2. Materials and Methods

2.1 Materials

Juvenile milkfish with standard length (SL) 13.41±1.07 cm were bought from a tackle shop in Keelung, Taiwan. Larval milkfish at 3-week old were bought from a fish hatchary in Pingtung, Taiwan. Juvenile milkfish were kept in seawater (ca 32 ‰ salinity) or freshwater in either 2- or 4-ton FRP tanks at 25-30℃ with a 12L12D hours photoperiod for at least two weeks prior to use. They were fed *ad libitum* with artificial milkfish feed twice a day. Larval milkfish were kept in seawater in 2 ton FRP tanks at 25-30℃ with a nature photoperiod. They were fed with green algae, egg yolk, artificial rotifer, and fish feed 3 times each day.

2.2 Experimental designs

In the osmoregulation experiment, the juvenile milkfish were first kept in seawater or freshwater for at least 2 weeks and then were transferred directly to either freshwater or seawater. The adipose eyelids, adipose eyelid chamber fluid, and blood were sampled when the milkfish were in the new salinity conditions 0 hour, 3 hours, 6 hours, 12 hours, 18 hours, 1 day, 2 days, 3 days, 4 days, 5 days, and more than 7 days later. The juvenile milkfish which had been kept in seawater for more than 14 days were used for collagen extraction, light transmission, and microspectrophotometry

(MSP) studies. The ontogenetic processes of adipose eyelid formation were observed on larval milkfish reared in seawater for a span of 60 days. The microspectrophotometry was used to examine the color vision abilities of retinal receptor cells.

2.3 Histological sectioning and staining

Three histological section methods, i.e., paraffin section, frozen section, and resin section were used in this study. In paraffin section, the adipose eyelids and retinas sampled from juvenile milkfish and the whole heads gathered from larval and young juvenile milkfish were fixed with 10 % formaldehyde solution in aqueous phosphate buffer (Malinckrodt LOT H121 B47754, USA) for 24 hours. The fixed heads were then bathed in decalcification solution (Osaka 676400, Japan) for 3 days and the solution was changed every day. The fixed adipose eyelids, retinas, and decalcified heads then underwent a series of dehydration process in 50 %, 50 %, 70 %, 80 %, 95 %, 100 %, 100 % alcohol solution, they stayed for 1 hour in each step. The dehydrated samples were transferred first into xylene for 1 hour twice, and then into liquid paraffin in 60 ℃ inside an oven for two days. The samples were embedded and sectioned (Leica RM2245, Germany) at a thickness of 5 μm. These sections were stained with the following methods: Periodic Acid-Schiff (PAS), Hematoxylin-Eosin (H&E), Alcian Blue pH 1.0 (Luna, 1968), Pico-Ponceau with Hematoxylin (Gurr,

1956), Aldyhyde Fuchsin-Alcian Blue (Sheehan, 1980) and Orcein. However, due to its special requirement for the Alcian Blue pH 2.8 staining (Putt, 1971), the adipose eyelids had to be fixed in a special solution of formalin: 100% alcohol = 1: 9, then the tissues were directly dehydrated in 100% alcohol.

In frozen section, the adipose eyelids were fixed with 4 % paraformaldehyde in aqueous phosphate buffer in 4 ℃ for 10 min. After fixation, the adipose eyelids were immersed in 100 % acetone and then in 100 % alcohol, these two steps were carried out in -20 ℃ for 10 min each. Finally, the adipose eyelids were soaked in 30 % sucrose in 4 ℃ for 2 hours to offer cryoprotection of the tissues. The samples were embedded in tissue freezing medium (Jung 020106926, Germany) and sectioned at 10 μm (Leica CM1900, Germany). The cryo-sections were adhered onto the polysine-coating slides (Menzel GmbH & Co KG J2800AMNZ, Germany). The cryo-sections were treated with Oil Red O for lipids staining and were also used for immunostaining.

