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Ionocyte Distribution in Gills of the Euryhaline Milkfish, *Chanos chanos* (Forsskål, 1775)

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Chun-Nian Chen, Li-Yih Lin and Tsung-Han Lee (2004) Ionocyte distribution in gills of the euryhaline milkfish, *Chanos chanos* (Forsskål, 1775). *Zoological Studies* **43**(4): 772-777. As a marine euryhaline teleost, the milkfish, *Chanos chanos*, is considered a good model fish for studies of ionoregulation. In preparation for studies of the effects of seawater-freshwater adaptation on the biochemistry and morphology of the milkfish gill, the present investigation describes different distributions of ionocytes on the gill epithelium of seawater- versus freshwater-adapted fish. Paraffin sections of filaments revealed afferent, interlamellar, and efferent regions of the epithelium. Round eosinophilic epithelial cells (5~10 μm in diameter) were exhibited in the interlamellar epithelium near the afferent side of the filament. Due to their identical size and location, histochemical (osmium-zinc iodide) and immunofluorescent (Na, K-ATPase) staining was used to demonstrate that these eosinophilic, Na, K-ATPase-immunoreactive (NKIR) cells are ionocytes. It is thus hard to examine the apical openings of ionocytes by scanning electron microscopy in the gill epithelium of the milkfish as compared to those in the other teleosts, because most ionocytes are distributed in the interlamellar regions of the filaments. Further confocal micrographs showed abundant NKIR cells on the filamental epithelia of both seawater- and freshwater-adapted fish. However, NKIR cells were rarely observed on the lamellar epithelia of gills in seawater-adapted individuals, while they were commonly found in freshwater-adapted milkfish.
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Ionocytes (i.e., chloride cells, mitochondria-rich cells) are thought to be a primary site of osmotic regulation in gills of euryhaline teleosts (Marshall 2002). Gill ionocytes appear mostly in the epithelia of filaments, and when necessary, may appear on lamellae (Sakamoto et al. 2001). Several techniques, including electron microscopy, histochemical staining, and immunocytochemical staining, have been used for identifying this cell type (Wilson and Laurent 2002). However, not all detection techniques are optimal due to the differential distribution of ionocytes in the gill epithelia of various species. Plus, inappropriate application of these methods may be misleading in the understanding of ionocytes. Thus, it is crucial to clarify the distribution of ionocytes in gills of milkfish by a combination of available techniques before further studies can be conducted.

Biochemical and morphological studies of teleostean gills have revealed a close relationship between ionocytes and Na, K-ATPase (NKA) activity. NKA is a widespread membrane-bound enzyme that generates an electrochemical gradient for Na^+ from the plasma to the cytoplasm of ionocytes to drive Na^+ inward across the basolateral membrane (Marshall 2002). NKA is composed of a catalytic α-subunit with a molecular weight of about 100 kDa and a smaller glycosylated β-subunit with a molecular weight of approximately 55 kDa (Mercer 1993). Na, K-ATPase-immunoreactive (NKIR) cells are not only abundant in the gill filament epithelium of both freshwater- and seawater-adapted teleosts, but also appear on the lamellar epithelium in several fresh-

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water-adapted euryhaline species (Sakamoto et al. 2001, Varsamos et al. 2002).

Salinity adaptation by euryhaline teleosts is a complex process involving a set of physiological responses to environments with differing ionoregulatory requirements. The current model of metabolic and cellular adaptation of euryhaline teleosts to varying environmental salinities is based on studies conducted on a restricted number of species including freshwater cichlids (*Oreochromis* spp.), intertidal killifish (*Fundulus* spp.), and diadromous fish, such as salmonids (salmon) and anguillids (eels), while few studies have focused on marine euryhaline teleosts, e.g., flounder, sea bream, and mullet (for reviews, see Marshall and Bryson 1998). The milkfish, *Chanos chanos* (Forsskål, 1775), is a marine inhabitant (Ferrais et al. 1988) and is extremely euryhaline throughout its life history (Bagrinao 1994). It is strong when exposed to experimental conditions, and adapts well to abrupt changes in salinity (Swanson 1998). These characteristics make the milkfish a good model species for studies of ionocytes as well as osmotic regulation in euryhaline marine teleosts. In light of the scarcity of existing data on marine fish, this work examined the distribution of gill ionocytes of milkfish using different methods and compared the localization of ionocytes in seawater- and freshwater-adapted individuals.

