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Haloplasticity of Black Seabream (*Mylio macrocephalus*): Hypersaline to Freshwater Acclimation

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ABSTRACT Black seabream (*Mylio macrocephalus*) were acclimated to various salinities (50, 33, 12 and 6‰) for eight months. Acclimation of fish to 6‰ for eight months allowed successful adaptation to freshwater (0‰) for a further 21 days without mortality. This is the first report of freshwater acclimation of a "true" marine fish for an acceptable experimental duration. Osmoregulatory and metabolic strategies were characterized via alterations in branchial chloride cell (CC) numbers and surface ultrastructural morphometrics along with changes in serum chemistry, muscle moisture, liver glycogen and branchial, renal, hepatic and intestinal enzyme activities. Branchial CC numbers were elevated in 50 and 6‰ environments; however, freshwater acclimation resulted in return to low numbers. Branchial Na^+-K^+-ATPase activity was generally higher in 50 and 33‰ environments and exhibited a declining trend in 12 and 6‰ environments. Freshwater acclimation resulted in a marked elevation in branchial Na^+-K^+-ATPase activity. Elevated CC exposure areas were typically found at salinity extremes. Serum Na^+, Cl^- and muscle moisture content did not vary between groups acclimated from 50 to 6‰. Freshwater acclimation resulted in significant hyponatremia, hypochloremia and muscle hydration. Branchial ICDH activity was lowest in a 12‰ environment and highest at salinity extremes. Renal Na^+-K^+-ATPase exhibited lower activity in 12 and 6‰ and was markedly elevated in 0‰. Enzyme activities of both liver and kidney indicated elevated gluconeogenic activity in freshwater-adapted fish. Total intestinal Na^+-K^+-ATPase activity tended to decline in lower salinities; however, lowest activity was found in fish adapted to 12‰. Na^+-K^+-ATPase activities in different segments of the intestine may reflect the osmoregulatory role of this organ in varying salinities. The data indicated efficient homeostatic control in *Mylio macrocephalus* acclimated from hypersaline to freshwater environments and clearly demonstrates the haloplasticity of this marine fish species. *J. Exp. Zool. 283:226–241, 1999.*

Teleost models of cellular and metabolic adaptation to varying environmental salinity have long been based on studies conducted on a limited amount of euryhaline species that are capable of adapting to both freshwater (FW) and seawater (SW) conditions. Dominating the species studied are diadromids and as such, general models of response could be characterized as "salmono and/or anguillocentric." This occurs and persists despite a growing body of literature that suggests many "true" marine fish are capable of adapting to salinities much more dilute than typical SW (Woo and Wu, '82; Wu and Woo, '83; Dutil et al., '92; Mancera et al., '93; Provencher et al., '93; Lambert et al., '94; Munro et al., '94; Gaumet et al., '95; Woo and Chung, '95; Woo and Kelly, '95).

The Sparidae (seabream) are generally considered "true" marine fish and, although they do not migrate between FW and SW, are an integral part of non-estuarine dependent nekton in tropical and sub-tropical regions (Day et al., '89). As such, these fish, like other species of fish that frequent estuarine environments, are likely to experience a degree of salinity variation, where efficient osmoregulatory strategies will have a bearing on the regulation of salts during natural exposure to salinity fluctuation.

A large gap in our knowledge of how marine fish adapt to salinities other than SW manifests in the form of the effect of salinity on the branchial chloride cell (CC). In diadromous fish, move-

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ment from FW to SW generally results in an increase in CC populace (Utida et al., '71; Thomson and Sargent, '77; Langdon and Thorpe, '84); however, little work has been done on other fish species. Tilapines are currently a popular model in studies concerned with the response of CCs to salinity alterations, yet despite concurrence with diadromid models, where elevations in CC numbers occur in response to higher salinity environments (Kültz and Jürss, '93), more recent evidence has revealed contrary results (Van Der Heijden et al., '97). To the best of our knowledge, only Hootman and Philpott ('78) have addressed the effects of salinity transfer on the CCs of a non-estuarine dependent marine fish (*Lagodon rhomboides*). Yet, the goal of said authors was to design a technique for the rapid isolation of CCs with good ultrastructural integrity, and no attempt was made to determine alterations in CC populace or the relationship the cells maintained with the external environment.

In light of the paucity of current information on marine fish hyperosmoregulation, a comprehensive characterization of the adaptive response of marine fish to varying salinities is warranted. The present set of experiments were conducted in order to characterize the adaptive response of a marine teleost (*Mylio macrocephalus*) acclimated to salinities ranging from hypersaline (50‰) down to and including a hyposmotic environment of 6‰ and to determine whether the response of this marine fish species deviated from the diadromid paradigm. After long-term hyposmotic acclimation, fish were subjected to FW (0‰) exposure in order to clarify whether successful homeostatic control could be maintained for time periods consistent with generally acceptable experimental acclimation periods.

MATERIALS AND METHODS

Fish and culture conditions

A stock of *Mylio macrocephalus* juveniles (1.02 ± 0.09 g) were netted in Tolo Harbor, Hong Kong. The fish were meristically identified and held in SW tanks. In groups designated for low salinity conditions, salinity was reduced via gradual flushing of SW with dechlorinated tapwater over a period of one week until the final experimental salinities were achieved (12‰ and 6‰). Hypersaline water was obtained by evaporating SW to a salinity of 50‰. Hypersaline culture conditions were reached via gradual flushing of SW with hypersaline SW over a period of one week until a final salinity of 50‰ was obtained. The ionic composition of the water in each experimental condition is shown in Table 1. Water was fully aerated and the temperature ranged from 22 to 24°C. Fish were fed ad libitum once daily with diets formulated according to Woo and Kelly ('95). After samples were taken from fish held in hyposmotic conditions of 6‰, remaining fish were subjected to further salinity reduction, 1‰/day, until FW conditions were reached. The culture period of fish held in salinities of 50‰, 33‰, 12‰ and 6‰ was eight months and fish exposed to FW were held for a further three weeks.

Blood and tissue sampling

The fish were unfed 24 hr prior to sacrifice and blood taken from the caudal vessels via syringe. Blood was allowed to clot at room temperature and serum collected after centrifugation. The first right branchial arch was removed and a portion fixed in 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 0–4°C. The rest of the arch was used for enzyme analysis. A standardized portion of muscle (full flank dorsal to the lateral line) was removed for compositional analysis and whole kidney, liver and intestine were removed, blotted dry and weighed, allowing the calculation of renosomatic [(kidney wt./fish wt.) × 100], hepatosomatic [HSI = (liver wt./fish wt.) × 100] and viscerosomatic indices [VSI = (viscera wt./fish wt.) × 100] respectively. The intestine was separated into the esophagus, stomach, pyloric ceca, midgut and rectum. The

TABLE 1. Ionic composition of water in experimental conditions[1]

Salinity	Na^+ (mM)[2]	Cl^- (mM)[3]	Ca^{++} (mM)[2]	K^+ (mM)[2]	Mg^{++} (mM)[2]
50‰	673	776	16.5	18.4	68.4
33‰	430	515	10.4	11.0	43.1
12‰	146	172	4.9	4.6	16.7
6‰	78	85	2.3	2.6	7.8
0‰	8	0.6	0.4	0.3	0.1

[1]All data are expressed as mean values (n = 3).
[2]Determined using atomic absorption spectrophotometry (Hitachi).
[3]Determined via titration using a chloridometer (Corning-eel).

esophagus was defined as the region of the intestine anterior to the proximal gastric pylorus and pyloric ceca were removed from the gastric region. The midgut was defined as the region of the intestine posterior to the distal gastric pylorus and anterior to the proximal rectal pylorus. The rectum was the portion of the intestine posterior to the proximal rectal pylorus down to the anus. All serum and tissue samples were quick frozen in liquid nitrogen and stored at –70°C until analysis.

