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METAMORPHOSIS IN THE SUMMER FLOUNDER *PARALICHTHYS DENTATUS*: CHANGES IN GILL MITOCHONDRIA-RICH CELLS

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Summary

Salinity tolerance changes during larval development and metamorphosis in the summer flounder (*Paralichthys dentatus*) and other teleosts. The physiological mechanisms responsible for osmoregulation during these early stages of development are not well understood. This study characterized changes in ultrastructure, intracellular membranes and immunoreactive Na^+/K^+-ATPase of mitochondria-rich cells (MRCs) in the gills of summer flounder during metamorphosis. Gill ultrastructure at the start of metamorphosis revealed only one type of MRC, which had weak reactivity to osmium and lacked a well-defined apical pit. In juveniles, two types of MRCs were observed: light-staining MRCs (LMRCs) with weak reactivity to osmium, and dark-staining MRCs (DMRCs) with strong reactivity to osmium and positioned adjacent to LMRCs. Compared with MRCs at the start of metamorphosis, the mitochondria of juvenile MRCs appeared smaller, with more transverse cristae and electron-dense matrices. Changes in MRCs during metamorphosis were also accompanied by increased immunoreactive Na^+/K^+-ATPase. These findings suggest that gill MRCs develop during the metamorphosis of summer flounder as the gill takes on an increasingly important osmoregulatory role.

Key words: chloride cell, mitochondria-rich cell, metamorphosis, osmoregulation, flounder, *Paralichthys dentatus*.

Introduction

Summer flounder (*Paralichthys dentatus*) are teleosts that metamorphose from pelagic larvae into benthic juveniles (Keefe and Able, 1993; Schreiber and Specker, 1998). Metamorphosis in this species of flatfish is accompanied by changes in salinity tolerance (Schreiber and Specker, 1999) and takes place as the fish move from the ocean to estuarine nursery grounds (Able and Kaiser, 1994).

Since the discovery of mitochondria-rich cells (MRCs) in the gill filaments of the eel (Keys and Wilmer, 1932), these cells have been shown to be the principal extra-renal sites for active salt extrusion in juvenile and adult saltwater-living teleosts (Foskett and Scheffey, 1982). Two morphologically distinct types of gill MRC have been described for juveniles and adults living in salt water. Seawater ‘chloride cells’ are characterized as rich in mitochondria and possessing a well-developed tubular system that is an extension of the basolateral membrane. ‘Accessory cells’ are observed adjacent to the apical region of chloride cells and share some of the ultrastructural features of chloride cells (see reviews by Pisam and Rambourg, 1991; Evans et al., 1999). Na^+/K^+-ATPase ion pumps have been located on the tubular system of MRCs (Karnaky et al., 1976; Hootman and Philpott, 1979). Parr–smolt transformation and saltwater adaptation in salmonids are accompanied by both increased gill Na^+/K^+-ATPase activity and enhanced immunoreactivity to Na^+/K^+-ATPase in the MRCs (Uchida et al., 1996; Ura et al., 1997). Furthermore, saltwater adaptation in several species is accompanied by ultrastructural changes in the tubular system and mitochondria (Shirai and Utida, 1970; Pisam, 1981; Hwang and Hirano, 1985; Uchida et al., 1996).

Several studies have shown that the yolk sac and skin of early larval teleosts contain MRCs with osmoregulatory functions (Alderice, 1988; Ayson et al., 1994; Kaneko et al., 1995; Shiraishi et al., 1997). Since epidermal MRCs in a flatfish (*Pleuronectes platessa* L.) degenerate during metamorphosis as the skin thickens (Roberts et al., 1973), the developing gill probably takes on an increasingly important osmoregulatory role during this period. Although there have been several studies on gill MRCs of early larval teleosts (Shelbourne, 1957; Hamada, 1968; Katsura and Hamada, 1986; Hwang, 1989, 1990; Li et al., 1995; Tytler and Ireland, 1995), and MRC changes have been described for an agnathan during metamorphosis (Peek and Youson, 1979), little is known about MRC development in the gills of teleosts during metamorphosis. In particular, it is not clear whether MRCs experience changes in ultrastructure or osmoregulatory physiology during metamorphosis, or how many types of MRC are present before the end of metamorphosis.

The purpose of this study was to characterize changes in gill MRCs during summer flounder metamorphosis. Gill MRCs were assessed for changes in ultrastructure using transmission electron microscopy, changes in intracellular membranes by reactivity to an osmium-based cytological stain, and localization and characterization of immunoreactive Na^{+}/K^{+}-ATPase.

Materials and methods

Fish maintenance

Summer flounder (*Paralichthys dentatus*) were obtained from the University of Rhode Island Narragansett Bay Campus summer flounder hatchery and raised as previously described (Schreiber and Specker, 1998). Fish were maintained in filtered Narragansett Bay sea water (30‰ salinity) at room temperature (21–23 °C) in 38 l aquaria, and were fed *Artemia* brine shrimp. Fish were killed by anesthetizing in 0.2 % 2-phenoxyethanol (Sigma) prior to tissue fixation.

Developmental stage classification

Fish at different developmental stages, as previously described by Schreiber and Specker (1998), were classified according to the position of the translocating eye. Metamorphic climax (MC) may be divided into three developmental stages consisting of early MC (eMC; the translocating right eye is at the dorsal midline), midMC (mMC; most of the translocating eye is past the dorsal midline), and late MC (lMC; the entire translocating eye is past the dorsal midline). In the juvenile stage, the right eye is completely translocated and the dorsal canal has closed. Fish were collected at various ages and screened for the appropriate developmental stage using a dissecting microscope.