MATERIALS AND METHODS

Fish and experimental environments

Juvenile (2.5 mo after hatching) milkfish, *C. chanos*, weighing (31.0 ± 11.8 g) were obtained from a local fish farm. Fish were raised in 10% brackish water (as they were at the fish farm) at 27 ± 1°C with a daily 12 h photoperiod for over 1 mo with the exception of those used for confocal laser scanning microscopic comparisons of Na, K-ATPase immunolocalization, which were adapted to either freshwater (0‰) or seawater (35‰) for at least 2 wk before sampling. Fish were fed a daily diet of commercial pellets ad libitum.

Scanning electron microscopy

Excised gills were processed as described in Lee et al. (1996a). In brief, tissues were fixed at 4°C in phosphate-buffered 4% paraformaldehyde plus 5% glutaraldehyde (pH 7.2) for 12 h and then in 1% osmium textroxide (pH 7.2) for 1 h at 4°C. Tissues were dehydrated in ascending concentrations of ethanol from 50% to absolute, then in 100% acetone, and were dried using a Hitachi HCP-2 critical-point drier (Japan). After sputter-coating with a gold-palladium complex for 3 min using an Eiko 1B-2 vacuum evaporator (Japan), specimens were examined with a Hitachi S-2500 scanning electron microscope.

Histological observations

The middle portion of the 1st gill arch was removed and fixed in 10% neutral formalin for 24 h. After serial dehydration in ascending percentages of ethanol, the gills were infiltrated with xylene and embedded in paraffin, sectioned at 5 μm, and stained in hematoxylin and eosin. Cross and longitudinal sections of the gill filaments and osmium-zinc iodide-stained sections (described in the following paragraph) were observed using an Olympus BX50 light microscope (Japan) and photographed with a Polaroid DMC CCD camera (USA).

OZI histochemical identification of ionocytes

An osmium-zinc iodide (OZI) method (Schreiber and Specter 1999) was used to histochemically observe MR cells in gills of the milkfish. For fixation, the zinc iodide (ZnI_2) solution had to be prepared immediately before use (0.24 g ZnI_2 powder (Aldrich, Germany) in deionized water to an 8 ml total volume). Excised gills were fixed in the dark for 24 h and rinsed with deionized water for 4~6 h before serial alcohol/xylene dehydration and embedding in paraffin. Sections at 5 μm (Leica, RM2010, Germany) were glued onto glass slides coated with poly-L-lysine (Sigma, St. Louis, MO, USA). After paraffin removal (with 100% xylene for 2 min x 3 times), sections were mounted with Histokitt mounting medium (Assistent, Germany) and covered with a cover slip. According to Watrin and Mayer-Gostan (1996), ionocytes with numerous plasma membrane infoldings produce a black color with OZI staining.

Na, K-ATPase antibody

A mouse monoclonal antibody (α5) against the α-subunit of avian Na, K-ATPase (Takeyasu et al. 1988) was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA).

Immunofluorescent staining of NKIR cells

These procedures were modified from Lee et al. (1998). Gills were excised, and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min at 4°C. Samples were then immersed in acetone followed by ethanol at –20 °C for 10 min each. After immersion in 30% sucrose in PBS for 1 h at room temperature, gills were embedded in Tissue-Tek® OCT compound (Sakura, Japan). Cross-sections of the filament were cut at 10 μm and mounted on poly-L-lysine-coated slides. To detect the distribution of Na, K-ATPase-immunoreactive (NKIR) cells, sections were blocked with 3% BSA in PBS for 30 min at room temperature, washed in PBS, and incubated with a monoclonal antibody (α5) of the Na, K-ATPase α-subunit for 2 h at room temperature. After washing with PBS, slides were incubated with FITC-conjugated secondary antibody (Jackson, USA) for 1 h at room temperature. Negative control experiments were conducted, using mouse whole serum (Sigma) instead of the primary antibody to confirm the above positive results (data not shown).