Preparation of gill cell isolates

Using the edge of a glass slide, the branchial epithelia was scraped free from the underlying cartilage into lysis-medium (9 parts 0.17 M NH_4Cl, 1 part 0.17 M Tris/HCl pH 7.4; from Yust et al., '76) according to Verbost et al. ('94). Incubation in this medium at room temperature for 10–15 min resulted in erythrocyte lysis and tissue fractionation. Following fractionation, the cells were drawn through a pipette (3-mm bore) 10 times and sieved through nylon mesh (125, 80 and 45 μm). The resulting cell isolates were drawn through a syringe (needle bore 1 mm) 10 times and washed twice in chilled Ca^{++} and Mg^{++}-free Hanks balanced salt solution (HBSS) of the following composition (mM): NaCl: 137, KCl: 5.4, Na_2HPO_4: 3.4, Na HCO_3: 4.2, KH_2PO_4: 0.4, glucose: 5.6; pH 7.4. After resuspension in Ca^{++} and Mg^{++}-free HBSS, a portion of the isolate was tested for viability employing Trypan Blue (Sigma, St. Louis, MO) exclusion (Sharpe, '88). Cells unstained by Trypan Blue after 10 min incubation were considered viable. CCs were selectively stained with the vital mitochondrial-specific fluorescent dye DASPMI [2-(p-dimethylaminostyryl)-1-ethylpyridiniumiodine] (Aldrich, Milwaukee, WI). The cell isolates were incubated in Ca^{++} and Mg^{++}-free HBSS containing 25 μM DASPMI for 15 min at 0–4°C in the dark. Cell isolates were washed twice with Ca^{++} and Mg^{++}-free HBSS and viewed under a microfluorophotometric microscope (Nikon, microphot-fx, Tokyo, Japan). The proportion of CCs in cell isolates was determined by viewing the cell isolates with and without fluorescent illumination on a hemocytometer. A number of viewing fields were counted for each fish (approximately 200 cells) and each site was chosen randomly before fluorescent excitation.

Scanning electron microscope (SEM) studies and morphometric analysis

Fixed gill filaments were washed twice in 0.1 M phosphate buffer (pH 7.4) and dehydrated in a graded acetone series (50–100%). Critical point drying was achieved using two 10-min baths of tetramethylsilane (Sigma). After air drying, filaments were mounted on copper stubs using double-sided non-conductive tape. Samples were given a fine coating of gold using a sputter coater (Edwards, model S150B, Crawley, England). All observations were conducted using a scanning electron microscope (Jeol, model JSM-5300, Tokyo, Japan). Photomicrographs of 4 afferent filament surfaces from each fish were taken parallel to the stub at the point of separation from the septum, near the base of the lamellae. CCs were easily distinguished from pavement cells and mucous cells using criteria previously established for seabream which combined both SEM and TEM studies (Kelly, '97). Quantification of CC apical area, CC fractional surface area and CC exposure numbers were conducted using photomicrograph images taken at 2000× mag and subsequent computer assisted image analysis (Quantimet 500, Leica, Cambridge, England) (Greco et al., '96).

Serum chemistry

Serum Na^+ was measured, after appropriate dilution, using atomic absorption spectrophotometry (Hitachi, Tokyo, Japan). Serum Cl^- was determined via titration using a chloridometer (Corning-eel, Halstead, England) and glucose levels were assessed using a glucose oxidase-peroxidase reaction (Sigma bulletin 510). Serum protein was determined according to Hartree ('72) using bovine serum albumin (Sigma) as a standard.

Tissue composition and enzyme activities

Liver glycogen was determined using amyloglucosidase (Murat and Serfaty, '74). Muscle moisture was determined after drying tissue overnight at 105°C and muscle lipid was measured gravimetrically.

Liver tissue was homogenized in ice-cold buffered seabream saline solution of the following composition (mM): NaCl: 124, KCl: 3.3, $MgCl_2$: 1.7, $CaCl_2$: 1.2, $NaHCO_3$: 10, glucose: 10; pH 7.4. The homogenate was centrifuged at 12,000 × g for 10 min and the supernatant used for enzyme analysis. Kidney, gill and intestinal tissues were homogenized in ice-cold sucrose-EDTA-imidazole (SEI)/sucrose-EDTA-imidazole-deoxycholic acid

Fig. 1. Representative scanning electron micrographs of gill filament tissue from *Mylio macrocephalus* acclimated to (**A**) 50‰, (**B**) 33‰, (**C**) 12‰, (**D**) 6‰ and (**E**) 0‰ environments. Representative chloride cell apical openings are indicated by an asterisk. Scale bars = 10 μm.

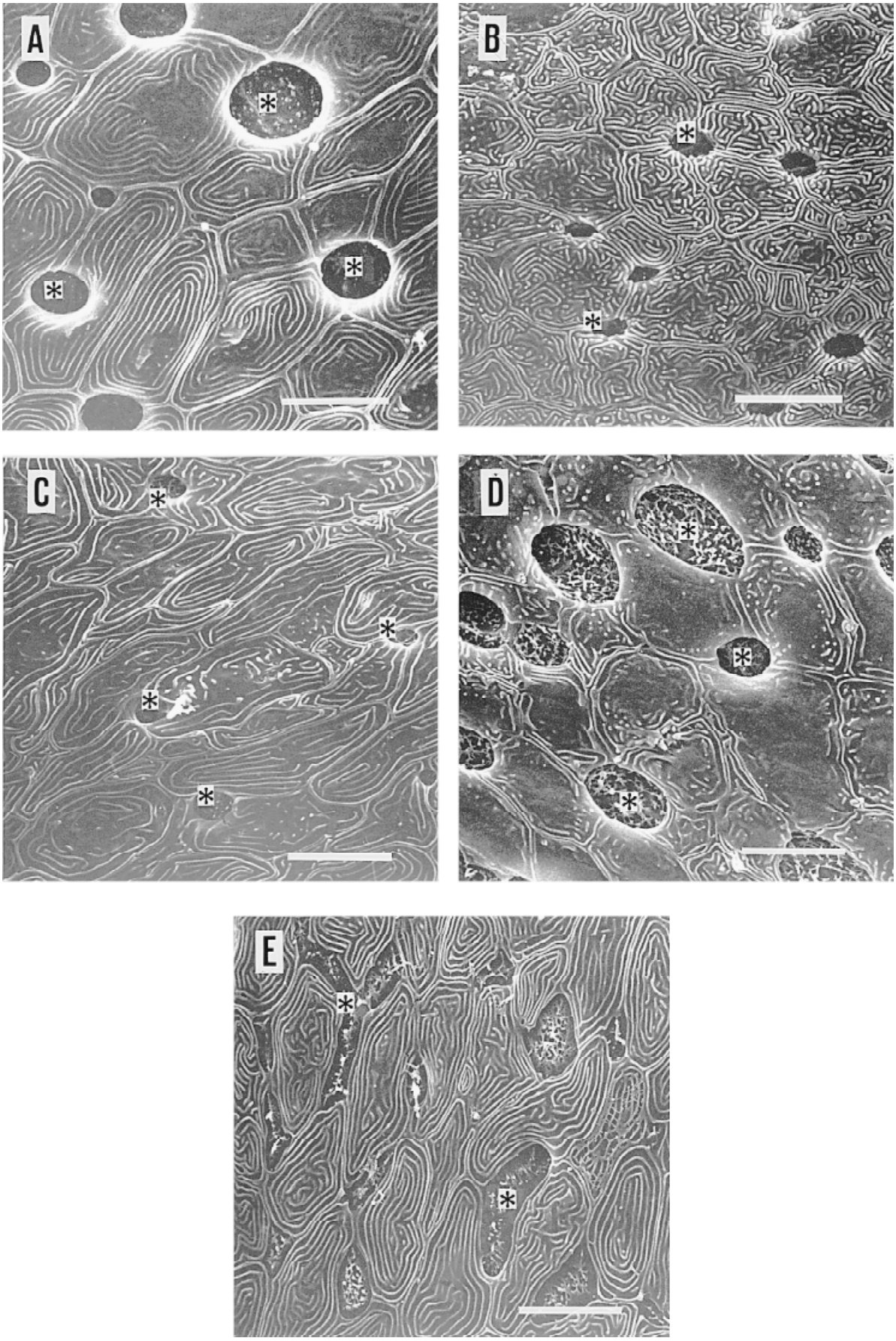

Figure 1.

