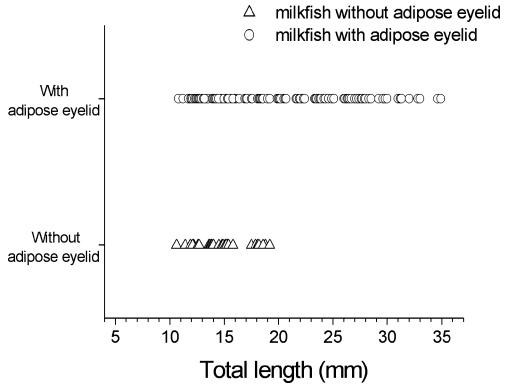

Fig. 4. Relationship between milkfish total length and the development of the adipose eyelids.

the cell bodies of the ganglion and neuroglial cells were found (Fig. 5A). Because processing from the visual cells to the ganglion cells was convergent, the nuclei in the ONL, INL, and GCL gradually became fewer. Radial paraffin sections stained with H&E revealed that the milkfish has a duplex retina, with only one type of cone cell (single cone); double cone and twin cone cells were absent. Paraffin sections of light- and dark-adapted retinas revealed movement of the screening pigments inside the visual cells. In the light-adapted eyes, the pigments invaded the rod and cone layer, and rods were positioned higher than the cones; in the dark-adapted eyes, the pigments retreated, and the cones moved to the periphery of the rod and cone layer (Fig. 5B).

DISCUSSION

The larval milkfish has violet, blue, green, and red cone photoreceptors (Fig. 1A). As the larvae grow into juveniles,

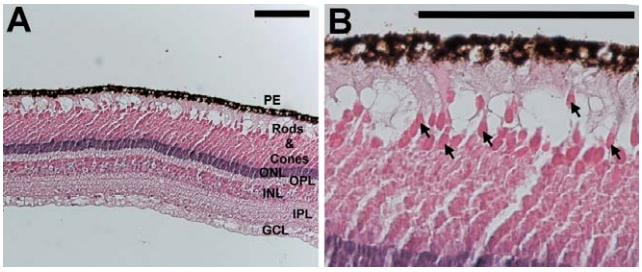

Fig. 5. **(A)** Transverse paraffin section showing layers of the retina of the milkfish lying beneath the pigment epithelium (PE): rod and cone layer; outer nuclear layer (ONL); outer plexioform layer (OPL); inner nuclear layer (INL); inner plexiform layer (IPL); and ganglion cell layer (GCL). See the Results section for descriptions of the layers. **(B)** Enlargement of part of the rod and cone layer in panel (A), showing single cone cells (arrows) and rod cells (asterisks). H&E staining. Scale bars, 100 μm.

the violet cones disappear, and the blue cones shift about 30 nm to shorter wavelengths (Fig. 1B). There are two possible explanations for this shift. First, because different light wavelengths have unequal transmission rates in water, fishes that live at different depths will experience different light conditions. When a fish migrates to different depths as it grows, it should shift its visual spectrum accordingly to match the photic conditions in the new surroundings. Second, a spectrum shift should also coincide with a diet shift to maximize the capture of prey. For example, juvenile pollack (*Pollachius pollachius*) and juvenile yellow perch (*Perca flavescens*) are planktivorous and have violet and UV photoreceptors. When they grow and undergo a dietary shift, the violet and UV vision disappear (Shand et al., 1988; Loew et al., 1993). Visual spectrum shifts thus can be affected by changes in both the ambient light spectrum and feeding habits.

Larval milkfish inhabit the inshore region, where they are planktivorous and ingest prey by swallowing. The violet cones could offer a higher-contrast image of the zooplankton, making it easy for the fish to perceive them (Tovée, 1995; Britt et al., 2001; Johnsen and Widder, 2001; Siebeck and Marshall, 2001). Juvenile milkfish cease preying on plankton; their prey changes from zooplankton to cyanobacteria, benthic invertebrates, and pelagic fish eggs and larvae (FishBase; http://www.fishbase.org/search.php). The specialized gill rakers of the juvenile milkfish help them capture prey by filtering. This change in food organisms makes the violet cones unnecessary. Juvenile milkfish (SL>25 cm) live in the open sea, where the water is clearer than in nearshore and coastal waters; the blue cone spectral shift from 470 nm to 440 nm functions to receive shorter-wavelength light, which better penetrates the water of the open sea (Sogandares and Fry, 1997). The MSP data demonstrate that a shift in the visual spectrum reflects the change in feeding habits and diet during the ontogeny of the milkfish.

