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Developmental and Environmental Regulation of Chloride Cells in Young American Shad, *Alosa sapidissima*

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ABSTRACT Location, abundance, and morphology of gill chloride cells were quantified during changes in osmoregulatory physiology accompanying early development in American shad, *Alosa sapidissima*. During the larval-juvenile transition of shad, gill chloride cells increased 3.5-fold in abundance coincident with gill formation, increased seawater tolerance, and increased Na^+,K^+-ATPase activity. Chloride cells were found on both the primary filament and secondary lamellae in pre-migratory juveniles. Chloride cells on both the primary filament and secondary lamellae increased in abundance (1.5- to 2-fold) and size (2- to 2.5-fold) in juveniles held in fresh water from August 31 to December 1 (the period of downstream migration) under declining temperature. This proliferation of chloride cells was correlated with physiological changes associated with migration (decreased hyperosmoregulatory ability and increased gill Na^+,K^+-ATPase activity). Increases in chloride cell size and number of fish in fresh water were delayed and of a lower magnitude when shad were maintained at constant temperature (24°C). When juveniles were acclimated to seawater, chloride cell abundance on the primary filament did not (though size increased 1.5- to 2-fold), but cells on the secondary lamellae disappeared. Na^+,K^+-ATPase was immunolocalized to chloride cells in both fresh water and seawater acclimated fish. The disappearance of chloride cells on the secondary lamellae upon seawater acclimation is evidence that their role is confined to fresh water. The proliferation of chloride cells in fresh water during the migratory-associated loss of hyperosmoregulatory ability is likely to be a compensatory mechanism for increasing ion uptake. *J. Exp. Zool. 290:73–87, 2001.*

The mechanisms of osmoregulation in teleosts acclimated to seawater (SW) have been aggressively studied and are relatively well understood. Chloride cells in the gill have been convincingly shown to be the site of ion excretion in fish acclimated to hypersaline environments (Keys and Willmer, '32; Foskett and Scheffey, '82). Euryhaline fish can tolerate a wide range of salinities during their life history, and chloride cells have been demonstrated to enlarge and proliferate upon acclimation to increased salinity in a number of teleosts (Karnaky, '86). Chloride cells are rich in Na^+,K^+-ATPase (Karnaky et al., '76; McCormick, '93; Ura et al., '96) and this enzyme is essential for ion excretion in SW acclimated fish. The most widely accepted model of Na^+ and Cl^- secretion by the gills is based on the presence of Na^+,K^+-ATPase on the basolateral membrane of the chloride cells which drives the transport of sodium (paracellularly) and chloride via an electroneutral cation-chloride cotransport system in concert with a passive apical chloride channel (Silva et al., '77; Foskett et al., '83a).

There is strong circumstantial evidence for the role of branchial chloride cells in sodium and chloride uptake (Laurent and Dunel, '80; Goss et al., '92) but the current understanding of this process is less complete than that of ion excretion. (Here "chloride cells" are identified by morphology and mitochondrion-richness, not by function.) Chloride cells of fresh water (FW) acclimated fish are generally smaller and fewer in number than in SW acclimated fish. These cells are either latent SW excretory cells or, more likely, cells with a significant physiological role in FW (and perhaps both). The presence of chloride cells in stenohaline FW species such as the cyprinids *Cyprinus*

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carpio and *Carassius auratus* supports this view (Lee et al., '96).

Models of sodium and chloride uptake generally include a Na^+/H^+ exchange and HCO_3^-/Cl^- exchange on the apical surface of the gill. Initial models included an apical Na^+/H^+ exchanger based upon the proportional transport of H^+ and Na^+ across the teleost gill (Goss and Wood, '90). More recent evidence supports the uptake of sodium through an apical channel, driven by the electrochemical gradient generated by H^+-ATPase (on the apical surface) and Na^+,K^+-ATPase (on the basolateral membrane) (Perry, '97; Claiborne, '98). Chloride cells have also been strongly implicated in acid–base metabolism (Goss et al., '92) and in calcium uptake (McCormick, '92; Flik et al., '95; Perry, '97; Marshall and Bryson, '98).

For fish that travel between fresh water and seawater, the appropriate chloride cell function and morphology must be coordinated with the salinity of the environment. Anadromous fish enter seawater at a particular life history stage, and the timing of entry is often predictable. Changes in the osmoregulatory mechanisms of anadromous fish are necessarily linked to developmental and environmental cues at the time of juvenile migration. While this is basic to the biology and management of many species, only salmonids have been extensively studied. The interaction between development, environment and osmoregulation is considered here for American shad (*Alosa sapidissima*).

As temperatures rise in spring, adult shad enter and spawn in the fresh water of rivers along the Atlantic coast of North America. Young shad must hyperosmoregulate as they develop and grow within the river system. Shad develop the ability to enter full-strength SW at the time of their larval–juvenile transition, months prior to their usual downstream migration. Development of seawater tolerance coincides with gill development (Shardo, '95; Zydlewski and McCormick, '97a) and increased gill Na^+,K^+-ATPase activity (Zydlewski and McCormick, '97a). It is likely that increased seawater tolerance and increased gill Na^+,K^+-ATPase activity during larval–juvenile transition are due to the proliferation of chloride cells capable of ion excretion.