(SEID) buffer (McCormick, '93). Kidney, gill and intestinal homogenates were centrifuged at 5,000*g* for 1 min and an aliquot removed for the analysis of Na^+-K^+-activated adenosinetriphosphatase activity. Kidney and gill suspensions were re-centrifuged for a further 10 min at 12,000*g* and the supernatants used for all other enzymes assays. All centrifugation steps were carried out at 0–4°C in a refrigerated centrifuge (Beckman model GS-15R, Palo Alto, CA) and homogenization conducted using an Ultra-turrax homogenizer. The protein content of supernatants were determined according to Hartree ('72) and enzyme activities are expressed as protein specific activities. The activities of all enzymes were measured at 25°C.

Na^+-K^+-activated adenosinetriphosphatase (E.C. 3.6.1.3.; Na^+-K^+-ATPase) was measured, in gill, kidney and intestinal regions according to McCormick ('93). Glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49.; G6P-DH) activity was measured according to Löhr and Waller ('74), lactate dehydrogenase (E.C. 1.6.4.3.; LDH) according to Bergmeyer and Bernt ('74) and isocitrate dehydrogenase (E.C. 1.1.1.42.; ICDH) according to Bernt and Bergmeyer ('74).

Statistical analysis

All data are presented as means ± S.E.M. and were subjected to either a one-way ANOVA or, in the case of Na^+-K^+-ATPase activity in intestinal segments, a two-way ANOVA. Subsequent significance was delineated using a Student-Newman-Keuls multiple comparison test (Sigmastat software, Jandel Scientific, San Rafael, CA).

RESULTS

Chloride cell morphology, numbers and morphometrics

The surfaces of the gill arch were covered with a mosaic of polygonal pavement cells with obvious, concentrically arranged surface ridges and the apical area of the CCs were demarcated as distinct openings along the borders of adjacent pavement cells (Fig. 1). The CCs were commonly found exposed on the afferent surfaces of the filament, on the interlamellar surfaces and around the base of the respiratory lamellae. Apical openings were completely absent from the efferent filament surface. In 50- and 33‰-adapted fish, CC openings were deep, round to oval invaginations (or crypts) with faintly discernible microvillous extensions on the internal surface (Figs. 1A, B, and 2A). Fish adapted to 12‰ exhibited several types of CC apical morphology (Fig. 1C). These consisted of the typical SW type, as described previously, along with cells that were the same shape but elevated to a greater extent. In some cases elevation rendered the CCs almost planar with the respiratory surface and the microvillous extensions present on the apical surface of the cells were more distinct. Fish adapted to 6‰ primarily displayed CCs that were planar with respiratory surface and microvillous extensions protruding from the cell surface were more numerous and extended (Figs. 1D, 2B). Despite this, a number of cells still exhibited a tendency to be slightly invaginated (Fig. 1D). Acclimation to FW resulted in a fundamental alteration in the shape of the CC apical surface. Unlike all the cells previously described, where the openings were round to oval, cells in 0‰-adapted fish were angular and often greatly elongated in appearance (Figs. 1E, 2C, D). The "freshwater-type" CC apical surfaces were elevated planar to the respiratory surface and while some had a dense network of microvillous extensions, others were smooth.

Gill cell isolate viability always remained acceptably high, ranging from 83.5% to 95.2% and CCs could be easily distinguished by DASPMI staining (Fig. 3A, B). In gill cell isolates, CC ratios were significantly ($P < 0.05$) elevated in 50‰ and 6‰ environments (Fig. 4i). CC exposure numbers were only significantly elevated in fish adapted to 6‰, when compared to fish held in 12 and 0‰ (Fig. 4ii). The apical area of CCs was significantly elevated in 50‰ and further elevated in low salinity environments of 6 and 0‰ (Fig. 4iii). The fractional surface area of CCs in fish acclimated to 6‰ was significantly greater than all other groups (Fig. 4iv). Acclimation of fish to 50 and 0‰ environments resulted in significantly elevated CC fractional surface area when compared to fish held in seawater and 12‰ conditions.

Serum chemistry and muscle composition

Serum Na^+ and Cl^- remained unaltered in a salinity range of 50–6‰ (Fig. 5i). Acclimation to 0‰ resulted in significant ($P < 0.05$) hyponatremia and hypochloremia. Serum protein levels tended to elevate in low salinity environments and circulating glucose levels in 0‰-adapted fish were significantly elevated ($P < 0.05$) when compared to fish held in salinities of 12 and 6‰ (Fig. 5ii). Freshwater adaptation resulted in significant ($P < 0.05$) muscle hydration while muscle lipid levels were not significantly affected (Fig. 6).