In resin section, the retinas were first cut into small pieces, each of them was about 5 mm × 5 mm. The specimens were fixed in 12 % glutaraldehyde solution for 90 min then were transferred to 12 % glutaraldehyde solution overnight. After fixation, the samples were rinsed with distilled water for 5 min twice and went through serial dehydration procedures in 50 %, 70 %, 90 %, 95 %, 100 %, and 100 % alcohol 5 min in each alcohol solution. The dehydrated samples were transferred into acetone

solution for 1 hour twice before they were bathed in acetone: low viscosity embedding medium (EMS Spurr's kit, USA) =3:1, 1:1, 1:3 for 4 hours in each step. After treating with pure Spurr's medium for 4 hours twice, a new Spurr's medium was used to embed the specimens and were cured inside a 70 ℃ oven for 12 hours. The embedded samples were sectioned at 1 μm (Microm HM350, USA) and sections were stained with Toluidine Blue O.

All the stained sections were observed under a microscope (Nikon E600, Japan) and microphotography was made with a digital camera (Nikon Coolpix 4500, Japan).

2.4 Scanning electron microscopy (SEM)

There were two parts of fixation in SEM. The first part of fixation of adipose eyelids was performed in P_4G_5 solution (4 % paraformaldehyde and 5 % glutaraldehyde in 0.1 M phosphate buffer) for 12 hours. After washing with 0.1 M phosphate buffer, the second fixation step was carried out in 1 % O_SO_4 solution for 2 hours. The fixed specimens underwent a serial dehydration steps in 30 %, 50 %, 70 %, 80 %, 95 %, 100 %, and 100 % alcohol before they were dried in critical point dryer (Hitachi HCP-2, Japan). The dried samples were coated with a layer of gold in an ion coater (Cressington 108, UK) then the outer and inner layer of adipose eyelids were observed and photographed under a scanning electron microscope (FEI Quanta 200, Holland).

2.5 Preparation of adipose eyelid collagen

The method for extracting the collagen from milkfish adipose eyelids followed the protocols previously used for the extraction of collagen from fishes (Sadowska et al., 2003; Nagai et al., 2004; Ogawa et al., 2004). All the preparations were made at 20℃ except the centrifugal procedures were at 4 ℃. The pooled 7 g adipose eyelids sample were collected and then washed by distilled water. These adipose eyelids were soaked in 0.1 N NaOH solution for 3 days to remove pigments and noncollagenous proteins, and the 0.1 N NaOH was changed daily. After cleaning adipose eyelids with distilled water, they were minced into small pieces. The whole preparation was bathed 48 hours in 140 ml 0.5 M acetic acid solution that was mixed with 0.7 g pepsin after this step, the collagen was dissolved in acetic acid solution and its trimer structure was dissociated by pepsin. Two days later, the solution was centrifuged at 10,000 ×g for 1 hour. The precipitate was then repeated with the previous dissociation and centrifuge processes one more time. The supernatants were collected twice, and the collagen in the supernatants was salted out by adding the 2.7 M NaCl to a final concentration of 0.9 M. The solution was left overnight in room temperature and was then centrifuged at 10,000 ×g for 25 min. The resulting precipitates were dissolved in 0.5 M acetic acid again then were dialyzed against 0.1 M acetic acid (Pierce 68700, USA) for removing the salt. The purified collagen was obtained after the solution was lyophilized with the

use of Labconco (77510-03, USA) system.

2.6 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) method

SDS-PAGE was used for identifying which type of collagen that was extracted from milkfish adipose eyelids. In this experiment, the Mini-PROTEAN 3 cell (Bio-Red 165-3301 & 165-3302, USA) was used for preparing electrophoresis gel (lower: 8 % separating gel and upper: stacking gel) and gel electrophoresis. The pure collagen was added with sample buffer to the concentration of 1 μg/μl and so did the calf skin collagen type I (FLU 27664, USA). These two sample buffers were denatured at 100℃ for 10 min. Each 10 μl of the protein marker (BIO-RED 161-0303, USA), denatured adipose eyelid collagen sample buffer, and denatured calf skin collagen type I sample buffer was loaded into the wells of the gel. The electrophoresis was started at 60 volt for 1 hour and then the voltage was increased to 120 volt until the electrophoresis was completed, all were under automatic control from the system. After the electrophoresis, the gel was drawn out from the glass space and stained in Coomassie Blue R-250 solution for 20 min. The stained gel was destained by destaining buffer (100 ml acetic acid, 300 ml methanol, and 600 ml distilled water) until the gel background was limpid.