Electron microscope observations of gill MRCs

The ultrastructure and location of gill MRCs was observed using transmission electron microscopy (TEM) of ultrathin sections. Whole fish in various stages of metamorphosis or the dissected gill arches of juveniles were fixed either in osmium–zinc iodide (OZI) solution (see below) and stored in 70 % ethanol, or in 1 % formaldehyde, 3 % glutaraldehyde in 0.1 mol l^{-1} sodium cacodylate buffer (pH 7.2) for 48 h at 4 °C. The tissues were washed three times with 0.1 mol l^{-1} sodium cacodylate buffer (20 min each). The formaldehyde/glutaraldehyde-fixed tissues were postfixed in 1 % osmium tetroxide in 0.1 mol l^{-1} sodium cacodylate buffer for 3 h at 4 °C. All tissues were dehydrated through a graded series of ethanol (10, 25, 50, 75, 90, 95 %) at 4 °C for 10 min at each step, then allowed to warm to room temperature. This step was followed by four changes of absolute ethanol (15 min each), then two changes of propylene oxide (5 min each). All samples were embedded in Spurr’s low viscosity resin (Spurr, 1969). Ultrathin sections were cut with a diamond knife using a DuPont/Sorvall MT2-B ultramicrotome and mounted on copper grids. Thin sections (1 µm) were also cut for observation by light microscopy. Ultrathin sections were stained with 2 % uranyl acetate in 50 % methanol followed by lead citrate (Venable and Coggeshall, 1965), and some of the OZI-fixed sections were left unstained.

Histochemical detection of gill MRCs

To observe MRCs of gill filaments in metamorphosing and juvenile summer flounder, whole fish 34–48 days post-hatch, or the dissected gill arches of juveniles (approximately 120 days post-hatch), were placed into freshly prepared osmium-zinc iodide (OZI) solution (0.4 % osmium tetroxide, 25 mg ml^{-1} metallic iodine and 50 mg ml^{-1} zinc powder) for 12 h in the dark (see Garcia-Romeu and Masoni, 1970; Avella et al., 1987). The samples were rinsed with deionized water, dehydrated to 70 % ethanol and embedded in paraffin. Sections (5 µm thick) were cut and examined by light microscopy (×400 and ×1000). MRC-rich regions of the gill filaments were identified by a black coloration. After sectioning, representative slides from each fish (1 slide/fish) were pooled together and scored for staining intensity as ‘weak’, ‘moderate’ or ‘strong’ by two observers who did not know the origin of the slides. Although the histochemical nature of the OZI reaction is unclear (see Clark and Ackerman, 1971), the fixation-coloration process reduces osmic acid into osmium, and cellular reactivity has been previously attributed to lipids and lipid moieties derived from lipoprotein (see Maillet, 1968; Niebauer et al., 1969). It has been generally assumed that this method specifically stains gill MRCs, due to reactivity with phospholipids in the extensive tubular system (see Madsen, 1990a,b; McCormick, 1990; Zydlewski and McCormick, 1997).

Immunocytochemical detection of gill MRCs

MRCs of gill filaments were detected immunocytochemically using a mouse monoclonal antibody IgG raised against the highly conserved α subunit of the avian Na^{+}/K^{+}-ATPase pump (Takeyasu et al., 1988; Kone et al., 1991). The monoclonal antibody (called α5 by D. M. Fambrough) was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences, under contract NO1-HD-7-3263 from the NICHD. This antibody has been used previously by Witters et al. (1996) to localize MRCs on gill filaments from rainbow trout. Whole fish (34–48 days post-hatch) or the dissected gill arches of a juvenile (120 days post-hatch) were fixed in freshly prepared 4 % paraformaldehyde for 24 h at 4 °C. The tissues were then dehydrated to 70 % ethanol, embedded in paraffin and sectioned at 5 µm thickness. Immunocytochemical labeling of Na^{+}/K^{+}-ATPase was adapted from the method of Witters et al. (1996) using the following procedure. Tissues were rehydrated at room temperature, fixed and permeabilized with ice-cold 95 % ethanol (10 min) and rinsed in phosphate-buffered saline (PBS) (3 min). Areas for immunocytochemical staining were circumscribed with a hydrophobic pen. Endogenous peroxidase activity was quenched by incubation with 3 % H_2O_2 (3 min), followed by a rinse with deionized water (3 min), and a rinse with PBS (3 min). The tissues were incubated for 2 h with 0.007 $\mu g\,ml^{-1}$ of the primary antibody (mouse anti-chicken, $\alpha 5$). The tissues were then rinsed in PBS (3 min) and incubated with biotinylated rabbit anti-mouse IgG (3 min) and then avidin and biotinylated horseradish peroxidase complex (1.5 min) (both from Vector Laboratories, Inc., Burlingame, CA, USA). Positive reactions were revealed by incubating the tissues with 0.05 % diaminobenzidine, 0.01 % H_2O_2 solution (3 min). The tissues were rinsed in demineralized water, mounted and observed by light microscopy (magnification, ×400 and ×1000). The optimal concentration of primary antibody (0.007 $\mu g\,ml^{-1}$) was chosen in advance after testing the immunocytochemical procedure on adjacent sections from the gill filaments of metamorphosing and juvenile summer flounder using a series of antibody dilutions ranging from 2.5–0.005 $\mu g\,ml^{-1}$. Sections processed within the same assay were scored for staining intensity and the activity was categorized as ‘weak’, ‘strong’ or ‘very strong’ by two observers who did not know the origin of the slides. The homogeneity of stain within immunoreactive regions was classified as ‘mottled’ or ‘homogeneous’.