During the period of seaward migration, shad lose the ability to osmoregulate effectively in FW (evidenced by decreased plasma chloride) resulting in increased mortality (Zydlewski and McCormick, '97b). The nature of this developmental loss of hyperosmoregulatory ability is not understood but may be linked to migratory behavior. Decreases in plasma chloride and increases in gill Na^+,K^+-ATPase activity are exaggerated by decreasing temperature. Increased gill Na^+,K^+-ATPase activity during this period is hypothesized to be a response to increased osmoregulatory demands; an increase in ion uptake machinery. If this hypothesis is correct, an increase in ion uptake cells (chloride cells) would be observed through the period of downstream migration.

In this study, changes in abundance, morphology and distribution of branchial chloride cells were quantified during the larval–juvenile transition period using classical histological techniques. The purpose of this approach was to clarify the relationship between chloride cell differentiation, increased gill Na^+,K^+-ATPase activity, gill development and the onset of seawater tolerance during this transition. Gill chloride cell morphology and abundance were also quantified in juveniles subjected to declining and constant temperature regimes in fresh water or seawater through the period of downstream migration. The goal was to characterize the effects and interactions of these environmental factors and development on gill chloride cells during the period of downstream migration. Gill Na^+,K^+-ATPase activity and the immunolocalization of this enzyme in the gill tissue is used to infer chloride cell function during this transitory life history stage prior to and subsequent to seawater entry.

MATERIALS AND METHODS

Rearing of shad through the larval–juvenile transition: SW tolerance and chloride cells

Rearing, SW challenges, and physiological sampling for American shad were carried out as described in Zydlewski and McCormick ('97a). Briefly, adults were captured in the Connecticut River on June 6, 1993, artificially spawned, and reared at the Conte Anadromous Fish Research Center, Turners Falls, MA. Hatching occurred on June 13, and young shad were maintained on river water under simulated natural photoperiod (SNP). The larval–juvenile transition was completed by 45 days post-hatch. Iso-thermal SW challenges entailed the transfer of 24 fish into either a 60-l test tank (35 ppt) or a control tank (FW) with matched flow and temperature. Ten shad were transferred to 300-l tanks for a SW challenge at 72 days post-hatch. Surviving fish were counted after 24 hr (in all tests) and sacrificed in accordance with animal care protocols (anesthetized with MS-222; 100 mg/l, pH 7.0). These protocols were adhered to in all methods where fish were sacrificed.

Gill tissue samples were taken for measurement of Na^+,K^+-ATPase activity (n = 24), gill development (n = 8), and analysis of chloride cell size and density (n = 3 to 8) from different individuals in the same cohort of shad maintained in FW (18–45 days post-hatch).

Rearing of shad through the period of migration; effects of temperature and salinity

Fish used to describe gill histology in this study are the same fish used for physiological analysis in Zydlewski and McCormick ('97b). Pre-migratory juvenile shad were captured in August 1994 in the Connecticut River, divided into four 1,100-l tanks (60 fish per tank), and maintained on river water. All groups were maintained under SNP and fed salmon feed (No. 2, Zeigler Bros., Gardners, PA). Sampling included the removal of gill tissue for measurement of Na^+,K^+-ATPase activity and analysis of chloride cell size and density. Four fish from each tank were sampled on August 31 (estimated 70–80 days post-hatch based on length and mass; Crecco and Savoy, '85; Zydlewski and McCormick, '97a) after which the tanks were converted to closed (recirculating) systems. Over the next 10 days, the salinities of two tanks were elevated in parallel to 32 ppt salinity (SW). The remaining two tanks were maintained at 0 ppt (river water; 5.9 ppm Na^+, 2.1 ppm Cl^-, conductivity 185 $\mu S \cdot cm^{-1}$). One SW tank and one FW tank were maintained at 24°C for the entire experiment. The temperatures of the other two tanks were maintained within 0.5° of the daily measured Connecticut River temperature (simulated natural temperature; SNT). Over the course of the study, corresponding FW and SW groups did not differ by more than 1°C.

At approximately four-week intervals, 10 fish were sacrificed (anesthetized with MS-222; 100 mg/l, pH 7.0) and sampled. Length and mass (mean ± SE) were 6.7 ± 0.1 cm and 2.3 ± 0.2 g at the onset of the experiment. At the end of the experiment (December 1), length and mass did not differ between salinity treatments but did differ between temperature treatments. Length and mass were 9.0 ± 0.1 cm and 7.3 ± 0.6 g for the SNT (both FW and SW) groups and 10.1 ± 0.2 cm and 11.0 ± 0.7 g for the 24°C groups, respectively.