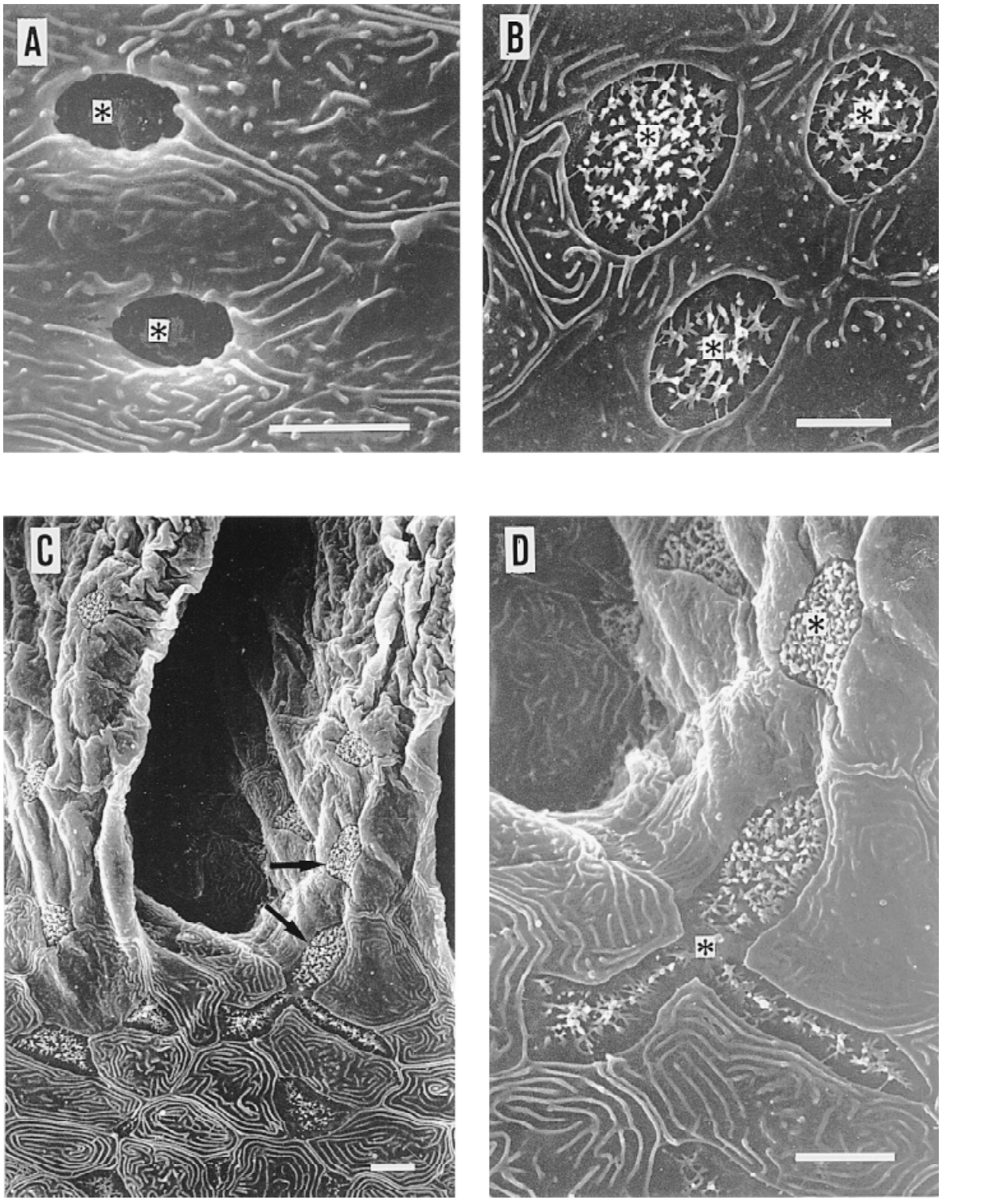

Fig. 2. Scanning electron micrographs of gill filaments from *Mylio macrocephalus* acclimated to (**A**) 33‰, (**B**) 6‰ and (**C**) and (**D**) 0‰ environments. All views show details of apical openings. In A note the presence of a typical seawater apical invagination which is replaced in low salinity environments (B–D) with an elevated condition and numerous microvillous extensions. In 0‰ (C and D) the normally round to oval openings become elongated and angular. Representative chloride cell apical openings are indicated by an asterisk and the arrows in C indicate the cells seen enlarged in D. Scale bars = 5 μm.

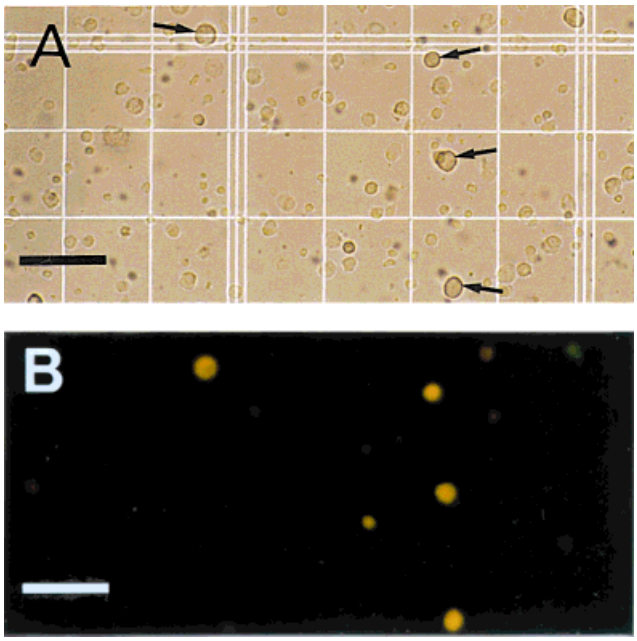

Fig. 3. Micrographs showing part of DASPMI-stained gill cell isolates (**A**) without and (**B**) with fluorescent excitation. In micrograph A the chloride cells are indicated by arrows and in micrograph B the same cells can be seen fluorescing. Scale bar = 50 μm.

Branchial enzymes

Branchial Na^+-K^+-ATPase activity significantly decreased in 6‰ when compared with the activity expressed by 33- and 50‰-acclimated fish (Table 2). A significant elevation of Na^+-K^+-ATPase activity occurred when fish were adapted to 0‰. Branchial G6P-DH activity was found to be highest in 33‰-adapted fish (Table 2). The activity of branchial G6P-DH was significantly lower in 50 and 12‰ environments and elevated in 6 and 0‰. Branchial LDH activity lowered in low salinity environments while ICDH activity was significantly greater in salinity extremes of 50 and 0‰ (Table 2).

RSI and kidney enzymes

Despite an apparent elevation of RSI in 50 and 0‰ environments, no statistical difference could be observed (Table 3). Acclimation to 12 and 6‰ caused a significant reduction in kidney Na^+-K^+-ATPase activity, whereas 0‰-adaptation saw a marked and significant elevation in the activity of this enzyme (Table 3). All other enzymes, G6Pase, G6P-DH, LDH and ICDH, remained unaffected in a salinity range of 50–6‰ (Table 3). Acclimation to 0‰ resulted in significant elevations in the activities of G6Pase, G6P-DH and LDH. The activity of ICDH remained unaltered in all environments (Table 3).

HSI and liver glycogen and enzymes

HSI tended to elevate in lower salinity environments (Table 4). Liver glycogen levels were significantly lower ($P < 0.05$) in fish adapted to 50‰ (Table 4). Hepatic G6Pase and ICDH activity were significantly elevated in 0‰-adapted fish, but did not significantly vary among other groups (Table 4). G6P-DH activity significantly ($P < 0.05$) elevated in lower salinity environments of 12 and 6‰ but slightly reduced in fish adapted to 0‰ (Table 4). In contrast, LDH activities tended to reduce in environments of 12 and 6‰ and were markedly elevated in fish adapted to 0‰ (Table 4).

VSI and intestinal Na^+-K^+-ATPase activity

VSI was significantly elevated in 50‰ when compared to groups adapted to 33, 6 and 0‰ (Table 5). Total intestinal Na^+-K^+-ATPase activity was greater in environments with a higher salt content and appeared to exhibit lowest activity in 12‰ (Fig. 7). The highest activity of Na^+-K^+-ATPase was found in the esophageal region of the fish acclimated to 50‰ (Table 5). In this region the activity was significantly greater than that found in the esophageal region of fish adapted to all other environments. Furthermore, the activity of Na^+-K^+-ATPase in the esophageal region of 50‰-acclimated fish was significantly greater than the activities found in all other regions of the intestine taken from the same fish. In fish adapted to 33‰, the activity of intestinal Na^+-K^+-ATPase tended to be higher in the midgut and rectal regions of the intestine (Table 5). In fish adapted to low salinity environments highest Na^+-K^+-ATPase activity occurred in the stomach (Table 5).

DISCUSSION

Euryhaline or diadromid fish acclimated from FW to an elevated salinity invariably display an increase in branchial CC numbers (Utida et al., ’71; Thomson and Sargent, ’77; Langdon and Thorpe, ’84; Kültz and Jürss, ’93; Avella et al., ’93), the functional importance of which has been reported in numerous studies where CC density has a marked effect on successful SW adaptation (Ouchi, ’85; Franklin, ’90; Madsen, ’90). Despite this, recent reports suggest that increased salinity may not always induce increased CC numbers. Acclimation to FW has been reported to augment CC populace in both mullet (Ciccotti et al., ’94) and tilapia (Van Der Heijden et al., ’97) while