Experimental designs

Gill MRC detection in juveniles

Juveniles were sampled to locate MRCs of summer flounder using electron microscopical, histochemical and immunocytochemical techniques as described above. The gill arches from one juvenile (approximately 120 days post-hatch) were fixed in either OZI solution or the formaldehyde/glutaraldehyde fixative for analysis by TEM. OZI-fixed gills were observed with TEM to evaluate the reactivity of different MRC organelles to OZI. Gill arches from a second juvenile (also approximately 120 days post-hatch) were sampled for immunocytochemical detection of Na^+/K^+-ATPase activity or reactivity to OZI by light microscopy. The locations of immunoreactive Na^+/K^+-ATPase and OZI-reactive regions of the gill filaments observed by light microscopy were compared with the locations of MRCs using TEM to confirm the MRC-specificity of these immunocytochemical and histochemical assays.

Development of MRC ultrastructure during metamorphosis

At 25 days post-hatch, 50 premetamorphic larvae were removed from their stock tanks and placed into a 38 l aquarium. When the first 20 % of the fish had developed to eMC (37 days post-hatch), three fish at this stage were collected at random and placed in the formaldehyde/glutaraldehyde fixative, and three were placed in OZI solution for analysis by TEM. The ultrastructure of MRC from these fish was compared with that of 120-day-old juveniles sampled in the above experiment, in order to determine whether MRC ultrastructure and reactivity of intracellular membranes to OZI change with developmental stage.

Development of MRC OZI reactivity and Na^+/K^+-ATPase immunoreactivity during metamorphosis

At 25 days post-hatch, premetamorphic larvae were removed from stock tanks and placed into two aquaria (38 l each; 150 fish per aquarium). Fish that were in the most advanced stages of development and represented at least 20 % of the sampling population were sampled at 34 days post-hatch (for eMC and mMC), 41 days post-hatch (for lMC) and 48 days post-hatch (for juveniles). On sampling days, fish from each aquarium were pooled together into buckets (15 l). Fish of the same developmental stage were randomly selected for detection of gill immunoreactive Na^+/K^+-ATPase (three fish per stage) and OZI reactivity (six fish per stage) by light microscopy, in order to determine whether MRC immunoreactive Na^+/K^+-ATPase and reactivity to OZI change with developmental stage.

Results

Identification of MRCs in larval summer flounder necessitated establishing the characteristics of MRCs in juvenile-staged flounder. 5.0 μm sections were examined by light microscopy, and OZI staining (Fig. 1A) and immunoreactive Na^+/K^+-ATPase (Fig. 1B) were found to be predominantly localized at the junctions of the primary and secondary lamellae and in interlamellar spaces, and occasionally on the upper half of the secondary lamellae in gill filaments of juveniles. Thin sections (1 μm) of OZI-stained juvenile filaments revealed the presence of two types of MRCs: dark-staining MRCs (DMRCs), and light-staining MRCs (LMRCs) that had OZI staining intensity similar to the surrounding non-MRC cells (Fig. 2).

MRCs in juvenile gill filaments were identified using transmission electron microscopy (TEM) by their prominent mitochondria, extensive tubular network, and frequent presence of an apical pit when in contact with the epithelial surface (Fig. 3A,C). The differences in OZI staining between DMRCs and LMRCs observed by light microscopy (Fig. 2) corresponded to similar differences in OZI staining observed by TEM: LMRCs had similar electron density to other non-MRC filament cells, and DMRCs were more electron dense than all other filament cells (Figs 3B,D). Frequently, well-defined apical pits were shared by LMRCs and DMRCs, with the latter exhibiting a flanking position in the pit. In both DMRCs and LMRCs, OZI reactivity appeared as electron-dense deposits localized particularly close to the mitochondria and, to a lesser degree, the tubular system and nuclear envelope.

Gill MRC ultrastructure at the start of metamorphosis was different to that at the juvenile stage. In contrast to juveniles (Fig. 4C), only one type of MRC was present in eMC, and these consisted of electron-lucent cells which lacked a well-defined apical pit, resembling neither juvenile LMRCs nor