Rearing of shad for immunohistochemistry; localization of gill Na^+,K^+-ATPase

Actively migrating juvenile shad were captured on the Connecticut River at Turners Falls, MA (198 km from the ocean) at a by-pass structure at the Cabot Station hydroelectric facility operated by Northeast Utilities on October 1, 1997. Fish were transported to CAFRC where they were divided into two 1,100-l circular tanks. One tank was maintained as a flow through system with river water, while a second tank was maintained as a closed system with particle and biological filtration at 24 ppt SW. The salinity in the SW tank was increased to 32 ppt over two days. Temperatures for the tanks were matched at 15°C. Shad were fed Biokyowa C-1000 feed daily. Sampling for immunohistochemistry followed at least 14 days of acclimation to laboratory and salinity conditions. Sampling was completed by December 31 for the FW group and by January 31 for the SW group.

Determination of gill Na^+,K^+-ATPase activity

Gill Na^+,K^+-ATPase activity was determined using the microplate method described by McCormick ('93) as validated for shad by Zydlewski and McCormick ('97a). Briefly, gill tissue was removed and immersed in 100 µl of ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and stored at –80°C. Gill samples were thawed immediately prior to assay and homogenized in 125 µl of 0.1% sodium deoxycholate in SEI buffer. The homogenate was centrifuged to remove insoluble material. Specific activity of Na^+,K^+-ATPase was determined in duplicate by measuring ATPase activity with and without 0.5 M ouabain in a solution containing 4 U/ml lactate dehydrogenase, 5 U/ml pyruvate kinase, 2.8 mM phosphoenolpyruvate, 0.7 mM adenosine triphosphate (ATP), 0.22 mM nicotinamide adenine dinucleotide (reduced) (NADH), 50 mM imidazole, 45 mM NaCl, 2.5 mM $MgCl_2$, 10 mM KCl, pH 7.5. Kinetic analysis of ATP hydrolysis was measured at 25°C by monitoring the decrease in [NADH] at 340 nm using a 96-well plate reader. Protein concentration of the gill homogenate was determined in triplicate using the bicinchoninic acid (BCA) method (Smith et al., '85; BCA Protein kit, Pierce, Rockford, IL) using bovine serum albumen as standard. In vitro activity of gill Na^+,K^+-ATPase is expressed as µmol ADP·mg protein^{-1}·hr^{-1}.

Measurement of gill development through the larval–juvenile transition

Determination of gill index was carried out as described in Zydlewski and McCormick ('97a). Briefly, gill arches were removed and placed into freshly mixed Champy-Maillet's fixative (0.2% os-

mium tetroxide, 25 mg/ml iodine and saturated metallic zinc) for 16 hr (Avella et al., '87). The tissue was rinsed with de-ionized water and dehydrated to 70% ethanol for storage. The gill tissue was examined at 1.2× to 4× magnification. The longest primary filament of the first gill arch was measured by obtaining a digital image via a CCD video camera (MTI CCD-72, Michigan City, IN) and applying a computerized image analyzing system (Image-1, Universal Imaging, West Chester, PA). A stage micrometer was used for calibration. Gill index was defined as length of the longest primary filament · fish fork length^{-1} ·100.

Analysis of chloride cell size, density, and shape in larval and juvenile shad

The first gill arch of the left side was excised immediately and placed into freshly mixed Champy-Maillet's fixative for 16 hr (Avella et al., '87). This method stains phospholipids, and because of their extensive tubular system, chloride cells are stained black. For this study, all positively stained cells using this method are defined as chloride cells. The tissue was rinsed with deionized water and dehydrated to 70% ethanol for storage. The tissue was subsequently dehydrated to 100% ethanol, rinsed three times with toluene (7 min each), and embedded in Tissue prep paraffin (Fisher Scientific, Chicago, IL). Seven-micron sections were placed on warmed gelatin-subbed slides. Paraffin was cleared with toluene, and slides were mounted with Permount (Fisher Scientific) for later analysis.

Five frames (200 μm × 150 μm) of the gill filament were observed for each individual fish at 400× using a Nikon Diaphot microscope. Images were obtained via a CCD video camera (MTI CCD-72) and a computerized image analysis system was applied (Image-1, Universal Imaging). The number of positively stained cells (chloride cells) were then counted, and the lengths of the analyzed region (dorsal and ventral sides of the primary filament) were recorded. A stage micrometer was used for calibration. For each side of the primary filament, the number of intersected secondary lamellae was counted, providing the average length of a "lamellar interval." A "lamellar interval" is defined as the distance from the proximate surface of a secondary lamella to the proximate surface of the next distal lamella on one side of a primary filament and is reported in microns.

Abundance of positively stained cells on the primary filament and secondary lamellae was tallied separately. Positively stained cells on the secondary lamellae were conservatively identified; if a cell touched the primary filament, the cell was classified as being on the primary filament. The number of each type of cell was expressed as cells per millimeter (of one side of a primary filament, dorsal or ventral). Chloride cell number was also calculated per lamellar interval.

Individual chloride cell size (area in μm^2) and "shape factor" (SF) were analyzed by "thresholding" the image gray levels to define cell borders. Borders were corroborated with the optical image through the microscope and discrepancies were corrected by hand. SF is defined as $4\pi A/P^2$ (where A is area and P is perimeter) such that values approaching 1 indicate a near-circular shape and lower values indicate a more elongated shape.