2.7 Ontogenetic development of the adipose eyelids

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

2.8 Analyses of adipose eyelid chamber fluid and plasma

1 ml syringe and 1.5 ml micro-centrifuge tubes used for phlebotomization were coated with 0.1 % heparin (RDH 39201, USA) to prevent blood coagulation. 1 ml blood was sampled from juvenile milkfish caudal vein. After centrifugation at 10,000 ×g, 4℃ for 10 min, the plasma was separated from the corpuscles. The plasma was stored -20 ℃ until the osmolarity and ion concentrations were measured. The syringe

with 27-G needle was used to sample the adipose eyelid fluid. The withdrawal of chamber fluid was carried out by penetrating the needle through the adipose eyelid to the chamber. The fluids were kept under the same condition as that of plasma until they were analyzed.

The plasma and adipose eyelid fluid osmolarities were measured with an osmometer (Wescor 5520 VAPRO, USA) and each sample was measured 3 times to obtain an averaged reading. The chloride ion concentrations of plasma and adipose eyelid fluid were determined with the Ferricyanide method (Franson 1985). After a 10,000-fold dilution of plasma and chamber fluid, each diluted sample was mixed with 200 μl ammonium iron sulfate (RDH 40620, Germany) solution (60 g $NH_4Fe(SO_4)_2 \cdot 12H_2O$ in 100 ml 6 N HNO_3) and 100μl mercury thiocyanate (Alfa Aesar J14H05, USA) solution (1.5 g $Hg(SCN)_2$ in 500 ml 95 % ethanol). The optic density (OD) values of the samples were measured at 460 nm by a spectrophotometer (Hitachi U-2001, Japan). The standard chloride solutions (concentrations: 0, 0.25, 0.5, 1, and 2 ml/L) used for calibration were made with distilled water and concentrated chloride solution (Merck 119897, Germany).

The sodium and potassium concentrations were measured with an atomic absorption spectrophotometer (Hitachi Z-8000, Japan). The plasma and adipose eyelid fluid were diluted 5000 times for sodium concentration determination and 400 times for potassium concentration measurement. The standard curves of sodium and

potassium were established with the use of a sodium standard solution (Merck 119507, Germany) at concentrations of 0, 0.25, 0.5, 1, 2, and 4 mg/L and a potassium standard solution (Merck 170230, Germany) at concentrations of 0, 0.25, 0.5, 1, and 2 mg/L, respectively.

The adipose eyelid fluid was first diluted 4000 times with distilled water and then mixed with protein assay reagent (Bio-Rad, 500-0006 USA). The optic density values at 595 nm were measured with a spectrophotometer (Hitachi U-2001, Japan). The protein standard curve was generated by diluting the protein standard (Sigma P0834, USA) to 0, 2, 4, and 8 μg/μl with different optic density values. The Minitab software was used to analyze these data (One-Way ANOVA), and each value was compared to the final salinity treatment condition (Tukey's pairwise comparisons).

2.9 Immunostaining for Na^+/K^+ ATPase (NKA) and $Na^+/K^+/Cl^-$(2) cotransporter (NKCC)

The cryo-sections of the eyelid tissue were washed by PBS solution for 5 min twice to remove the tissue freezing medium. The specimens were soaked in 10 % H_2O_2 for 10 min to block possible endogenesis peroxidase activity then rinsed with PBS solution again. The antibodies for NKA (DHSB α5, USA) and NKCC (DHSB T4, USA) were diluted 1:3000 and 1:1000 respectively and then were reacted with frozen section samples at room temperature for 3 hours. After 3 hours of reactions, the PBS

solution was used to wash the specimens for 5 min twice. The reagent A (ready-to-use HRP polymer conjugate) of the commercial kit (Zymed 879963, USA) was applied to the sections at room temperature for 30 min, then the samples were rinsed again by PBS solution. Finally, the sections were reacted with the reagent B (ready-to-use AEC *Single Solution* chromogen) of the kit at room temperature for 10 min, and then rinsed with PBS solution to finish the immunostaining processes. The background was counterstained with hematoxyline and the slices were observed and microphotographs were taken under a microscope. For the confocal observation purpose, after the α5 antibodies were reacted with frozen section samples at room temperature for 3 hours, these samples were incubated with the secondary antibody anti-mouse IgG conjugated with the fluorochrome fluorescein (FITC) (Pierce 31547, USA) at room temperature for 2 hours. These sections were observed and microphotographs were taken under a confocal microscope (Leica TCS-NT, Germany).