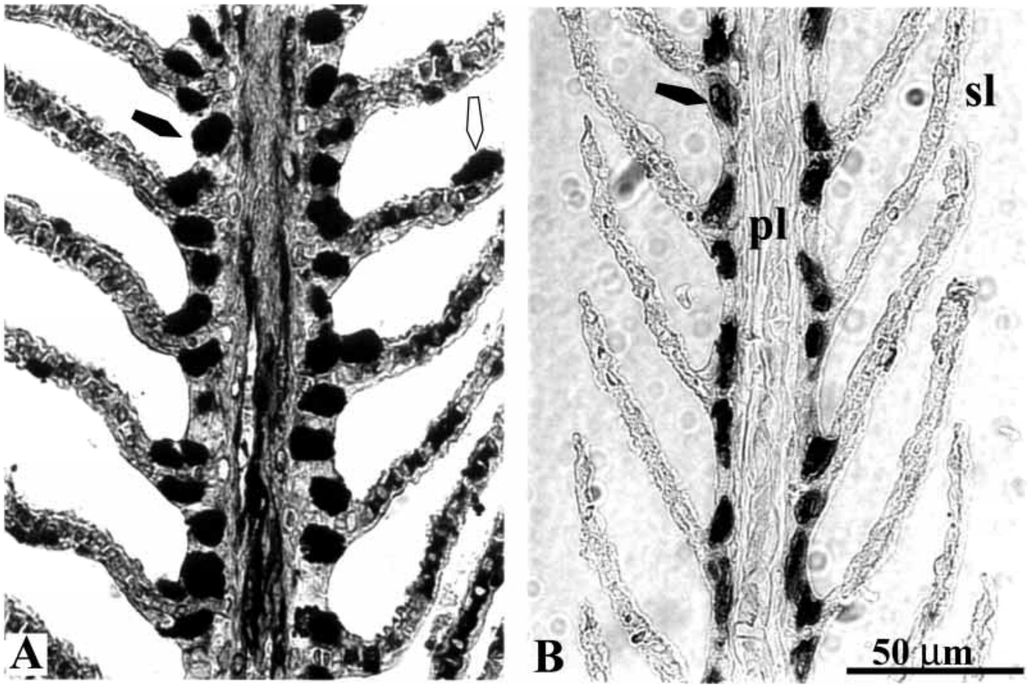

Fig. 1. Localization of mitochondria-rich cells (MRCs) in gill filaments from a 4-month-old juvenile summer flounder, identified histochemically with osmium-zinc iodide (A) and immunocytochemically with Na^+/K^+-ATPase antibody (B). MRCs are observed at the junctions of the primary lamella (pl) and secondary lamellae (sl) (filled arrows), and only occasionally on the secondary lamella (open arrow). Gill sections (5 µm thick) were observed with a light microscope.

DMRCs (Fig. 4A). Apical regions of cells in eMC ranged from slightly concave to convex. OZI staining of MRC organelles in eMC was weak and did not appear to differ in intensity from adjacent tissues (not shown).

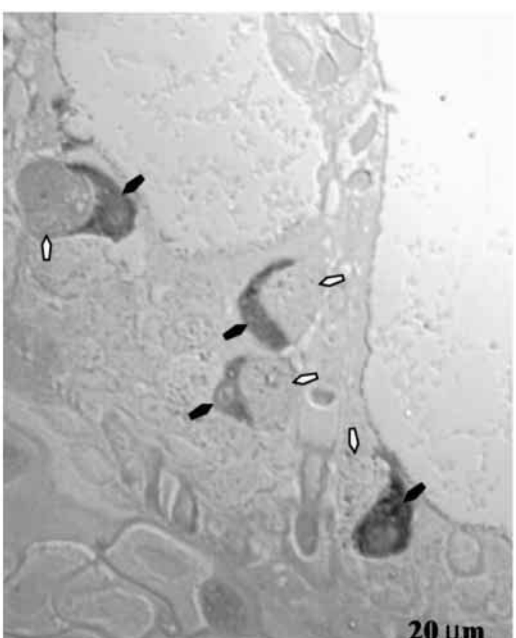

Fig. 2. Thin section (1 µm thick) of osmium-zinc iodide-stained gill filaments from a 4-month-old juvenile summer flounder viewed with a light microscope. Staining intensity of light-staining mitochondria-rich cells (LMRCs) (open arrows) is not different from that of surrounding non-MRCs, whereas dark-staining MRCs (DMRCs) (filled arrows) stain strongly.

The size and organization of the mitochondria changed with metamorphosis. MRCs in eMC had globular-shaped, densely packed mitochondria, which occupied a large cross-sectional area relative to the cytoplasm (Fig. 4B). However, fish in the juvenile stage appeared to have more elongated and less densely packed mitochondria, which occupied a smaller cross-sectional area relative to the cytoplasm compared to eMC (Fig. 4C). Unlike mitochondria in eMC, the mitochondria of juvenile MRCs were arranged approximately parallel to the long axis of the cell. Mitochondria in eMC were characterized by an electron-lucent matrix, with many villous cristae projecting into the matrix at all angles. The matrices of mitochondria from a juvenile were more electron-opaque compared with those in eMC, and the mitochondria possessed larger numbers of transverse cristae. Although the size of MRC mitochondria was not measured in a way that could be subjected to statistical test, profiles of juvenile mitochondria appeared to be generally smaller than those from eMC.

OZI staining of gill MRCs changed dramatically during metamorphosis. For the six fish sampled in each group, OZI reactivity was predominantly weak during eMC, moderate during mMC and strong during lMC and at the start of the juvenile stage (Figs 5, 6).

Immunoreactive Na^+/K^+-ATPase in MRC of fish entering metamorphosis was different from that at the end of metamorphosis. In contrast to MRCs in lMC and juvenile stages, MRCs in eMC were weakly immunopositive for Na^+/K^+-ATPase activity, and the staining was distinctly mottled in appearance (Figs 7, 8). These observations were consistent for the three larvae examined from each developmental stage.