Immunolocalization of Na^+,K^+-ATPase in the gills of juvenile shad

Shad were anesthetized in MS-222 (pH 7.0, buffered with 12.2 mM sodium bicarbonate), and the caudal peduncle was severed to partially exsanguinate the gills. The gill tissue was removed and dissected from the gill arch. Preparation of the tissue was a modification of Ginns et al. ('96). The tissue was placed into fixative (80% absolute methanol/20% dimethyl sulfoxide) at –20°C for 12 hr. The tissue was warmed to 4°C for subsequent procedures. The tissue was rinsed in phosphate-buffered saline (PBS: 274 mM NaCl, 5.4 mM KCl, 8.6 mM Na_2HPO_4, 2.8 mM KH_2PO_4, pH 7.8) and then gradually brought up to 30% w/v sucrose in PBS over 1 hr prior to cryosectioning in Histo Prep Embedding Media (Fisher Scientific, Pittsburgh, PA). Sections (10 μm) were placed on a warmed polylysine-subbed slide. The sections were rinsed twice with high-salt phosphate-buffered saline (HSPBS: 500 mM NaCl, 5.4 mM KCl, 8.6 mM Na_2HPO_4, 2.8 mM KH_2PO_4, 1% w/v BSA, pH 7.3) for a total of 15 min, then incubated in a glycine wash (50 mM glycine, 1% w/v BSA in PBS) for 20 min. The sections were incubated with primary antibody (rabbit polyclonal antibodies against the α subunit of Na^+,K^+-ATPase, primary antisera diluted 1:500 in 0.2% NaN_2 and 1% w/v BSA in PBS; Ura et al., '96) for 24 hr. The slides were then rinsed in HSPB five times for 1 hr prior to a 2-hr incubation with the secondary antibody [Cy3 labeled goat anti-rabbit IgG(H+L) diluted 1:500 in 0.2% NaN_2 and 1% w/v BSA in PBS; Kirkegaard and Perry Laboratories, Gaithersburg, MD]. Finally, the slides were rinsed four times with PBS

and mounted with a coverslip prior to viewing with a Nikon Diaphot microscope (Omega XF-34 filter block, excitation 535 nm, emission 590 nm; Brattleboro, VT). Controls omitting the primary antibody and both antibodies were performed and yielded no staining.

Statistics

Significance of statistical analysis is reported at the $P < 0.05$ level. A two-way ANOVA was used for comparison of FW and SW groups over time in the SW acclimation study. If factors or interactions were significant, a one-way ANOVA was used for multiple group analysis. In all analyses, significance with a one-way ANOVA analysis was followed by a Newman–Keuls post-hoc test. Intervals about a mean are reported as plus/minus one standard error (SE).

RESULTS

Seawater tolerance and chloride cells through the larval-juvenile transition

Twenty-four-hour survival in 35 ppt SW increased from 0% (at 18 and 26 days post-hatch) to 25% at 36 days and 89% survival by 45 days post-hatch (Fig. 1). This rapid increase in SW tolerance from 36 to 45 days post-hatch was coincident with completion of the larval–juvenile transition. The density of chloride cells in the gill tissue increased progressively during this period, and by 45 days post-hatch it was 3.5-fold higher than at 18 days post-hatch. From 18 to 36 days the secondary lamellae were incompletely formed, thus it was difficult to characterize a cell as being positioned on the primary or secondary lamellae. Chloride cells present on the secondary lamellae were 38% of the total number of positively stained cells in juveniles (45 days post-hatch). This ratio was not significantly different from 70- to 80-days post-hatch juveniles captured in the field (38%; Fig. 1). There was no significant change in cell area or shape through the larval–juvenile transition (Table 1). The increases in chloride cell density and seawater tolerance are matched by a 2.8-fold increase in gill index from 0.9 at 18 days to 2.5 by 45 days. Additionally, a 30-fold increase in gill Na^+,K^+-ATPase activity occurred over this same period of development (Table 1).