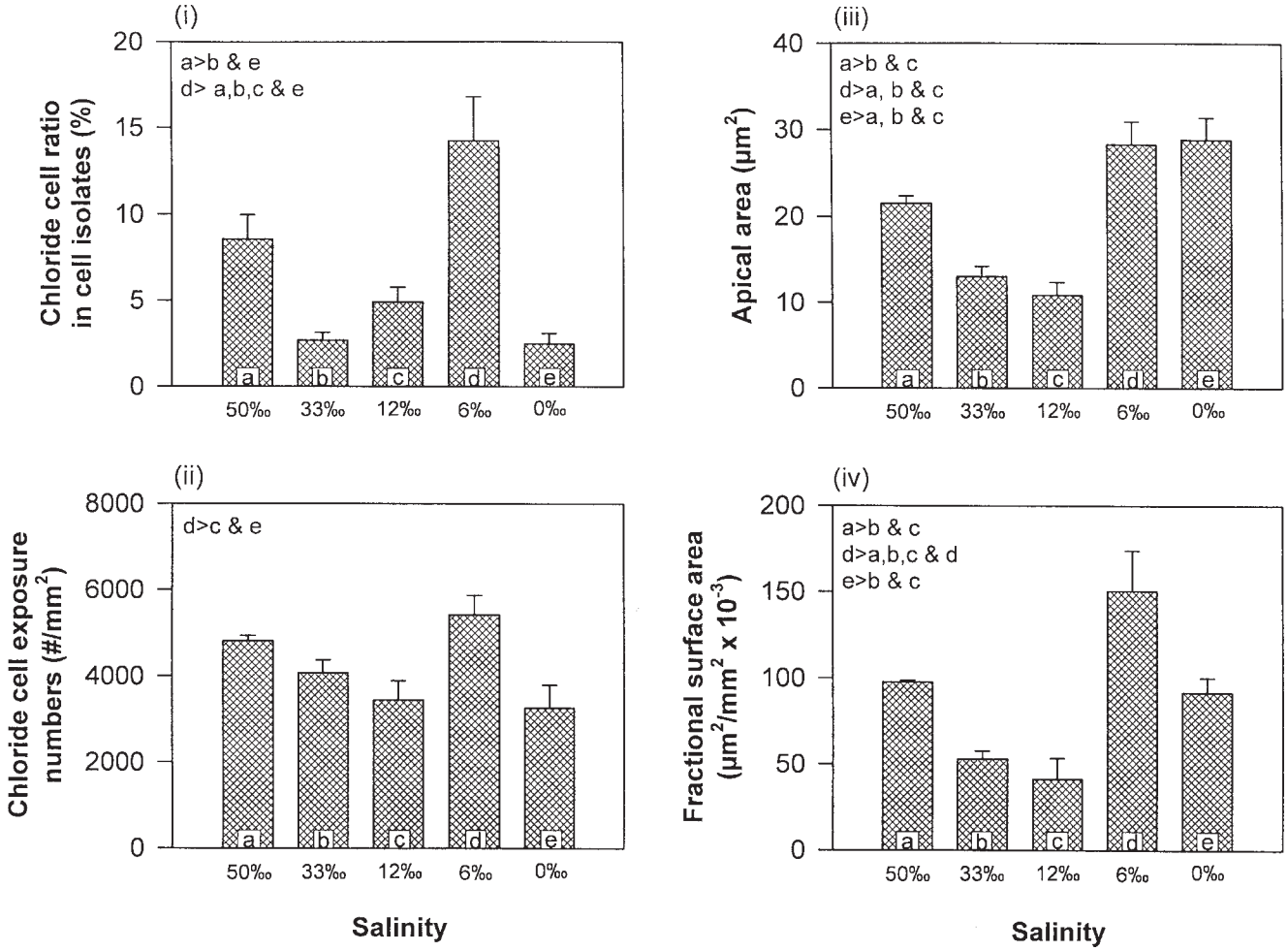

Fig. 4. Effect of salinity on chloride cell (**i**) ratio in cell isolates, (**ii**) exposure numbers, (**iii**) apical area and (**iv**) fractional area in the branchial epithelium of *Mylio macrocephalus*. Data are expressed as mean values ± S.E.M. Significance ($P < 0.05$) between groups a–e is denoted in the top left hand corner of each graph. n = (i) 5 or (ii–iv) 4.

Uchida et al. ('96) made an important distinction between the filamental and lamellar chloride cell populace of SW adapting chum salmon fry, reporting a decrease in total CC populace coupled with an increase in filament CC size and branchial Na^+-K^+-ATPase activity. Furthermore an elevated CC populace appears to be a necessity for successful acclimation of FW fish to ion poor conditions (for review see Perry, '97). Undoubtedly the CC is equally effective at eliminating ions as well as soliciting ion uptake. As such, it would seem that an increase in branchial CC numbers found in *Mylio macrocephalus* adapted to 6‰ may be analogous to that of FW fish adapted to ion poor conditions. Consistent with this hypothesis is an increase in CC apical area, fractional surface area and exposure numbers. The dual function of CCs as the driving force behind ion elimination and solicitation is emphasized by increased numbers in both 50 and 6‰ environments. A 0‰-induced reduction in CC numbers, however, seems contradictory as a further decline in environmental ion content may be expected to elicit a further increase in CC numbers. The tissues of fish in 0‰, however, were responding in an entirely different manner. Although a trend of increased CC exposure was sustained, CC numbers declined and Na^+-K^+-ATPase activity markedly elevated. It is this marked elevation in branchial Na^+-K^+-ATPase activity that separates the response of these fish from all other groups tested, and most likely holds the key to successful homeostatic control in *M. macrocephalus* under such conditions. As yet, the role of branchial Na^+-K^+-ATPase in FW fish escapes complete definition. Despite this, in *M. macrocephalus* adapted to 0‰, it seems very likely that elevated gill Na^+-K^+-ATPase plays an important role in ionic homeostasis.

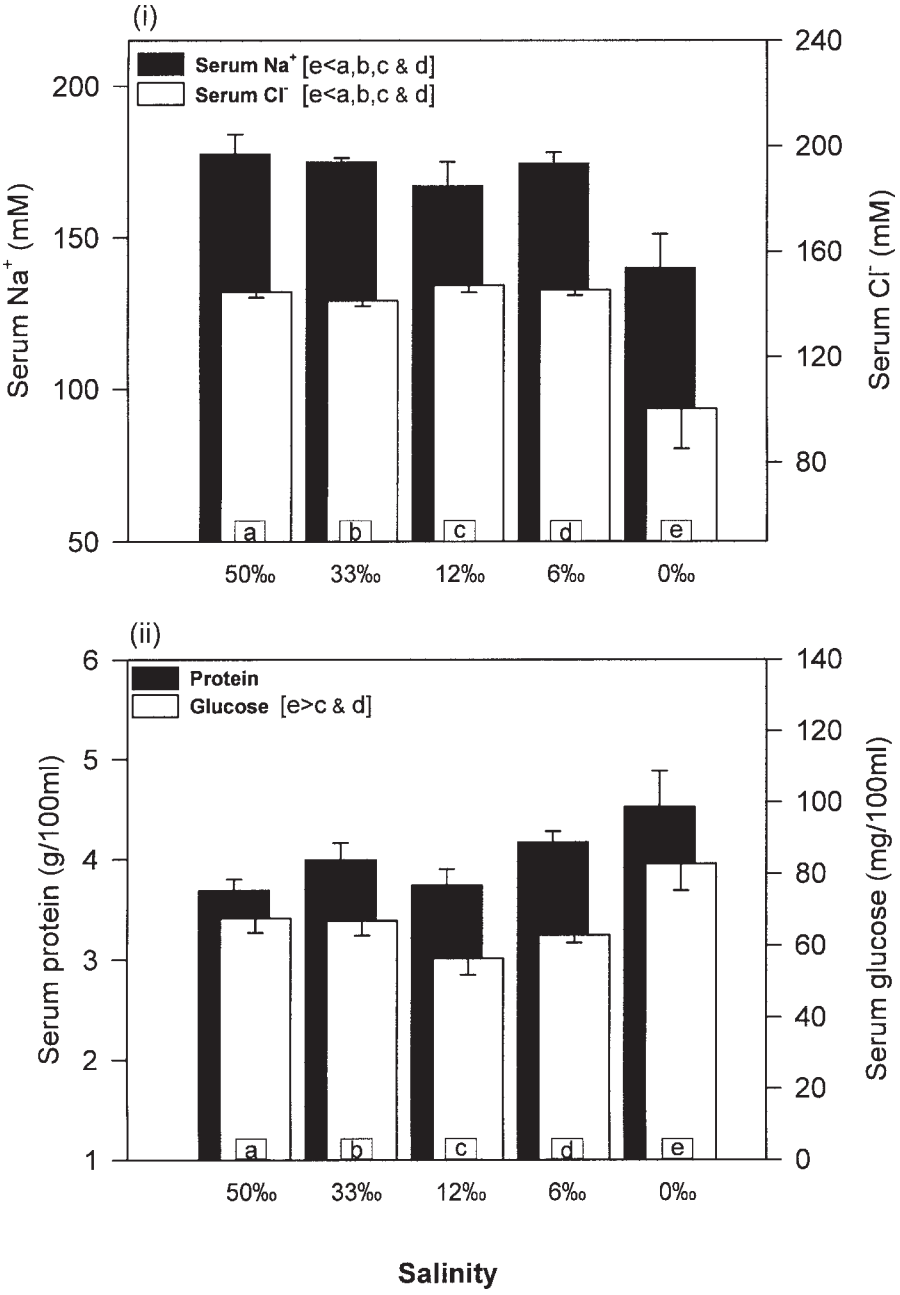

Fig. 5. Effect of salinity on serum (**i**) Na^+ and Cl^- and (**ii**) glucose and protein in *Mylio macrocephalus*. Data are expressed as mean values ± S.E.M. Significance ($P < 0.05$) between groups a–e is denoted in the top left hand corner of each graph. n = 7.