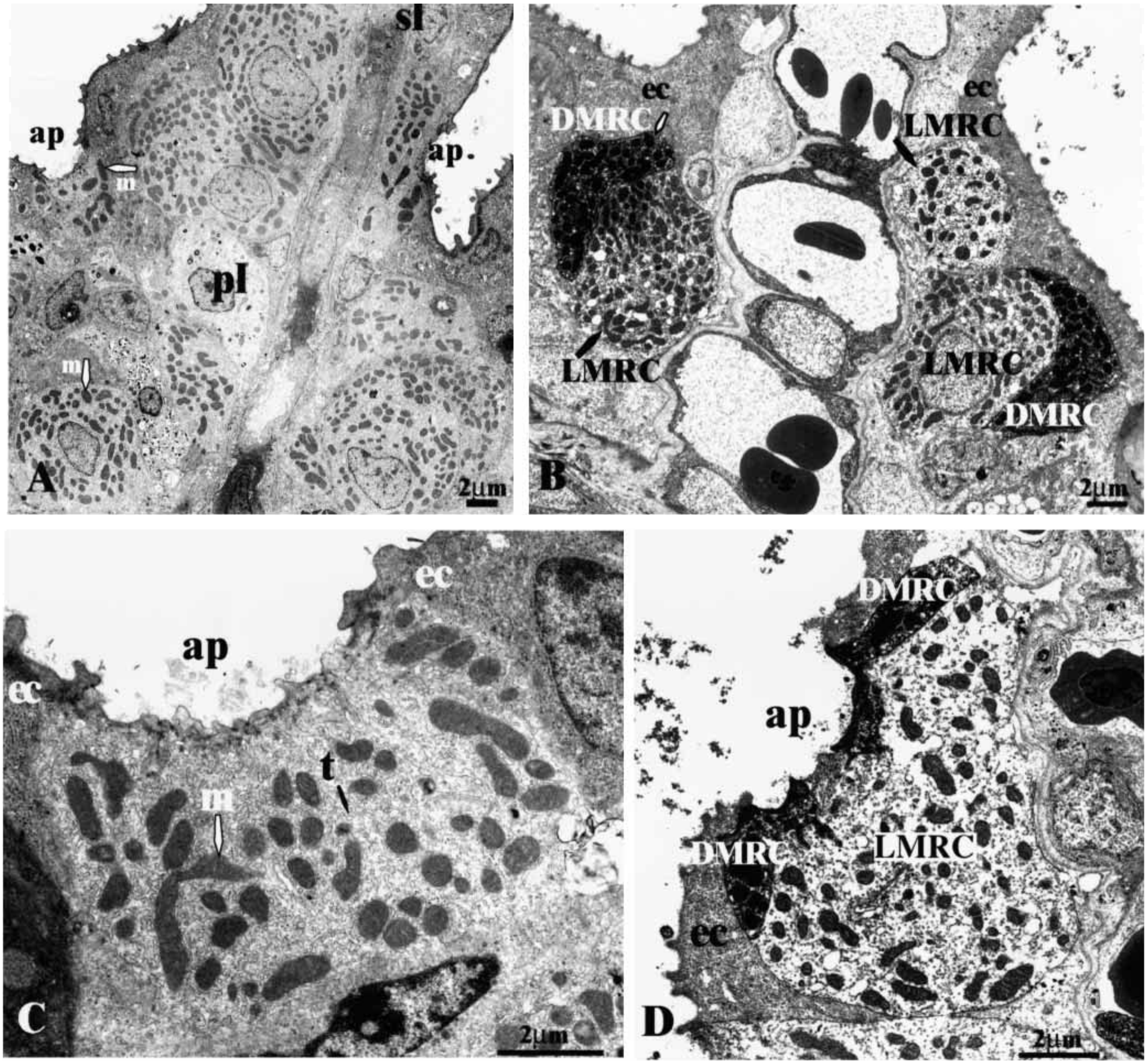

Fig. 3. Ultrastructural features of gill mitochondria-rich cells (MRCs) in juvenile summer flounder. Gill filaments of a 4-month-old juvenile summer flounder were stained without osmium-zinc iodide (OZI) (A,C) or differentially stained with OZI (B,D). MRCs had prominent mitochondria (m), an extensive tubular system (t), and the presence of a well-developed apical pit (ap) when in contact with the external environment. Two types of MRC were revealed by differential OZI staining: light-staining MRCs (LMRCs), usually flanked by either epithelial cells (ec), or dark-staining MRCs (DMRCs). OZI reactivity appears as electron-dense deposits localized particularly close to the mitochondria and, to a lesser degree, the tubular system, nuclear membrane and smooth endoplasmic reticulum.

Discussion

The most important finding from this study is that during summer flounder metamorphosis gill MRCs change from a single ‘larval’ type with uniform ultrastructure into two ‘juvenile’ types with different ultrastructures. Compared with larval MRCs, juvenile MRCs show changes in intracellular membranes and increased immunoreactive Na^+/K^+-ATPase. These changes, which coincide with the development of increased salinity tolerance (Schreiber and Specker, 1999), suggest that gill MRCs develop during summer flounder metamorphosis as the gills take on an increasingly important osmoregulatory role.

Our finding that gill MRCs are present in juvenile summer flounder was expected, considering the numerous reports of these cells in other juvenile and adult marine teleosts (see review by McCormick, 1995). Gill MRCs were located at the junctions of the primary and secondary lamellae and in the interlamellar spaces, and only occasionally on the secondary