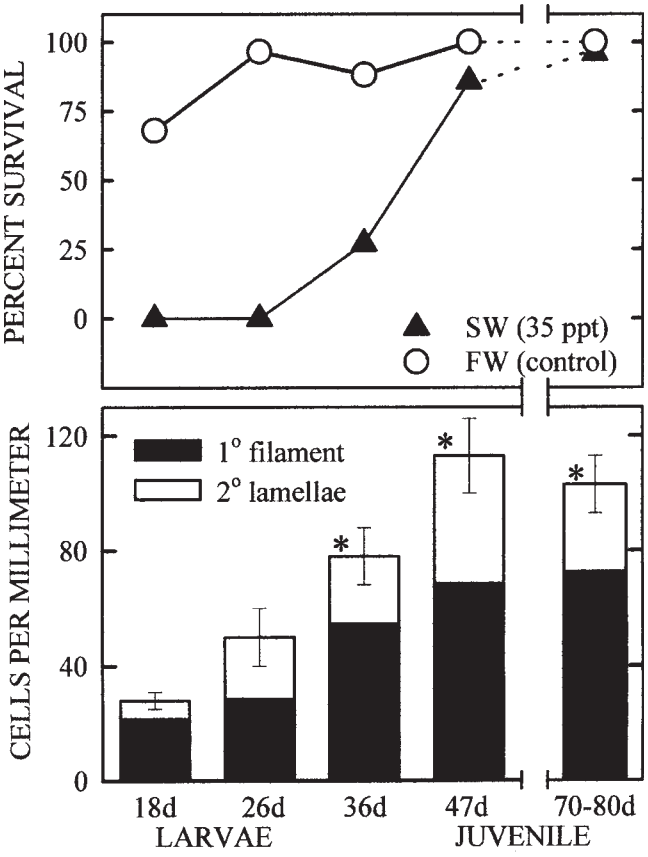

Fig. 1. **Top:** Percent survival of American shad reared in the laboratory 24 hr after isothermal transfer to 35 ppt SW or FW control at 18, 26, 36, and 45 days post-hatch (n = 24) and 72 days (n = 10). **Bottom:** Gill chloride cell density of American shad reared in the laboratory, at 18 days (n = 3), 26 days (n = 3), 36 days (n = 8), and 45 days post-hatch (n = 6), and field-captured juvenile shad (estimated 70–80 days post-hatch) expressed as chloride cells/mm for cells on the primary filament and secondary lamellae. Values are given as mean ± SE for the total number of chloride cells at each time point. An asterisk denotes a significant difference from 18 days post-hatch.

Effects of salinity on chloride cells

Representative Champy-Maillet prepared gill sections for FW and SW acclimated juvenile shad are shown in Fig. 2. In FW-acclimated shad, chloride cells are present on the primary filament and the secondary lamellae. In SW-acclimated shad, chloride cells are present almost exclusively on the primary filament. There is no obvious difference in the morphology of chloride cells on the primary filament in FW- and SW-acclimated fish. Chloride cells present on the primary filament are large columnar cells in full contact with the serosal and mucosal surfaces. The apical surface represents a small proportion of the cell perimeter. Chloride cells on the secondary lamellae in FW are short cells with a large exposed apical surface.

TABLE 1. Area (μm^2) and shape factor ($4\pi A/P^2$) for chloride cells on the primary filament and secondary lamellae, gill Na^+, K^+-ATPase activity (μmol ADP·mg $prot^{-1}\cdot h^{-1}$), and gill index (length of primary filament·fork $length^{-1}\cdot 100$) for shad reared in the laboratory through the period of larval–juvenile transition.[1]

Age post-hatch (Date)	Cells on primary filament		Cells on secondary lamellae		Gill Na^+, K^+-ATPase activity	Gill index
	Area	Shape	Area	Shape		
18 days (Jul 1)	44.7 ± 6.9 (3)	0.68 ± 0.01 (3)	40.2 ± 1.7 (3)	0.68 ± 0.02 (3)	0.14 ± 0.02[a] (24)	0.9 ± 0.1[a] (8)
26 days (Jul 9)	36.1 ± 5.0 (3)	0.68 ± 0.01 (3)	26.7 ± 1.7 (3)	0.67 ± 0.01 (3)	0.25 ± 0.03[a] (24)	1.6 ± 0.1[b] (8)
36 days (Jul 19)	36.0 ± 1.4 (3)	0.68 ± 0.01 (3)	33.3 ± 2.8 (3)	0.67 ± 0.01 (3)	3.88 ± 0.23[b] (24)	1.7 ± 0.1[b] (8)
45 days (Jul 28)	36.1 ± 1.6 (6)	0.64 ± 0.01 (6)	27.7 ± 1.5 (6)	0.62 ± 0.01 (6)	4.21 ± 0.20[b] (23)	2.5 ± 0.1[c] (8)

Note:Values are given as the mean ± SE, with n given in parentheses. Means followed by different letters are significantly different over time (one-way ANOVA and Newman–Keuls test, $P < 0.05$). Where no letters follow the means, there are no differences among groups.
[1]Fish at 18 days were larvae; the larval–juvenile transition was completed by 45 days.

Fresh water acclimation: effects of temperature on chloride cells

Chloride cell abundance on both the primary filament and the secondary lamellae increased in both FW groups over the 3-month experiment (Fig. 3). These increases were greater in the SNT group. On the primary filament, abundance increased 1.5-fold in the SNT group, reaching 114 cells/mm by December 1. Chloride cell abundance on the secondary lamellae increased 3.4-fold (to 99 cells/mm). These increases were delayed and of a lesser magnitude in the 24°C fish. At 24°C, chloride cell abundance increased 1.3-fold (to 95 cells/mm on the primary filament) and 2.5-fold (to 75 cells/mm on the secondary lamellae) by December 1.

Chloride cells on the primary filament and secondary lamellae increased in size under both FW temperature treatments (Fig. 3). The SNT group had the greater increase in size, gradually increasing 2.3-fold (from 26 μm^2 to 59 μm^2) over the three-month study. Chloride cells on the secondary lamellae also increased 2.3-fold (from 25 μm^2 to 59 μm^2). As with changes in chloride cell abundance, size increases were delayed and of a lesser magnitude in the 24°C fish. Chloride cell size (for both cells on the primary filament and secondary lamellae) did not increase until October. In the remaining two months, both cell classes increased 1.5-fold (to 37 μm^2 and 39 μm^2 for cells on the primary filament and secondary lamellae, respectively).