Confocal laser scanning microscopy

After being immunostained with Na, K-ATPase, some cryosections were counterstained with TRITC-labeled phalloidin (Sigma) for 30 min at room temperature to reveal the structures of the sections. Immunofluorescent-stained gill filaments were examined according to Wu et al. (2003) using a Leica TCS NT confocal laser scanning microscope equipped with an argon laser (488 and 514 nm) for excitation and attached to a Leica DMRE microscope. The stained images of Na, K-ATPase were obtained with the use of the FITC/Texas red filter set (BF 525/50 for FITC and BP 600/30 for Texas red) controlled by Leica TSC NT software. With the filter set, the emission wavelengths of FITC-conjugated antibody (green) and phalloidin (dark red) were separated and transmitted to different photomultipliers. Pictures from each photomultiplier were subsequently merged to simultaneously visualize the labels (yellowish green for double-stained cells).

RESULTS

In milkfish, each gill arch bears rakers on its rostral aspect and supports 2 rows of filaments extending posterolaterally (Fig. 1a) with an obvious branchial septum found just beneath the arch, which expands to join neighboring filaments. A row of leaf-like lamellae angling slightly toward the apex of the filament occurs on both sides of each filament from its base to its apex with 1 side lying near the leading edge of the filament (Fig. 1b). Longitudinal sections revealed bilaterally symmetrical lamellae branching from the central axis of the filaments, consisting mainly of capillaries and erythrocytes (Fig. 2a). In the cross-sections, filamental epithelium could be identified as afferent, interlamellar, or efferent regions according to the distribution of the arteriole system (Fig. 2b). Large

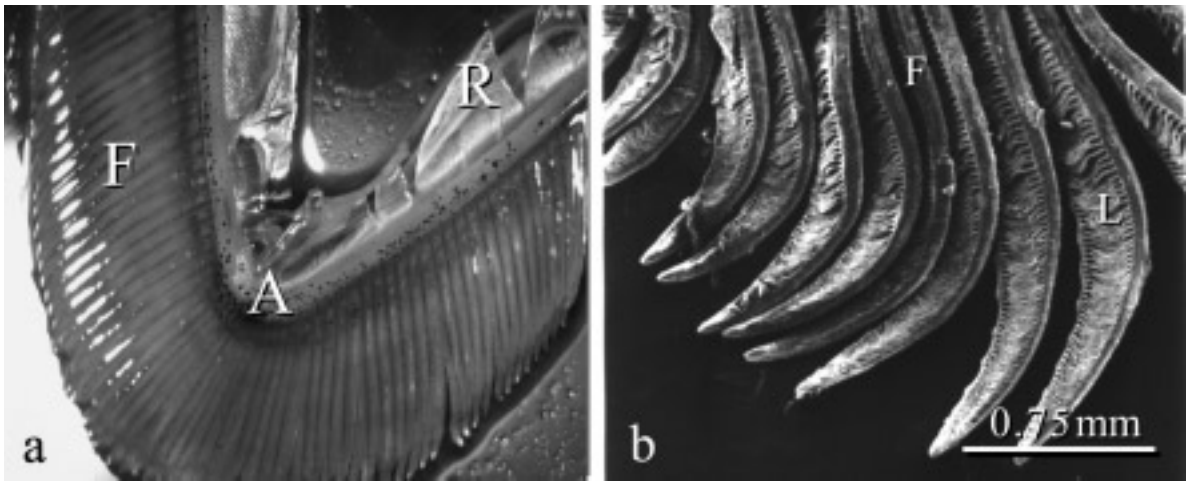

Fig. 1. The structures of milkfish gills. a. Stereomicroscopic observation shows that the gill consists of the arch (A), rakers (R), and filaments (F). The arch bears two rows of long, fine gill rakers on the rostral aspects as well as supporting two rows of filaments extending posterolaterally. b. Higher magnifications of the gill structures observed by the scanning electron microscope. Centrally located, flattened lamellae (L) branch bilaterally from the filament.