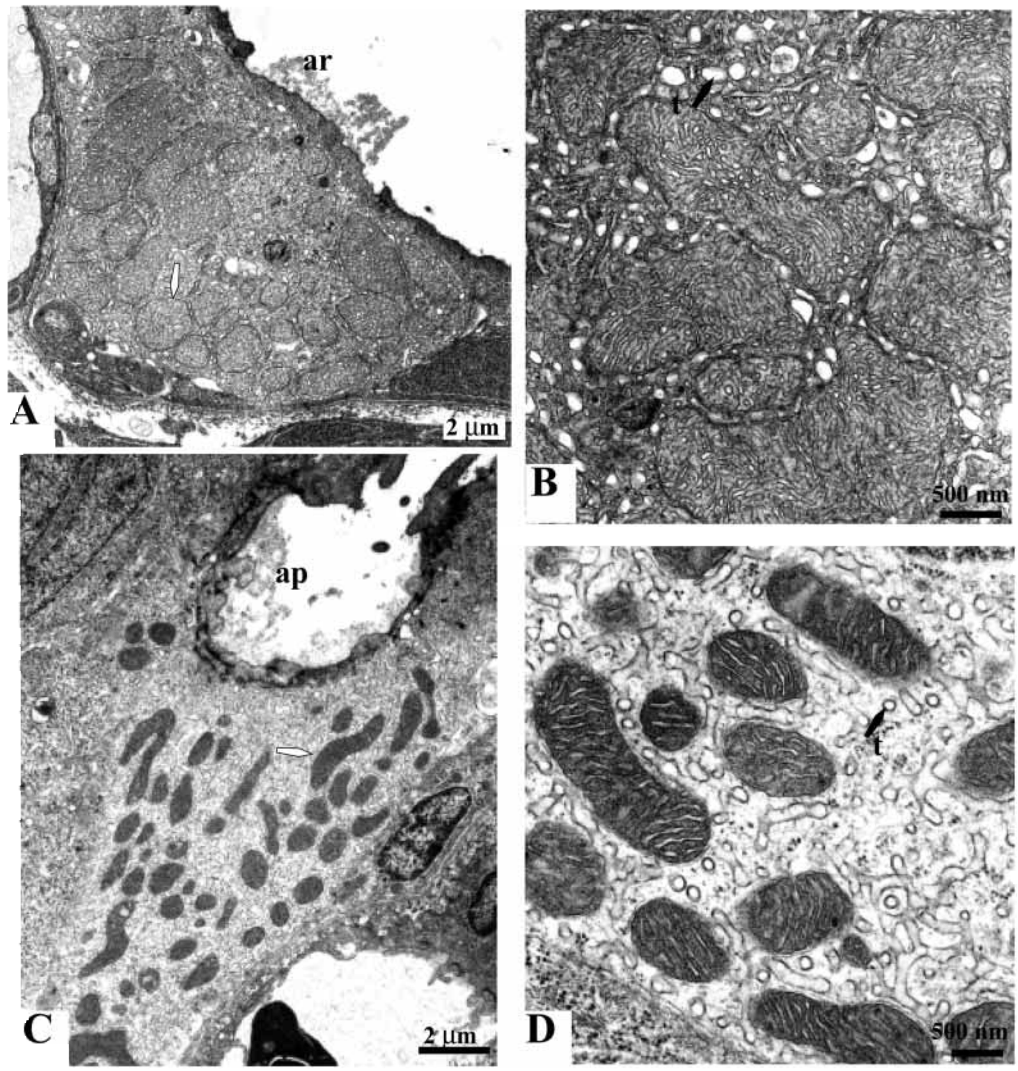

Fig. 4. Ultrastructural features of mitochondria of gill mitochondria-rich cells (MRCs) from summer flounder at early metamorphic climax (eMC; 37 days post-hatch) (A,B) and from a juvenile (120 days post-hatch) (C,D). Prominent mitochondria (open arrows) and tubular system (t) (filled arrows) are present in both MRCs. Mitochondria from MRCs in eMC appear large, globular-shaped, and occupy a large cross-sectional area of the cell. Juvenile MRC mitochondria appear smaller, longitudinally oriented, and occupy a smaller cross-sectional area of the cell. Apical regions (ar) of MRCs in eMC range from slightly concave to slightly convex, whereas some juvenile MRCs may possess well-defined apical pits. eMC, early metamorphic climax.

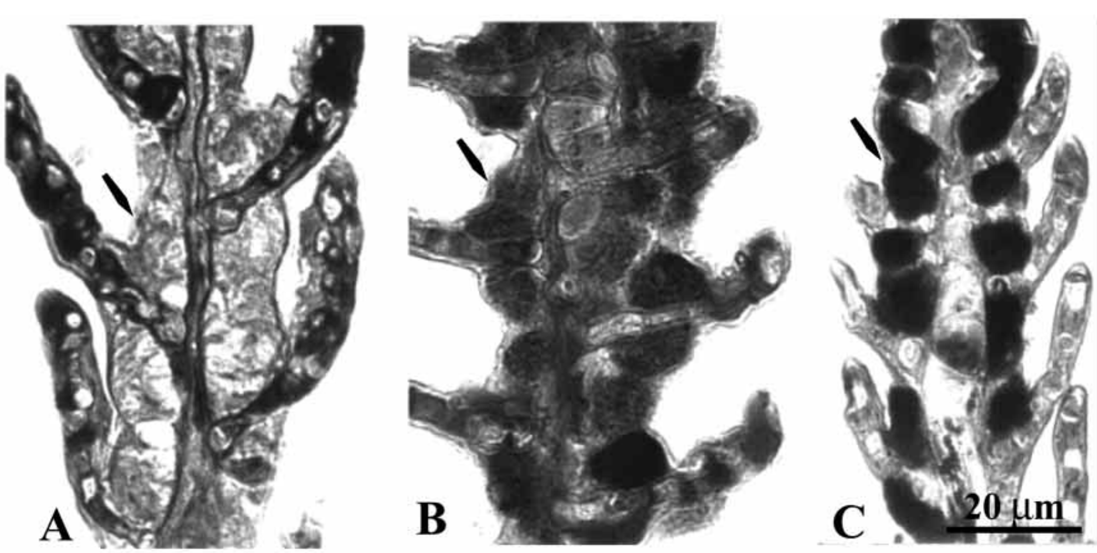

Fig. 5. Development of osmium-zinc iodide reactivity in gill mitochondria-rich cells (MRCs) (arrows) during summer flounder metamorphosis. Reactivity (arrows) is weak during eMC (34 days post-hatch) (A), moderate during mMC (34 days post-hatch) (B) and strong during lMC (41 days post-hatch) (C). Gills were observed using a light microscope. eMC, mMC, lMC, early, mid-, late metamorphic climax, respectively.