2.10 Western blotting for NKA and NKCC

The ion transporter proteins, NKA and NKCC, in the adipose eyelids were extracted for Western blotting purpose. The adipose eyelids were immersed in 500 μl homogenizing buffer (100 mM imidizole, 5 mM Na_2EDTA, 200 mM sucrose, 0.1 % sodium deoxycholate in ddH_2O) with 1/400 proteinase inhibitor (10 mg antipain, 5 mg leupeptin, and 1 g benzamidine in 5 ml aprotinin) added, the tissues were first

minced by a pair of dissecting scissors and then stored in a 1.5 ml Eppendrof tube. The treated adipose eyelids were then homogenized by a tissue homogenizer (Polytron PT-3100, Switzerland). After 4 ℃, 13000 ×g, and 20 min centrifugation, the ion transporter proteins should reside in the supernatant. The protein assay kit (Bio-Red 500-0006, USA), standard protein (Sigma 54010, USA), and a spectrophotometer (Hitachi U-2001, Japan) were used to measure the protein concentration in each specimen. The eleven specimens obtained at different sampling times from freshwater to seawater environment were diluted with homogenizing buffer to the same protein concentration and then each of them was mixed with the 5× sample buffer. The protein denaturing condition for NKA was set of 37 ℃ for 5 min and for NKCC was 100 ℃ for 10 min. The 40 μg protein of each specimens and a 5 μl of the protein marker (Fermentas SM0671, USA) were loaded into the wells of the electrophoresis gel (lower: 8 % separating gel and upper: stacking gel). The electrophoresis was started at 60 volt for 1 hour and then the voltage was gradually increased to 120 volt until the electrophoresis was completed. After the electrophoresis was done, the gel was removed from the glass space. The proteins in the gel were transferred to the PVDF membrane by the horizontal electrophoresis process (10 V, 400 mA) for 26 min in the semi-dry transfer (Bio-Red 221BR13821, USA). After 2 hours of blocking (5g non-fat milk powder in 100 ml TTBS) for non-specific binding, the PVDF membrane was soaked in TTBS for 10 min twice for

cleanness. The cleaned PVDF membrane was then reacted with 1/10,000 α5 or T4 antibodies in TTBS at room temperature for 2 hours. The α5-reacted PVDF membrane was then immunoreacted with the 0.2 μg/ml peroxidase-labeled anti-mouse IgG antibodies (KPL 074-1806, USA) in TTBS solution. The T4-reacted PVDF membrane was immunoreacted with the 0.2 μg/ml phosphatase-labeled anti-mouse IgG antibodies (KPL 075-1806, USA) in TTBS solution at room temperature for 2 hours. After TTBS washing and pH 9.5 Tri-HCl buffer (0.1 M Tris-base, 0.1 M NaCl, 0.05 M $MgCl_2$) reaction for 5 min, the Super Signal® West Pico Chemiluminescent Substrate (PIERCE 34080, USA) and the NBT/BCIP Kit (KPL 508118, USA) were used to mark the proteins that were conjugated with antibodies. The image was scanned and analyzed with a KodaK digital Science 1D, 1995.