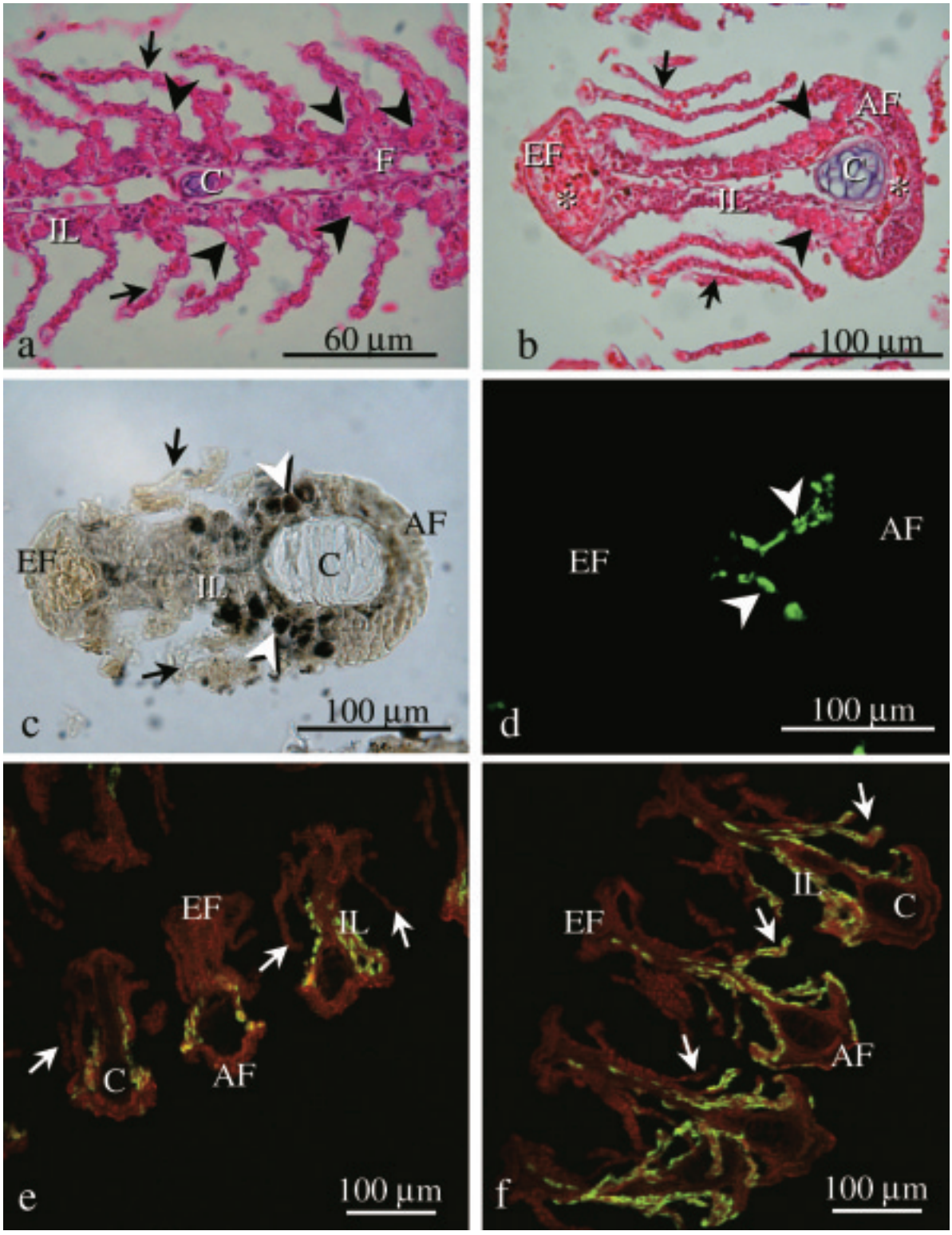

Fig. 2. Distribution of ionocytes in the gill epithelia of milkfish. Longitudinal (a) and cross (b) sections of the gill filament of milkfish displaying the structures of afferent (AF), interlamellar (IL), and efferent (EF) epithelia and bilaterally symmetrical lamellae (arrows) branch from the central axis of the filament (F). Big, oval to round epithelial cells (arrowheads in Figs. a and b) exhibited in the interlamellar regions of the filaments are identical to the positive reactions (arrowheads in Fig. c) to OZI, a method for detecting MR cells, and to Na, K-ATPase α-subunit (arrows in Fig. d). Furthermore, confocal micrographs of immunofluorescent staining of Na, K-ATPase α-subunit (yellowish green) counterstained with phalloidin (dark red) show that NKIR cells (arrowheads) are only found in filaments of SW-adapted milkfish (e), but on both interlamellar regions of the filaments and lamellae of the FW-adapted fish (f). Asterisk, erythrocytes; C, cartilage.

(5~10 μm in diameter), round, eosinophilic epithelial cells were observed on the interlamellar region near the afferent side (Fig. 2a, b). Histochemically, OZI-reacting cells (Fig. 2c) as well as Na, K-ATPase-immunoreactive (NKIR) cells (Fig. 2d) were identically localized to eosinophilic epithelial cells of the cross-sections of the filaments. Moreover, confocal micrographs of the filaments counterstained with phalloidin (dark red) illustrated that in both seawater- and freshwater-adapted fish, NKIR cells (yellowish green) occurred in the interlamellar epithelia near the afferent side of the filaments (Fig. 2e, f), while freshwater NKIR cells were also found in the lamellae (Fig. 2f).

DISCUSSION

Results of histological, histochemical, and immunofluorescent techniques demonstrated that euryhaline marine milkfish gill ionocytes were round, eosinophilic Na, K-ATPase (NKA) cells which were located on the interlamellar epithelium of gill filaments. Shirai and Utida (1970) also reported that chloride cells (i.e., ionocytes) are acidophilic in the gill epithelium of the eel, *Anguilla japonica*. In the present study, ionocytes in the gill