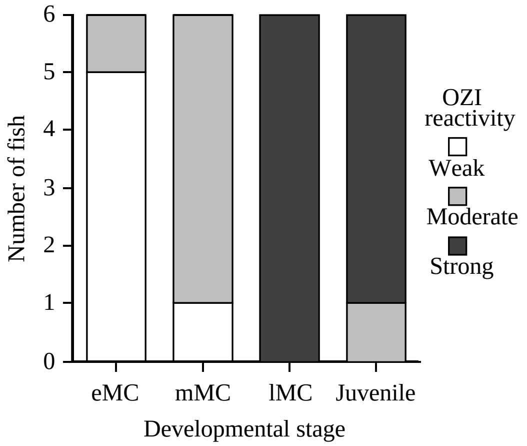

Fig. 6. Osmium-zinc iodide (OZI) reactivity of gill mitochondria-rich cells (MRCs) from metamorphosing and juvenile summer flounder (*N*=6 fish per stage), as determined by light microscopy. Fish in eMC and mMC were sampled at 34 days days post-hatch, lMC at 41 days post-hatch, and juveniles at 48 days post-hatch. eMC, mMC, lMC, early, mid-, late metamorphic climax, respectively.

lamellae. We identified juvenile flounder gill MRCs by their unique ultrastructure, strong reactivity to OZI and strong immunoreactive Na^+/K^+-ATPase, which is consistent with similar reports for juveniles and adults of other species (see McCormick, 1995; Uchida et al., 1996; Shikano and Fujio, 1998). In this study we also used these procedures to describe changes in gill MRCs during metamorphosis.

A single type of larval MRC, characterized in part by large mitochondria and the absence of well-defined apical pits, was identified at the start of metamorphosis. By the end of metamorphosis larval MRCs were no longer visible, but instead two types of juvenile MRC were present. Compared with larval MRCs, both juvenile types shared a well-defined apical pit and appeared to have smaller, more electron-dense mitochondria with more transverse cristae. Similar changes in mitochondria have not been reported before for MRCs in developing fish. However, Shirai and Utida (1970) have noted that when juvenile eels were transferred from fresh water to salt water the mitochondria matrices of MRCs also became more electron dense, with more transverse cristae, as they adapted to salt water. The differences in mitochondrial ultrastructure between larval and juvenile flounder MRCs may represent different stages of mitochondria development, though it remains unclear whether the larval MRCs observed in this study are in their terminally differentiated form or if they develop into juvenile MRCs. Only one study prior to this has addressed changes in gill MRC ultrastructure during fish metamorphosis, and this was for the anadromous sea lamprey, *Petromyzon marinus* L. (Peek and Youson, 1979). Unlike what was observed for summer flounder, changes in lamprey MRCs primarily involved the degeneration of ion-absorptive larval MRCs and differentiation of intermediate MRCs into the adult-type through the formation and proliferation of the tubular system. As with the summer flounder, changes in lamprey MRCs accompanied increased tolerance to a hyperosmotic environment.

Unlike larval MRCs, which all stained weakly with OZI, two types of juvenile MRCs were easily distinguishable by their differential OZI staining intensity. Light-staining juvenile MRCs (LMRCs) often possessed well-defined apical pits, which are a characteristic of seawater ‘chloride cells’ described for other species (Pisam and Rambourg, 1991). Dark-staining juvenile MRCs (DMRCs) exhibited many of the characteristics reported for seawater ‘accessory cells’, namely a more electron-dense cytoplasm compared with chloride cells, a crescent shape, smaller size and a tendency to flank the apical regions of chloride cells (Hootman and Philpott, 1980; Cioni et al., 1991; Pisam and Rambourg, 1991). Although changes from larval to juvenile MRCs in a marine teleost have not been described before, this process may be analogous to changes observed in juvenile and adult euryhaline fishes during transfer from fresh water to sea water. Three types of MRCs with unique ultrastructure are involved during this process in the guppy and other euryhaline teleosts: α- and β-MRCs are

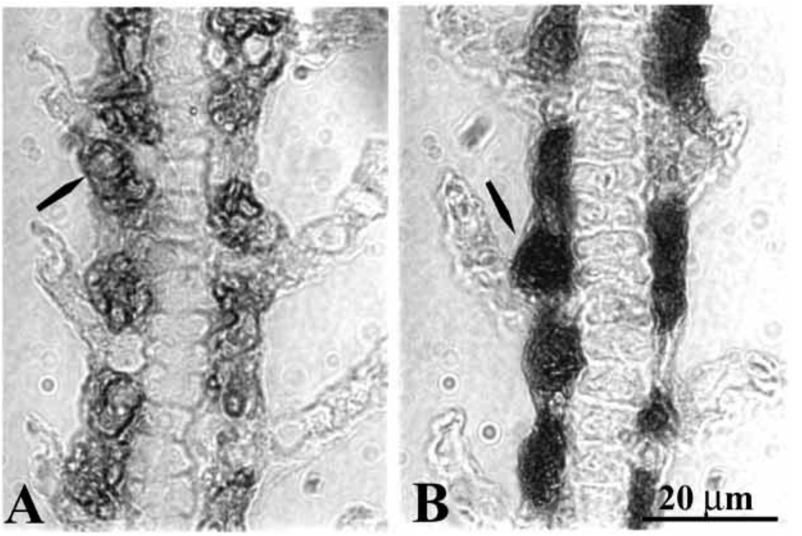

Fig. 7. Development of immunoreactive Na^+/K^+-ATPase in gill mitochondria-rich cells (MRCs) during summer flounder metamorphosis. MRCs in eMC (34 days post-hatch) had weak immunoreactivity and mottled staining (A, arrow), compared with MRCs in lMC (41 days post-hatch), which had strong immunoreactivity and homogeneous staining (B, arrow). Gills were observed using a light microscope. eMC, lMC, early, late metamorphic climax, respectively.