In the FW SNT group, chloride cells on both the primary filament and the secondary lamellae became more round, as indicated by an increase in SF. SF of chloride cells on the primary filament gradually increased from 0.64 to 0.73 over the course of the experiment (Fig. 4). Similarly, SF of cells on the secondary lamellae increased from 0.62 to 0.75. However, chloride cell SF did not change from initial values, regardless of position, in the 24°C group.

Seawater acclimation: effects of temperature on chloride cells

There was no change in the abundance of chloride cells present on the primary filament during SW acclimation. Although the average abundance of cells in the SNT group was greater than that of the 24°C group at all time points, these differences were not significant. In stark contrast, the abundance of chloride cells on the secondary lamellae decreased to negligible levels during the first month of acclimation in both temperature regimes. Because of the extremely low number of chloride cells present on the secondary lamellae in SW, analysis of cell measures (size and SF) is omitted below.

Acclimation to SW caused an increase in the size of chloride cells on the primary filament. The SNT group had a greater increase (2.2-fold to 57 μm^2) than the 24°C group (1.8-fold increase to 46 μm^2). Chloride cell area did not change in either group during the last 2 months of the experiment.

During SW acclimation, chloride cells on the primary filament became more round in both treatments. Chloride cell SF increased to peak levels 1 month after SW entry for both the SNT and 24°C groups (0.69 and 0.68, respectively). While SF for the SNT group remained high through the study, chloride cells in the 24°C group fell to initial levels by December 1.

Lamellar interval

There was no difference in lamellar interval lengths between groups at any time point, and only