Visual pigment contains chromophore and opsin proteins. Changes in both chromophore type and expression of various opsin genes could alter the visual spectrum (Brown et al., 1963; Bridges, 1967; White et al., 2004; Yokoyama, 1995). Most fish have all five groups of opsins: RH1, RH2, SWS1, SWS2, and LWS/MWS, with multiple subtypes

(Yokoyama, 2000), and two types of chromophores, based on vitamin A1 (retinal) or vitamin A2 (3, 4-dehdyroretinal) (Ueno et al., 2005). For example, the zebrafish has two red (*LWS-1* and *LWS-2*) and four green (*RH2-1*, *RH2-2*, *RH2-3*, and *RH2-4*) opsin genes. In-situ hybridization data indicate that both the red and green opsin genes initiate expression at shorter wavelength (*LWS-2*, *RH2-1*, and *RH2-2*), followed by expression at longer wavelengths (*LWS-1*, *RH2-3*, and *RH2-4*) (Takechi and Kawamura, 2005). Site-directed mutagenesis of these opsin genes shows that some residues are more critical than others to the maximum absorption spectra (λ_{max}) (Cowing et al., 2002; Shi and Yokoyama, 2003; Chinen et al., 2005). These critical residues offer the organism an avenue to evolve quickly to adapt to the ambient spectrum. Cone cell regeneration is not necessary in visual spectrum shifts, because the expression of different opsin genes in the same cone cell is observed when the visual spectrum shifts (Cheng and Flamarique, 2004).

Chromophores generally process A1 in marine fishes, and A2 (or both A1 and A2) in freshwater fishes, but vary in composition in most fish. Chromophore composition can be affected by many factors, including the life stage of the fish, season of the year, and habitat (Ueno et al., 2005). However, the visual spectrum shift from 470 nm to 440 nm observed in the milkfish may be due only to different opsin gene expression, for two reason: (1) A chromophore shift has a larger effect on longer wavelengths (as great as 60 nm) and a smaller effect on shorter wavelengths (as little as 5 to 10 nm) (Whitmore and Bowmaker, 1989; Bowmaker, 1995), but the red cones were statistically unchanged between the larval and juvenile stages; (2) The visual spectrum shift from 470 nm to 440 nm was too large to be accounted for by chromophore exchange (Parry and Bowmaker, 2000).

The milkfish is not born with adipose eyelids. The adipose eyelids begin to form in the larval stage (ca. TL=10 mm) and are completely formed before the milkfish reaches the juvenile stage (TL=20 mm) (Fig. 4). A similar phenomenon has also been observed in *Mugil cephalus* and *Trachurus trachurus*, whose adipose eyelids are not formed until the juvenile stage (Jacot, 1920; Rangaswamy, 1987; Artüz, 2000). Adipose eyelid contain type-I collagen fibrils, the same material that forms tendons and supplies them with tensile strength (Chang et al., 2009). In light of this property, the eyelids could be resilient in absorbing the force exerted by water during high speed cruising of milkfish. The milkfish becomes a powerful swimmer when it reaches the juvenile stage (TL>20 mm), and this may explain why the adipose eyelids are completely formed by this stage: to streamline the head for swimming at high speed, and to protect the head from force exerted by the water (Chang et al., 2008).

The retina of the milkfish is composed of both cone and rod cells, as observed. Teleosts have six types of cone cells: single, bilobed, double, twin, triple, and quadruple (Lyall, 1956; Lyall, 1957; Collins and MacNichol Jr, 1978; Fineran and Nicole, 1978). In most fishes in shallow seas, there is an increase in rod cells with increasing depth, but it is not always true that a decrease in the double and twin cones (Yew and Wu, 1979) is related to depth changes. The milkfish is considered a shallow sea fish, and its retina contains only single cones (Fig. 5B). In most fishes, the retina consists of cone mosaics, i.e., double or twin cones form squares, with single cones either in the middle or the corners of the squares (Lyall, 1957; Engstrom, 1963; Engström and Ahlbert, 1963). The two mechanisms for polarized vision in fish are the presence of double cones arranged in square mosaic units (e.g., salmonids), or the presence of bilobed cones (e.g., anchovies) (Fineran and Nicole, 1978; Flamarique and Hawryshyn, 1997; Flamarique and Hawryshyn, 1998; Hawryshyn, 2000; Flamarique and Harosi, 2002). Although polarized vision could help fishes detect zooplankton (Shashar et al., 1998; Flamarique and Browman, 2001; Sabbah and Shashar, 2006), milkfish seem unlikely to have this ability because they lack double cones.

The relative transmission spectrum of the adipose eyelid shows that the latter could block some part of UVB (Chang et al, 2009). From a comparison with the juvenile milkfish visual spectra (Fig. 1B), it is safe to state that the adipose eyelid does not interfere with the overall visual cell reactions. The adipose eyelid of the milkfish is transparent. UV vision has many meanings to fishes, but a contrasting condition is found in some coral reef fishes. Many coral reef fishes have ocular media that block UV, but at the same time possess UV-sensitive cones (Siebeck and Marshall, 2001; Losey et al., 2003; Nelson et al., 2003). Although the milkfish has a UV-penetrable adipose eyelid, it lacks UV vision.

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