4. Discussion

In terms of fish phylogenetic relationship, the monophyly of Teleostei is divided into four subdivisions: the Osteoglossomorpha, the Elopomorpha, the Ostarioclupeomorpha, and the Euteleostei. The fishes with adipose eyelids are found in the last three subdivisions except the most primitive subdivision, the Ostarioclupeomorpha (Arratia, 2001; Nelson, 2006). According to the cladogram (Nelson, 2006), the adipose eyelid character could be traced back to the coancestor of the Elopomorpha, and the Ostarioclupeomorpha. Since the fossil records of the Osteoglossomorpha, the Elopomorpha, and the Ostarioclupeomorpha were all found in the upper Jurassic (Frickhinger, 1995), the adipose eyelid is one of the fish ancient characters which evolves first in the upper Jurassic and exists until today.

For years, due to its translucent nature, all the known eyelids have been regarded as of adipose composition. In this study, the histology sections and SEM photos demonstrate that there are three layers in the eyelid, and they are either the epithelial or the connective tissue (Figure 1). Because of the negative reaction of the Oil Red O staining, no adipose tissue could be found in the adipose eyelid (Figure 3). The middle layer is considered the connective tissue because it is positive to the Pico-Ponceau with Hematoxylin staining (Figure 2). The connective tissue could have three kinds of fibers, i.e., the collagen fibers, the reticular fibers, and the elastic fibers. The possibility of the adipose eyelid contains elastic fiber is excluded by the negative reaction of the Orcein staining (Figure 4). Both of the collagen and reticular fibers are composed of collagen fibrils, but it is curious to know which type of the collagen fibril forms the connective tissue part of the eyelid. For example, the reticular fiber is usually formed by the type III collagen fibrils (Ross et al., 1995). SDS-PAGE reveals that the collagen extracted from the milkfish adipose eyelid is the type I collagen fibril which has two α1 chains and one α2 chain but not the type III collagen fibrils (Figure 11). The results revealed that the connective part of the eyelid is formed by the collagen fibers. Because of the aforementioned characteristics of the eyelid, hence the long accepted term "adipose" should be considered a misnomer. Perhaps the "connective eyelid" would be a proper name for the eyelid structure.

A series of the histochemical staining methods, the PAS staining (Figure 6), the Alcian Blue pH 2.8 staining (Figure 7), the Alcian Blue pH 1.0 staining (Figure 8), and the Aldyhyde Fuchsin-Alcian Blue staining (Figure 9), finally identified that the substances comprised of the middle connective tissue part is either epithelial sulfomucins or epithelial mucosubstances.

The milkfish is not born with the adipose eyelid. The adipose eyelid starts its formation from the larval stage (ca. TL=10 mm) and it is completely formed before the milkfish reaches the juvenile stage (TL ≧20 mm) (Figure 14). Similar phenomena are also observed in the *Mugil cephalus* and the *Trachurus trachurus*, whose adipose eyelids are not formed until the juvenile stage (Jacot, 1920; Rangaswamy, 1978; Artűz, 2000).

The chamber between the adipose eyelid and the eyeball is filled with liquid. The osmolarities of the liquid are always higher than those of the ambient waters (Figure 17) which clearly indicate they are under active osmoregulation. In the teleost fish ion regulation, many kinds of ion transport proteins which are expressed in the fish gills and kidneys have been identified to participate in the ions secretion or ions absorption mechanisms, for example, the Na^+ /K^+ ATPase (NKA), V-type H^+-ATPase, chloride ion channels likes CFTR, and Na^+ /K^+ /(2) Cl^- cotransporter (NKCC). The NKA is thought to participate in both ion absorption and ion secretion functions, whereas the NKCC is more associated with the ion secretion function. These proteins have differentially expressed in both different salinity environments and among fishes (Marshall, 2002; Tipsmark et al., 2002; Perry et al., 2003; Lin et al., 2006; Lionetto & Schettino, 2006; Prodocimo & Freire, 2006). The NKA expression in the milkfish eyelid is recognized by the immunostaing and Western blotting methods (Figures 25, 27, 28) and the NKA is expressed in the baselateral part of the epithelial cell of the outer multiple epithelial tissue as it is also expressed in the mitochondria-rich cell in fish gill whether in long-term freshwater adapted fish or long-term seawater adapted fish (Marshall, 2002). The NKCC expression is recognized by the Western blotting but the molecular weight data fail to reveal its true identity. The NKCC belongs to the cation-chloride cotransporters (CCC) family which contains: one Na^+ /Cl^-

cotransporters, two isoforms of NKCC, four isoforms of K^+ /Cl^- cotransporters, and two unknown function cotransporters. The CCC family has 12 transmembrane domains and the members of the CCC family are at least 100 KDa (Delpire & Mount, 2002). The Western blotting reveals that the T4 antibody conjugated proteins are single band which is about 50 KDa (Figure 29). The protein degradation seems impossible for there is only single band reaction in the Western blotting. Because the expressions of the T4 antibody conjugated proteins are correlative to the salinity transfer period, therefore, it is suggested that they are directly or indirectly involved in osmoregulation.