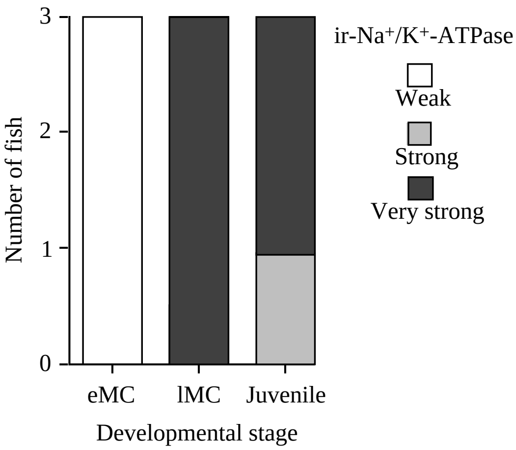

Fig. 8. Immunoreactive (ir)-Na^+/K^+-ATPase of gill mitochondria-rich cells (MRCs) from metamorphosing and juvenile summer flounder (*N*=3 fish per stage), as determined by light microscopy. Fish in eMC were sampled at 34 days days post-hatch, l-MC at 41 days post-hatch, and juveniles at 48 days post-hatch. eMC, lMC, early, late metamorphic climax, respectively.

present in fresh water, and seawater adaptation is accompanied by degeneration of β-MRCs, hypertrophy of α-MRCs into seawater chloride cells, and the appearance of seawater accessory cells (Pisam and Rambourg, 1991). In summer flounder, the appearance of juvenile MRCs corresponds with an increased hypo-osmoregulatory capacity (Schreiber and Specker, 1999), suggesting that juvenile MRCs may play a greater role in hypo-osmoregulation than larval MRCs.

OZI staining intensity of gill MRCs was weak at the start of metamorphosis and became very strong by the end. This probably reflects changes in MRC intracellular membranes (Madsen, 1990a,b; McCormick, 1990), particularly in juvenile DMRCs, which become the most strongly stained cells. The juvenile MRC organelles with the highest affinity for OZI are the mitochondria, and to a lesser degree the tubular system, and the nuclear envelope. Since larval MRCs appear to contain an unusually large cross-sectional area of mitochondria yet stain very weakly with OZI compared with juvenile MRCs, we suspect that the changes in OZI staining during metamorphosis reflect changes in membrane biochemistry. Osmium tetroxide, the active component of OZI, is thought to react with the double bonds of unsaturated fatty acids and lipoproteins (Niebauer et al., 1969; Hayat, 1970). Changes in OZI reactivity may indicate changes in membrane fluidity, an important parameter affecting cell ion transport capacity (Raynard and Cossins, 1991; Gibbs, 1998).

Gill immunoreactive Na^+/K^+-ATPase changed from a weak staining intensity and a mottled appearance at the start of metamorphosis to very strong, homogeneous staining at the end. These findings are analogous to observations made by Ura et al. (1997), who reported that gill immunoreactive Na^+/K^+-ATPase increased during the parr–smolt transformation in masu salmon (*Oncorhynchus masou*), and by Uchida et al. (1996), who showed that immunoreactive Na^+/K^+-ATPase was higher in chum salmon (*Oncorhynchus keta*) fry following transfer from fresh water to sea water. Some of the changes in MRC ultrastructure during metamorphosis may influence these changes in immunoreactive Na^+/K^+-ATPase. Since Na^+/K^+-ATPase is located on the tubular system (Karnaky et al., 1976; Hootman and Philpott, 1979), the presence of large mitochondria occupying large cross-sectional areas of larval MRCs at the start of metamorphosis probably displaces the tubular system compared with the smaller mitochondria of juvenile MRCs, and causes the mottled staining appearance of immunoreactive Na^+/K^+-ATPase at the start of metamorphosis. The increase in immunoreactive Na^+/K^+-ATPase from larval to juvenile MRCs may contribute to the increase in salinity tolerance at the end of summer flounder metamorphosis observed by us previously (Schreiber and Specker, 1999).

In summary, our findings for summer flounder suggest that during metamorphosis gill MRCs change from one larval to two juvenile forms. Whereas larval MRCs possess ultrastructural and histochemical characteristics that have not been previously reported for a marine teleost, juvenile MRCs appear similar to the seawater chloride cells and accessory cells that have been reported for other juvenile and adult teleosts. Some of the changes in MRCs during metamorphosis, such as increased immunoreactive Na^+/K^+-ATPase and the appearance of DMRCs and LMRCs, appear analogous to changes in other euryhaline teleosts during acclimation from fresh water to sea water. This is interesting considering that salinity tolerance develops during summer flounder metamorphosis, and the appearance of new juvenile-type MRCs probably contributes to osmoregulatory ability. Whether the larval MRC differentiates into both the DMRCs and LMRCs of juveniles deserves further investigation.

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