2.11 Light transmission measurements of adipose eyelid, cornea, and lens

The adipose eyelid, cornea, and lens were removed from the juvenile milkfish for light transmission measurements. They were first cleansed to remove either the blood or vitreous fluids with PBS solution (Sigma P4417, USA) and soaked in PBS solution in 4 ℃ prior to measurements. The measuring procedures were carried out as soon as the samples were prepared. A metal holding device was designed to secure the illumination fiber, specimen, and reading fiber at a horizontal position. The Ocean Optics 2000 spectrometer was used to measure the light intensities that passed

through the adipose eyelid, the cornea, or the lens from the wavelength of 300-700 nm. The light transmission data were obtained by comparing these data to the light intensities measured when the specimen was not present. Because lens was not of homogeneous nature, therefore the absolute light transmission could be altered by measuring it with different positions. However, the relative spectral transmission would not be altered by its positions. According to the method of Douglas & McGuigan (1989) all transmission curves were normalized at 700 nm which equaled to 100 %. Such a method allowed us to characterize the optic media wavelength at which 50 % transmission rate (T50) of these three different tissues were achieved.

2.12 Photoretinoscopy

The photoretinoscope was used to measure the refractive state of the eyes of juvenile milkfish. It was modified from the infrared photoretinoscope (Schaeffel et al., 1987) and was incorporated with the use of a digital camera (Nikon cooplix8700, Japan), a camera lens adapter, and a 622 nm LED light which was powered by two 1.5V batteries connected in series. The camera lens adapter was covered by a black board slightly over half of its aperture, and the LED light was configured at the center of the camera optical axis. The distance from the LED light to the upper margin of the black board was defined as the eccentricity *E*. The distance from the camera to the fish eye was defined as *A*; the radius of the pupil was *R*; and the ratio of the dark

fraction of the pupil to the total pupil diameter was *DF*. All of the dimensions were expressed in meters. The defocus *D* was calculated as

$D = E / (2 \times A \times DF \times R)$

In this study, A was 0.5 m and E was 10.5×10^{-3} m. If the eye is well focused, the photoretinoscope would show a photo with whole black pupil. If the eye is not well focused, the illuminated part of the pupil could be used to differentiate the milkfish eye was either myopic or hyperopic. The pupil with lower part illuminated is considered myopic and with upper part illuminated then it is hyperopic. The milkfish was kept in a narrow aquarium and was anesthetized with 0.3 mL/L 2-phenoxythanol (RDH 60248, Germany) to fix its gaze direction and reduce the eye accommodation. All equipments were set up in the dark room. After 10 min dark adaptation to allow dilation of the milkfish pupil, the eye was photographed by a photoretinoscope through the glass wall of the aquarium where the fish was held inside. The software Image J was used to determine the boundary between light and dark area in the pupil. The refractive data obtained were analyzed by Minitab software (One-Way ANOVA, Tukey's pairwise comparisons) to compare the changes of refractive states before and after the adipose eyelid was removed.

2.13 Microspectrophotometry (MSP)

The microspectrophotometry was used to measure the color reception of the

retinal cells of milkfish eyes. In order to protect the photoreceptor cells from light bleaching, all the following processes were carried out inside the dark room and a dim red light source was used to provide the needed illumination. After at least 6 hours of dark adaptation, the eyeballs of either larval or juvenile milkfish were enucleated and then dipped into ice-cold 6 % sucrose PBS solution. The eyeball was dissected to separate the retina under a night-vision dissecting microscope. The preliminary data showed that the cone and rod cells could maintain their activities in ice-cold 6 % sucrose PBS solution for up to 24 hours. A drop of 6 % sucrose PBS solution and a small piece of retinal tissues were placed onto a cover slip, and then a dissecting blade was used to tease the small piece of retinal tissues apart in order to dissociate the photoreceptor cells. After mounting these retinal cells between cover slips, the MSP measurement was performed in room temperature (20-23 ℃). The monochromator of the MSP allowed scanning of light wavelengths from 350 nm to 750 nm. The beam scanning was first performed at a clear background site near the visual cell to obtain the background values. The light beam was then moved to the outer segment of either cone or rod cell, and the second scanning was performed as the recorded values. The computer software then sunstracted the background values from the recorded values and to calculate the absorption spectrum of that particular retinal cell. In order to prove the viability of the retinal cell examined, right after the recording a strong bright light was shone on the examined cell for 2-min to bleach it. Then a new MSP reading

was followed, a no response of the bleached cell indicating the previous reading was valid. The rod cell and the different kinds of cone cell spectra of larval and juvenile milkfish were compared by Minitab software (One-Way ANOVA, Tukey's pairwise comparisons).