The presence of the ion transport protein may be the reason why the chamber fluid's osmolarities are always higher than those of ambient osmolaerities. The concentrations of the proteins in the chamber liquid are neither positively nor negatively correlated to the salinity translation period. These proteins seem not to be affected by the environment salinity, and it is speculated that they could be originated from the degenerated epithelial cell of the inner single layer epithelial tissue or it may be derived from the plasma (Figure 24). The goal of the osmoregulation in teleost fish is to maintain its plasma osmolality that is hyper-osmotic to the environment in freshwater and hypo-osmotic to the environment in seawater (Evans, 1993). However, the milkfish chamber fluid's osmolality is maintained hyper-osmotic to the environment in freshwater and slightly hyper-osmotic to the environment in seawater.

This phenomenon is similar to the body fluid osmolality of the elasmobranch fish in freshwater and seawater (Thorson et al., 1973; Piermarini & Evans, 1998). This study finds that the adipose eyelid chamber fluid of milkfish, one of the teleost fish, displays the osmoregulative character of the elasmobranch fish.

The functions of the adipose eyelid are long believed to protect the eyes from the impacts of water molecules as well as offering streamlining of the cephalic region during high speed cruising. Yet, how the adipose eyelid achieves these functions has never been addressed in details. The adipose eyelid is shown to have the type I collagen fibril which also forms the tendon and supplies the tendon with the tensile strength (Lodish et al., 2003). In light of this property, the eyelid could be resilient in absorbing the impacts by the water molecules during high speed cruising of milkfish. Furthermore, chamber fluid which is always hypertonic to the surrounding solution offers the turgor pressure to separate the adipose eyelid from the eyeball and therefore it acts as a damper to absorb the possible impacts from water molecules directly exerted to the eyes. In the course of the life history of the milkfish, it becomes a powerful swimmer when it reaches the juvenile stage, this may explain why the adipose eyelids are formed completely at this stage. In analogy, the protection mechanism of the eyelid is similar to the air cushion used in athletic’s shoes to protect the feet. The chamber liquid acts as the liquid cushion and the ion transport proteins would indirectly inflate it, at most two days, after transfer to a new ambient salinity.

The water goes through the adipose eyelid passively by the osmolarity gradient, then the water channel, aquaporin, may also be expressed in the adipose eyelid to control the water movement when the milkfish is in the hypertonic or hypotonic ambient just like the different expressions of aquaporin in the euryhaline fishes' gills could prevent water loss in hypertonic ambient and permit water absorption in hypotonic solution (Lignot et al., 2002; Watanabe et al., 2005; Deane & Woo, 2006). The presence of the aquaporin in the eyelids remains to be investigated.

The type I collagen fibril supports the tensile strength of the adipose eyelid by its strong intermolecular cross-linking between two α_1 chains and one α_2 chain which forms to stabilize the heterotrimeric of the type I collagen (Miles et al., 2002). Such a nature raises an additional question: How does the adipose eyelid become transparent and maintain its shape? Comparing with the cornea and lens, they are both transparent ocular structures. The cornea is affluent in extracellular matrix (ECM) which contains a number of different collagen types, and the cornea transparency is owing to the short-range arranged array of these collage fibrils which is maintained by the interaction between collagens and proteoglycans (Robert et al., 2001; Ihanamäki et al., 2004). The alteration of the fibril arrangement would destroy the natural cornea structure (Liu et al., 2003; Meek et al., 2003). The lens is composed of crystallins, and the transparency of it is due to the short-range interactions among three types of crystallin proteins, α-, β-, and γ- (Delaye & Tardieu, 1983; Benedek et al., 1999). Any disruption of the protein-protein interaction that reduces the solubility of the crystallin proteins would induce the cataract and turns the lens into milky white condition and obscures the vision (Fu & Liang, 2003; Pone et al., 2006). Therefore, adipose eyelid must have some mechanisms to maintain the transparency and the form while offering tensile strength at the same time. These mechanisms remaine to be investigated.

From the phylogenetic relationship and the cladogram (Nelson, 2006), the earliest fish which has the adipose eyelids is conjectured to be the concestor of the Elopomorpha and the Ostarioclupeomorpha which are also powerful swimmers. The possible benefits of being high speed swimmers are to escape from the predators and to catch the preys. Yet at the same time, it also increases the possibility of mechanical damages to the eyes because of impacts from high speed contact with water molecules. To overcome this challenge, high speed swimming fishes evolved to have the adipose eyelid and utilized the most common type of collagen fibrils, the type I collagen fibril, which are the major collagen fibrils to provide the needed protection (Miller & Rhodes, 1982; Saito et al., 2001).