The generally accepted model of branchial Na^+-K^+-ATPase response to SW adaptation is an elevation in activity (Kamiya and Utida, '68 [*Anguilla japonica*]; Kirschner, '69 [*A. anguilla*]; Jampol and Epstein, '70 [*A. rostrata*]; Zaugg and McLain, '70 [*Oncorhynchus kisutch*]; Langdon and Thorpe, '84 [*Salmo salar*]; Madsen and Naamansen, '89 [*O. mykiss*]; Pelletier and Besner, '92 [*Salvelinus fontinalis*]; Madsen et al., '95 [*Salmo trutta*]). Furthermore, increased Na^+-K^+-ATPase activity most often parallels an increase in branchial CC populace (Utida et al., '71; Thomson and Sargent, '77; Perry and Walsh, '89; Kültz and Jürss, '93). However, contradictions do occur in the literature. Seven species of teleost (*Chelon labrosus* and *Dicentrarchus labarax,* Lassere, '71; *Liza ramada,* Gallis and Bourdichon, '76; *Platichthys flesus,* Stagg and Shuttleworth, '82; *Opsanus beta,* Mallery, '83;

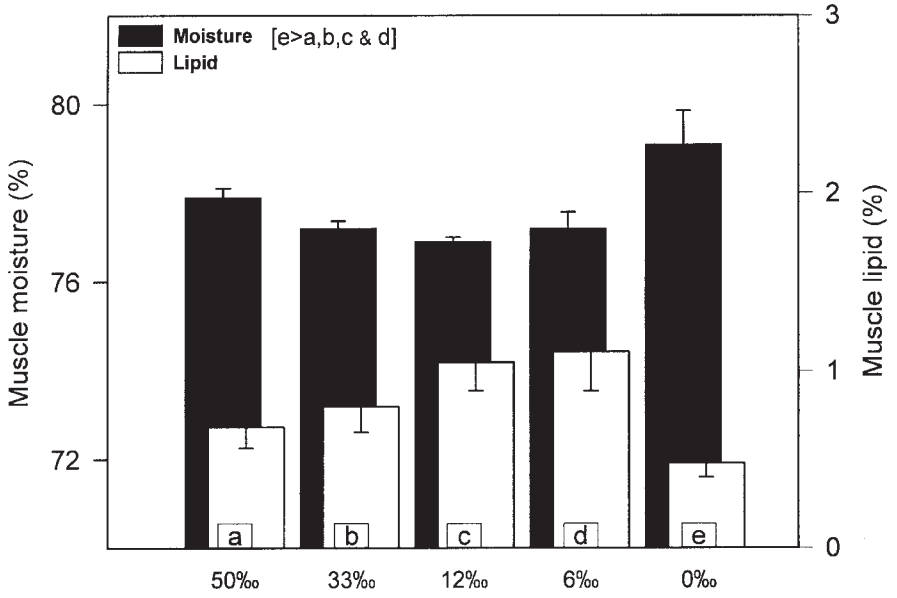

Fig. 6. Effect of salinity on muscle moisture and lipid content in *Mylio macrocephalus*. Data are expressed as mean values ± S.E.M. Significance ($P < 0.05$) between groups a–e is denoted in the top left hand corner of the graph. n = 7.

Mugil cephalus, Ciccotti et al., '94; *Pomacanthus imperator,* Woo and Chung, '95), five of which are notoriously euryhaline, exhibit a response that questions the applicability of a single model. That is, branchial Na^+-K^+-ATPase activity is greater in FW- or low salinity–acclimated fish than in SW fish. While *M. macrocephalus* exhibited a decline in branchial Na^+-K^+-ATPase activity in 6‰, adaptation to 0‰ elevated enzyme activity, and as such, the response of this fish appears to be consistent with the species mentioned above. From an ecophysiological standpoint, it may be of further importance to note that of the fish exhibiting this "alternative" Na^+-K^+-ATPase response, the majority are either marine or estuarine dependent marine species, suggesting that the ill-defined processes of low salinity adaptation in marine fish may yield a general model of response substantially different from the diadromid paradigm. In support of this, the present study revealed further deviations from the accepted model in that *M. macrocephalus* exhibited an uncoupling of the typical parallel relationship between branchial Na^+-K^+-ATPase activity and CC numbers. This occurred in fish acclimated to 6‰, where CC numbers were high and Na^+-K^+-ATPase activity low, and in 0‰, where CC numbers were low and Na^+-K^+-ATPase activity high. Clearly, further work on the duality of such a response is warranted.

In studies performed to date, and in the absence of appreciable gill cell gluconeogenesis (Mommsen, '84), teleost gills appear to rely primarily on circulating glucose and lactate as metabolic fuel, with the use of other substrates, such as amino acids, occurring to a lesser extent (Perry and Walsh, '89). A number of enzymes associated with the intermediary metabolism of gills exhibit elevated activity during salinity transfer (Langdon and Thorpe,

TABLE 2. Effect of salinity on gill enzyme activities of Mylio macrocephalus[1]

	50‰	33‰	12‰	6‰	0‰
Na^+-K^+-ATPase (μmoles NADH/min/mg)	45.4 ± 7^{ab}	59 ± 9^{b}	34 ± 5^{ac}	22 ± 6^{c}	192 ± 44^{d}
G6P-DH (μmoles NADPH/hr/mg	1.07 ± 0.03^{a}	2.13 ± 0.19^{b}	1.28 ± 0.11^{a}	1.64 ± 0.14^{c}	1.94 ± 0.11^{bc}
LDH (μmoles NADH/hr/mg)	150 ± 16^{a}	167 ± 45^{a}	118 ± 17^{ab}	86 ± 20^{b}	87 ± 10^{b}
ICDH (μmoles NADPH/hr/mg)	13.5 ± 1.5^{a}	9.1 ± 0.7^{b}	6.8 ± 0.5^{c}	9.0 ± 0.3^{b}	15.4 ± 1.0^{a}

[1]Data are expressed as mean values ± S.E.M. Within a row of data, values having different superscripts are significantly different ($P < 0.05$). The activities of all enzymes are expressed as protein specific activities.

TABLE 3. Effect of salinity on RSI and kidney enzyme activities of Mylio macrocephalus[1]

	50‰	33‰	12‰	6‰	0‰
RSI (%)	0.19 ± 0.03[a]	0.18 ± 0.01[a]	0.16 ± 0.01[a]	0.14 ± 0.01[a]	0.21 ± 0.02[a]
Na^+-K^+-ATPase activity (μmoles NADH/min/mg)	252 ± 19[a]	271 ± 14[a]	177 ± 18[b]	174 ± 10[b]	493 ± 46[c]
G6Pase activity (μmoles Pi/hr/mg)	17.4 ± 3.4[a]	19.1 ± 5.6[a]	21.6 ± 2.2[a]	21.1 ± 2.4[a]	38.9 ± 5.4[b]
G6P-DH activity (μmoles NADPH/hr/mg)	1.88 ± 0.14[a]	1.78 ± 0.10[a]	1.87 ± 0.05[a]	1.91 ± 0.10[a]	2.29 ± 0.07[b]
LDH activity (μmoles NADH/hr/mg)	15.5 ± 2.6[a]	12.9 ± 0.9[a]	15.6 ± 0.6[a]	18.2 ± 1.1[a]	44.8 ± 5.6[b]
ICDH activity (μmoles NADPH/hr/mg)	11.6 ± 1.3[a]	10.9 ± 0.8[a]	8.7 ± 0.6[a]	8.3 ± 0.3[a]	11.4 ± 1.0[a]

[1]Data are expressed as mean values ± S.E.M. Within a row of data, values having different superscripts are significantly different ($P < 0.05$). The activities of all enzymes are expressed as protein specific activities.