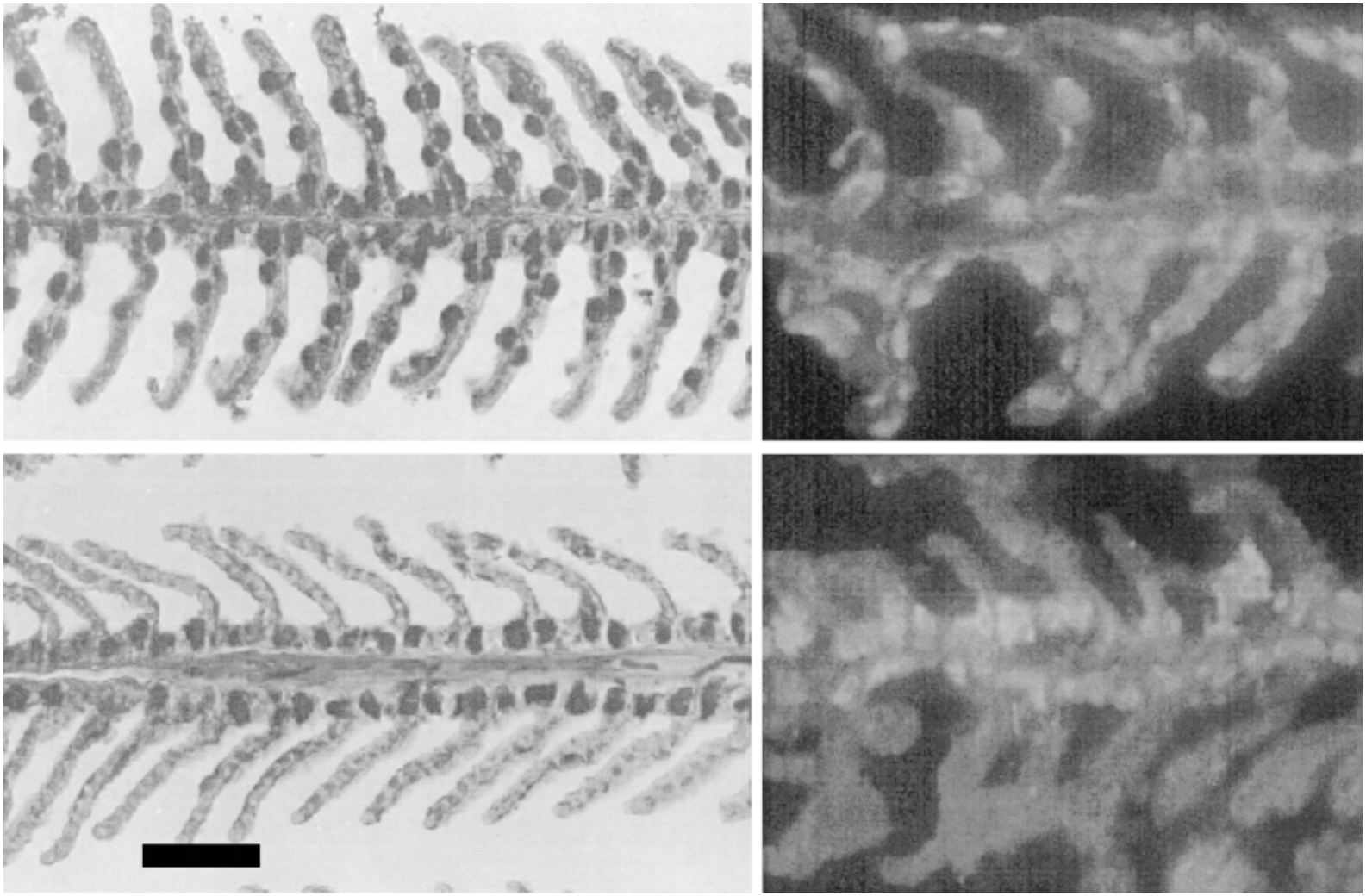

Fig. 2. **Left:** Histological preparations of gill filaments of FW acclimated (top) and SW acclimated shad (bottom) stained in Champy-Maillet fixative. **Right:** Fluorescent immunocytochemical preparations of American shad gill from FW- acclimated (top) and SW-acclimated (bottom) shad using a polyclonal antibody against a consensus sequence of the α subunit of Na^+,K^+-ATPase. The bar is 50 μm.

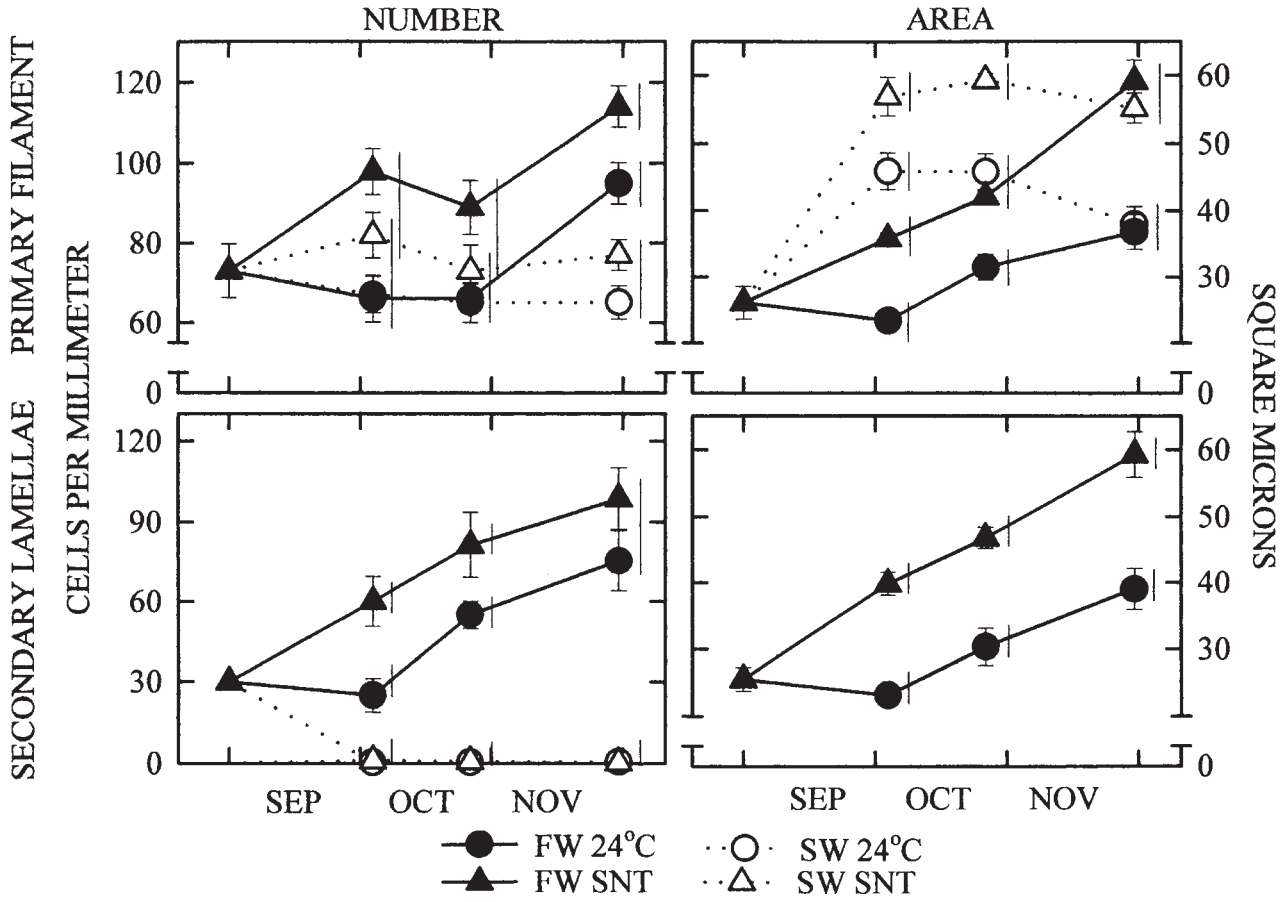

Fig. 3. Cell number in cells/mm (left) and area in μm^2 (right) of chloride cells measured for FW and SW (32 ppt) 24°C and SNT groups. Vertical lines indicate homogeneous subsets within a sampling date (one-way ANOVA, followed by Newman–Keuls test).

the SW SNT group showed any significant difference over the course of the experiment (Table 2). The length of lamellar intervals in the sample measured on October 27 was 13% higher than initial values. However, there was no difference in the December 1 sampling. Calculations of cell abundance were performed using both measures of cells/mm and cells per lamellar interval. No qualitative difference in analysis resulted, hence cell abundance data is presented solely as cells/mm.