The retina of milkfish is composed of both cone and rod cells as observed. The teleosts have five types of cone cells: single, double, twin, triple, and quadruple cones (Lyall, 1956; Lyall, 1957; Collins & MacNichol Jr, 1978). In most of the shallow sea fishes there is an increase of the rod cells with increasing depth, but it is not always true that the decrease of the double and twin cones (Yew & Wu, 1979) is related to depth changes. The milkfish is considered a shallow sea fish and its eyes contain only the single cone in its retina (Figure 16). In most of the fish, the retina is of cone mosaics, i.e., the double or twin cones form the squares with single cones set either in the middle or corner of the square (Lyall, 1957; Engstrom, 1963; Engström & Ahlbert, 1963). Lacking double and twin cones, the milkfish eye has a retina with irregular single cone arrangement. Because of lack of double cones, the polarized vision is not likely to have in milkfish (Flamarique et al., 1998). If the homing behavior in milkfish is true, how the milkfish navigate itself without polarization vision remains a question to be tackled. Many fish species are known to use various cues for navigation, such as the magnetic detection, the olfaction, the positive rheotaxis, the auditory, and the salinity preference all could guide fishes' orientation (Hawryshyn et al., 1990; Quinn & Dittman, 1990; Oguar et al., 1992; Dittman & Quinn, 1996; Stobutzki & Bellwood, 1998; Tolimieri et al., 2000; Atema et al., 2002; Leis et al., 2003). It would be of great biological significance to know which cues are used by milkfish in returning to their spawning sites.

The relative transmission spectrum of adipose eyelid shows that the adipose eyelid could block some part of UVB, and the light with wavelength shorter than 340 nm could not reach the retina (Figure 30). Comparing with the juvenile milkfish visual spectrum (Figure 34), it is safe to state that the adipose eyelid doesn't interfere with the overall visual cell reactions. For milkfish, the adipose eyelid is transparent.

The UV vision has many meanings to fishes, but a contradictory condition is found in coral reef fishes. Many of the coral reef fishes have UV blocking ocular media and possess UV sensitive cones at the same time (Siebeck & Marshall, 2001; Losey et al., 2003; Nelson et al., 2003). Although milkfish has a UV penetrable ocular medium, it doesn’t have UV vision.

The photoretinoscope data indicate that the adipose eyelid doesn’t participate in the eye focusing mechanism (Figure 32). Stewart (1962) hypothesized that the two possible fuctions of adipose eyelid are (1) to increase the focusing power and (2) to detect polarized light. In light of the aforementioned findings, the adipose eyelid may only perform simple task to protect fish eyeballs from mechanical damage, to absorb UV light and to streamline the cephalic region.

The larval milkfish has violet cones (λmax = 423.16± 9.12 nm), blue cones (λmax = 468.45± 9.78 nm), green cones (λmax = 506.56± 10.24 nm), and red cones (578.61± 10.39 nm) (Figure 33). Yet as the larva grows into juvenile stage the violet cones disappeared and the blue cones shift about 30 nm to shorter wavelength. As mentioned before, the visual spectrum shifts could be affected by the ambient light spectrum and the feeding habitats. The larval milkfish lives in the lagoons, mangroves, and estuary where the water is turbid and it is planktivorous who hunts the zooplankton by swallowing it. The violet cones could offer a higher contrast image of the zooplankton and make them easy to be perceived by the fish. The juvenile milkfish is no longer a planktivorous predator, in fact, the food habitat change from the zooplankton to the cyanobacteria, benthic invertebrates, and pelagic fish eggs and larvae. The specialized gill rakers of the juvenile milkfish help them to capture the preys by filtering action. The food organism change makes the violet cones no longer needed. Because the juvenile milkfish lives in the open sea where the water is more lucid than the near shore and coastal wetlands, the blue cones shift from 470 nm to 440 nm is meant to receive shorter wavelength light.

The visual pigment contains the choromophore and the opsin protein. Both of the choromophore type change and the different opsin gene expression all could alter the visual spectrum (Brown et al., 1963; Bridges, 1967; Yokoyama, 1995). Most fish have all five opsin genes: RH1, RH2, SWS1, SWS2, and LWS/MWS, with multiple subtypes (Yokoyama, 2000) and two types of the choromophores: vitamin A1- (retinal) and vitamin A2- (3,4-dehdyroretinal) based chromophores (Ueno et al., 2005). The visual spectrum shifts from 470 nm to 440 nm could be due to either the vitamin A1-/A2- based choromophore change or the different opsin gene expression or both.