'84; McCormick et al., '89; Soengas et al., '95) and Perry and Walsh ('89) demonstrated a linear correlation between Na^+-K^+-ATPase activity and CO_2 production from lactate in tilapia gill cell suspensions. To the best of our knowledge, however, no published report describes branchial enzyme activities of marine fish in varying salinities. Alterations in the activities of enzymes in the gills of *Mylio macrocephalus* do not appear to clearly mirror the changes that occur in gill Na^+-K^+-ATPase activity or CC numbers. It is noteworthy, however, that branchial ICDH activity, an enzyme of the citric acid cycle, representing the hub of the metabolism, is elevated at both salinity extremes and lowest in a near isosmotic environment of 12‰. This trend also appeared to manifest in renal and hepatic tissue, and may reflect a diminished metabolic cost of osmoregulation (Woo and Kelly, '95).

Salinity-induced alterations in the circulating electrolytes of euryhaline fish are well documented. Acclimation of marine fish to low salinity regimes usually results, when given enough time, in minor differences between pre- and post-transfer electrolyte levels (Woo and Fung, '81; Woo and Wu, '82; Dutil et al., '92; Mancera et al., '93; Provencher et al., '93; Munro et al., '94; Woo and Chung, '95). This is certainly the case in various species of seabream, where Na^+ and Cl^- levels either remain stable or marginally decline after "sub-seawater" acclimation (Woo and Fung, '81; Mancera et al., '93). In the present study, *Mylio macrocephalus* adapted to FW for three weeks exhibited an ≈19% reduction in serum Na^+ levels. The efficiency of the osmoregulatory processes in 0‰-adapted *M. macrocephalus* is evident when compared to the disparity found in Na^+ levels between FW- and SW-adapted eels, where SW levels were ≈164 mM and FW levels fell to ≈117 mM, and ≈29% reduction (Utida et al., '71). Furthermore, acclimation to a salinity range of 50–6‰ had no effect on final primary electrolyte levels, indicating maintenance of a tight extracellular Na^+ and Cl^- range. Further credence is given to efficient homeostatic control by stable muscle moisture content, where tissue hydration is only exhibited by fish in 0‰.

Salinity variation has distinct effects on the osmoregulatory function of renal tissue in fish. Yoon et al. ('93) described morphological changes while Oikari and Rankin ('85), Brown et al. ('80) and Salman and Eddy ('88) described changes in di-

TABLE 4. Effect of salinity on HSI, liver glycogen and enzyme activities of Mylio macrocephalus[1]

	50‰	33‰	12‰	6‰	0‰
HSI (%)	1.25 ± 0.18[a]	1.38 ± 1.10[ab]	1.87 ± 0.15[b]	1.71 ± 0.12[b]	1.78 ± 0.05[b]
Liver glycogen (mg/g)	4.12 ± 0.88[a]	7.75 ± 0.64[b]	8.05 ± 0.92[b]	10.14 ± 0.41[b]	9.92 ± 0.92[b]
G6Pase (μmoles Pi/min/mg)	23.5 ± 3.28[a]	21.7 ± 1.59[a]	17.2 ± 2.34[a]	15.7 ± 2.47[a]	38.1 ± 5.83[b]
G6P-DH (μmoles NADPH/hr/mg)	8.9 ± 1.0[a]	10.3 ± 0.9[a]	16.9 ± 1.3[b]	15.8 ± 1.3[b]	12.9 ± 1.4[ab]
LDH (μmoles NADH/hr/mg)	3.76 ± 0.26[a]	3.98 ± 0.43[a]	3.02 ± 0.36[ab]	2.38 ± 0.21[b]	5.20 ± 0.58[c]
ICDH (μmoles NADPH/hr/mg)	1.40 ± 0.20[a]	1.30 ± 0.09[a]	1.03 ± 0.14[a]	0.94 ± 0.15[a]	2.28 ± 0.35[b]

[1]Data are expressed as mean values ± S.E.M. Within a row of data, values having different superscripts are significantly different ($P < 0.05$). The activities of all enzymes are expressed as protein specific activities.

TABLE 5. Effect of salinity on VSI (%) and Na^+-K^+-ATPase activity (μmoles NADH/min/mg) in different regions of the intestinal tract of Mylio macrocephalus[†]

	Intestinal region					
Salinity	VSI	Esophagus	Stomach	Pyloric ceca	Midgut	Rectum
50‰	7.14 ± 0.40*	11.9 ± 2.2^{a2}	7.32 ± 0.70^{ab1}	7.53 ± 1.57^{a1}	7.31 ± 1.68^{a1}	4.41 ± 0.73^{ab1}
33‰	5.13 ± 0.16	5.58 ± 0.68^{b1}	5.07 ± 0.67^{a1}	4.37 ± 0.32^{a1}	7.89 ± 1.90^{a1}	8.77 ± 1.40^{a1}
12‰	5.94 ± 0.66	2.89 ± 0.37^{b1}	4.18 ± 0.42^{a1}	4.19 ± 0.87^{a1}	3.68 ± 0.57^{a1}	2.61 ± 0.27^{b1}
6‰	5.29 ± 0.27	4.44 ± 1.00^{b1}	9.00 ± 0.62^{b2}	5.19 ± 1.10^{a1}	4.10 ± 0.53^{a1}	4.17 ± 0.49^{ab1}
0‰	4.29 ± 0.71	3.8 ± 0.63^{b1}	8.33 ± 0.56^{b2}	3.82 ± 0.76^{a1}	3.46 ± 0.90^{a1}	2.45 ± 0.49^{b1}

[†]Enzyme activity is expressed as protein specific activity. n = 6. For Na^+-K^+-ATPase, different alphabetical superscripts denote significance ($P < 0.05$) within a column of data. For Na^+-K^+-ATPase, different numerical superscripts denote significance ($P < 0.05$) within a row of data. For VSI, *denotes significance ($P < 0.05$) within a column of data.

valent ion transport, glomerular filtration and urine production respectively. These processes, like those discussed for the branchial epithelium of fish, are likely to necessitate alterations in metabolic enzyme activities and/or availability of energetic substrates. Furthermore, kidney tissue is rich in Na^+-K^+-ATPase, the activity of which alters in response to salinity variation (Lassere, '71; Gallis and Bourdichon, '76; Venturini et al., '92). As such, it is surprising that few studies have addressed changes in the metabolism of the kidney relative to salinity adaptation (Jürss et al., '87; McCormick et al., '89; Soengas et al., '94). During SW adaptation, the major renal energetic cost is in the production of small amounts of concentrated urine (Furspan et al., '84). Increased energetic demand associated with this phenomenon have been assessed via the measurement of respiratory enzymes such as cytochrome C oxidase and citrate synthetase (McCormick et al., '89), and Soengas et al. ('94) reported substrate provision via enhanced glycogenolysis and gluconeogenesis in SW exposed rainbow trout. To date, and with the exception of Na^+-K^+-ATPase activity (Lassere, '71; Gallis and Bourdichon, '76; Venturini et al., '92), no studies have addressed the metabolic effects of salinity transfer in the SW to FW direction.

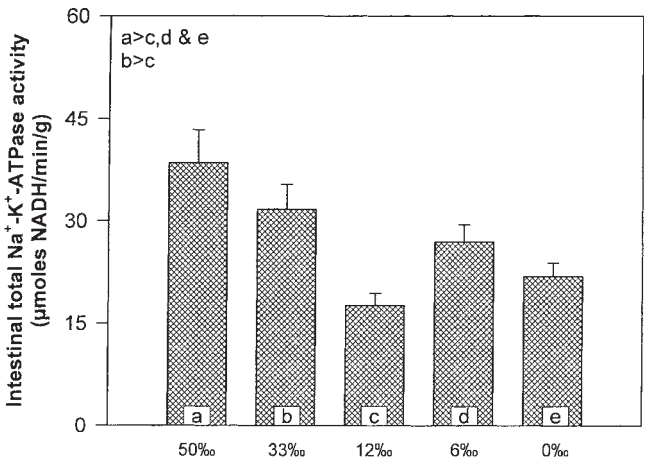

Fig. 7. Effect of salinity on total intestinal Na^+-K^+-ATPase activity in *Mylio macrocephalus*. Data are expressed as mean values ± S.E.M. Significance ($P < 0.05$) between groups a–e is denoted in the top left hand corner of the graph. n = 5.