epithelium of the milkfish were histochemically detected by the OZI method (Fig. 2c). Previous studies by transmission electron microscopy (Schreiber and Specker 1999) or light microscopy (Ciccotti et al. 1994, Madsen et al. 1994, Greco 1995, Watrin and Mayer-Gostan 1996, Caberoy and Quinitio 2000, Wilson and Laurent 2002, Lin and Sung, 2003) proved that OZI fluid (i.e., Champ Maillet's fluid) was useful for identifying ionocytes in the gill epithelium of teleosts because the stain reacts specifically with membranes, and the reaction with the membranous tubular systems of the ionocytes results in its selective blackening.

In milkfish, epithelial ionocytes occurred mostly on the interlamellar region near the afferent side of the filament (Fig. 2a, b), but rarely appeared on the trailing edge (afferent region) of the filament, as has been observed in other teleosts (Wilson and Laurent 2002). Hence, it is inappropriate to examine branchial ionocytes of milkfish by scanning electron microscopy as is done in other teleosts (Wilson and Laurent 2002, Katoh and Kaneko 2003). As mentioned above, ionocytes were abundant in gill filaments of both seawater- (SW) and freshwater (FW)-adapted teleosts. However, in stenohaline FW fish (Lee et al. 1996b) or FW-adapted euryhaline fish (Sakamoto et al. 2001, Varsamos et al. 2002), ionocytes appeared on both filaments and lamellae. In FW-adapted milkfish, Na, K-ATPase-immunoreactive (NKIR) cells (i.e., ionocytes) were also found in lamellae (Fig. 2f). The occurrence of lamellar ionocytes is thought to satisfy the physiological demand of ion uptake in some FW-adapted euryhaline teleosts (Uchida et al. 1996, Sasai et al. 1998, Hirai et al. 1999) but not in others (Laurent and Perry 1990, Lin and Sung 2003). Whether there is a functional differentiation between lamellar and filamental ionocytes is still under debate.

The present paper illustrates the distribution of ionocytes in gill epithelia of the milkfish. Based on this study, this marine-residing species will be used as a model fish for future ionoregulatory studies on euryhaline marine teleosts.

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