In *Mylio macrocephalus,* renal Na^+-K^+-ATPase is lower in fish adapted to 12 and 6‰; however, adaptation to 0‰ elicited a marked increase in activity, a phenomenon in line with previous observations of estuarine-dependent marine fish adapted to FW (Lassere, '71; Gallis and Bourdichon, '76; Venturini et al., '92). Elevated renal gluconeogenesis coupled with an elevation in glycolysis suggests that in situ production of glucose may provide the substrate required for elevated activity. In contrast, however, salinity has little effect on renal Na^+-K^+-ATPase activity in salmonids such as brown trout (*Salmo trutta*) (Madsen et al., '95). This may be an additional link in the alternative strategies previously discussed. An increase in renal G6P-DH activity of fish in 0‰ provides further evidence of an increased kidney metabolism in *M. macrocephalus,* yet the activity of renal ICDH did not appear to be affected. The reasons for this are unclear; however, a trend that mimicked the response of Na^+-K^+-ATPase was present.

The effect of salinity on the composition of hepatic tissue has previously been investigated in a number of seabream species (Woo and Fung, '81; Woo and Murat, '81; Woo and Wu, '82; Woo and Kelly, '95), as well as other marine fish (Woo and Wu, '82; Lambert et al., '94; Woo and Chung, '95). Despite an array of different nutritional variables and shorter adaptation periods, the general response of seabream in dilute media is elevated glycogen and lipid content, decreased protein content and elevated gluconeogenic strategies (dietary dependent). In line with the first of these characteristics, *Mylio macrocephalus* adapted to low sa-

linity regimes exhibited elevated liver glycogen content. In contrast, however, fish held in 6‰ do not exhibit elevated gluconeogenic strategies. Despite this, adaptation to 0‰ revived gluconeogenic strategies and this was coupled with an apparent increase in glycolytic enzyme activity and overall metabolic action in the form of hepatic LDH and ICDH respectively. Furthermore, the pentose phosphate shunt appeared to be activated in salinities of 12 and 6‰ indicating enhanced production of reducing power, a phenomenon consistent with previously described observations of elevated liver lipid levels in low salinity adapted seabream (Woo and Kelly, '95).

Salinity-induced alterations in circulating metabolites are also well documented in a number of seabream (Ishioka, '80; Woo and Fung, '81; Woo and Murat, '81; Woo and Wu, '82; Mancera et al., '93) and other marine species (Woo and Wu, '82; Dutil et al., '92; Provencher et al., '93; Munro et al., '94; Woo and Chung, '95). In general, no differences can be found in the levels of circulating glucose of seabream fully acclimated to low salinity environments (Woo and Fung, '81; Woo and Murat, '81; Woo and Wu, '82; Mancera et al., '93). Glucose levels found in *Mylio macrocephalus* within a salinity range of 50–6‰ are consistent with the above observations; however, the hyperglycemic response of *M. macrocephalus* in 0‰ indicated an increase in substrate mobilization that is well reflected by the apparent elevations in metabolic activity of the osmoregulatory organs. This hyperglycemia may also be closely linked to the increase in hepatic and renal gluconeogenesis given that liver glycogen levels do not significantly decrease. Serum or plasma protein levels were elevated in food-deprived red (*Chryrosphrys major*) and black seabream (*Mylio macrocephalus*) acclimated to low salinity environments (Woo and Murat, '81); Woo and Wu, '82). In silver seabream (*Sparus sarba*), however, fed (Kelly, '94) or food deprived (6 days starvation) (Kelly, '97) fish exhibited no significant salinity-induced alteration in circulating protein levels. Although serum protein levels were not significantly elevated in *M. macrocephalus* adapted to 0‰, an elevated trend is visible, a result that concurs with prior observations of Woo and Wu ('82). In light of the elevated status of both glucose and protein in 0‰-adapted fish, coupled with a reduction in primary electrolyte levels, it would seem likely that these substances fulfill the role of additional "osmotically active substances," suggested to be present in low salinity challenged *M. macrocephalus* by Woo and Wu ('82).

In a marine environment, fish drink SW and an increase in the intestinal absorption of Na^+, Cl^- and water, coupled with the ion eliminating capabilities of the gill and kidney, allows successful hydromineral balance. As such, elevated intestinal Na^+-K^+-ATPase activity is most often associated with SW adaptation (Jampol and Epstein, '70; McKay and Janicki, '79; Colin et al., '85). In contrast FW fish do not drink the surrounding water and electrolyte loss is compensated via ion uptake at the gills and from food. A progressive processing of seawater in the gut of fish, via in vivo observations of the luminal concentrations of monovalent ions, has been demonstrated in the gut of eel (Sharratt et al., '64) and trout (Shehadeh and Gordon, '69). These observations ultimately led to the discovery of the osmoregulatory importance of the esophagus (Kirsch and Laurent, '75), the permeability of which allows fast absorption of 50–70% of Na^+ and Cl^- ions ingested down an electrochemical gradient. Although this phenomenon may not necessitate high esophageal Na^+-K^+-ATPase activity in 33‰-acclimated *Mylio macrocephalus,* acclimation to 50‰ elevated Na^+-K^+-ATPase activity in this region. Indeed, the activity in the esophagus of *M. macrocephalus* adapted to 50‰ is greater than that found in all other intestinal segments assessed in 50‰–adapted fish and the esophageal regions of seabream adapted from 33–0‰. Furthermore, total Na^+-K^+-ATPase activity in fish adapted to varying salinities appeared to reflect the decreasing concentration of external ions. However, the pattern of Na^+-K^+-ATPase activity in different intestinal regions suggested that the site-specific importance of this enzyme varies greatly in different environments. That is, progressive desalination may take place in fish adapted to 50 and 33‰; however, the role of Na^+-K^+-ATPase in the desalination of intestinal fluid appeared to result in an elevation in Na^+-K^+-ATPase activity only in the posterior regions of the gastrointestinal tract. Fish adapted to 12‰ exhibited low enzyme activities throughout the intestine, possibly due to a diminished need to balance extracellular ion concentration due to the reduced ionic gradient in a near isosmotic environment. This phenomenon can be further supported by the low branchial and renal Na^+-K^+-ATPase activities found in 12‰-adapted fish and is often reflected in key metabolic enzymes such as branchial and renal ICDH activity, sug-

gesting an overall reduction in the metabolic work done by osmoregulatory organs. An interesting phenomenon is, however, the elevated activity of Na^+-K^+-ATPase found in the stomachs of fish adapted to 6 and 0‰. This may be related to the replacement of lost ions via food and may be supported by recent reports where an increase in the dietary salt load of a FW fish results in an increase in branchial Na^+ efflux rates (Smith et al., '95). In light of the ability of freshwater fish to replace lost ions via food, elevated Na^+-K^+-ATPase activity in the stomach may reflect the importance of ion provision from food in low salinity acclimated marine fish.

In conclusion, the results of the present study demonstrate the haloplasticity of black seabream (*Mylio macrocephalus*) and reveal efficient osmoregulatory strategies in a salinity range of 50–0‰. Successful FW acclimation, albeit after prolonged hyposmotic acclimation, is demonstrated for the first time and tissue reorganization indicated that both elevated branchial and renal Na^+-K^+-ATPase play a key role in successful homeostatic control. An uncoupling of the typical parallel relationship between CC numbers and Na^+-K^+-ATPase activity in fish acclimated to 6‰ suggests that an alternative model of osmoregulatory response to low salinity environments is present in this fish species.

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