Effect of salinity and temperature on gill Na^+,K^+-ATPase activity

Gill Na^+,K^+-ATPase activity increased in all groups over the course of the experiment (Table 3). Activity in the FW SNT group increased more than 3-fold from initial levels, peaking at 7.0 μmol ADP·mg protein^{-1}·hr^{-1} on October 27. The rate and extent of increase in gill Na^+,K^+-ATPase activity were lower in the FW 24°C group than in the FW SNT group. Activity peaked at 5.6 μmol ADP·mg protein^{-1}·hr^{-1} on December 1. In FW the number and size of cells on the primary filament were correlated with gill Na^+,K^+-ATPase activity ($P = 0.02$ and <0.001, respectively) with R^2 values of 0.07 and 0.18, respectively. The number and size of cells on the secondary lamellae were also correlated with gill Na^+,K^+-ATPase activity ($P < 0.001$ for both) with R^2 values of 0.19 and 0.18, respectively.

Gill Na^+,K^+-ATPase activity increased 3-fold within 1 month of acclimation to SW. Gill Na^+,K^+-ATPase of the SW groups did not change significantly during the remainder of the experiment, and there was no difference in the activity between the two SW temperature regimes at any time. In SW there was no relationship between the number of chloride cells and gill Na^+,K^+-ATPase activity ($P = 0.76$). While chloride cell size was correlated with gill Na^+,K^+-ATPase activity at the $P < 0.05$ level, the R^2 value is 0.001.

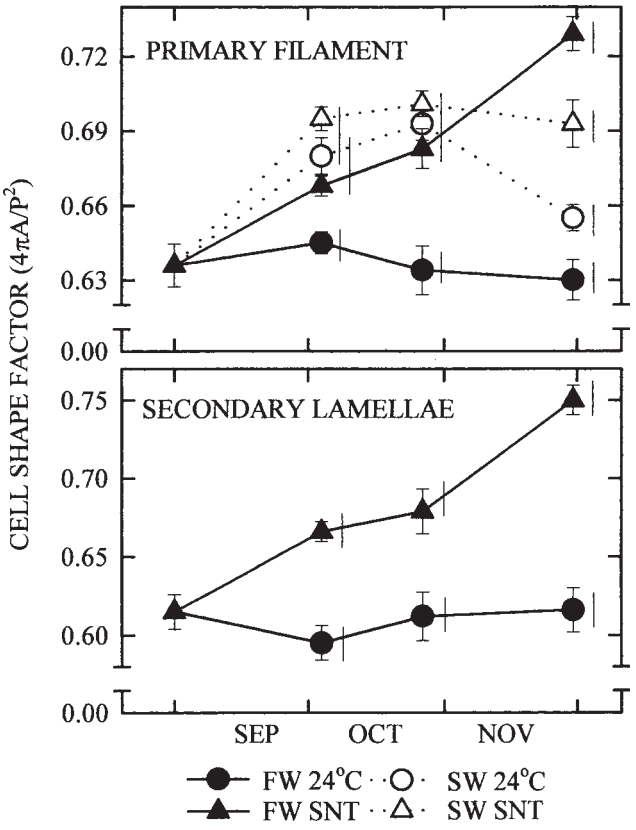

Fig. 4. Cell SF ($4\pi A/P^2$) of chloride cells of FW- and SW (32 ppt)-acclimated shad at 24°C and SNT. Vertical lines indicate homogeneous subsets within a sampling date (one-way ANOVA, followed by Newman–Keuls test).

Immunolocalization of Na^+,K^+-ATPase in the gill tissue

Representative staining for FW- and SW-acclimated shad is shown on the right side of Fig. 2. In FW-acclimated shad, chloride cells on both the primary filament and the secondary lamellae were positively stained and the qualitative intensity of staining was similar. In SW-acclimated shad, positively stained cells were restricted to the primary filament. Staining of cells in SW was of qualitatively equal intensity with cells stained positively in FW-acclimated fish. Cells stained on the primary filament in both FW and SW were large columnar cells which fully spanned the distance from the basal surface of the epithelium to the mucosal surface; apical exposure on the mucosal surface was present but narrow. Cells present on the secondary lamellae in FW were rounded but flattened and had extensive apical contact with the environment. The position, appearance, and size of these stained cells matched that of chloride cells stained by Champy-Maillet fixative.

TABLE 2. Lamellar interval length[1] for shad corresponding to Figs. 2 and 3

	Lamellar interval length			
Date	FW SNT	FW 24°C	SW SNT	SW 24°C
Aug 31 (n = 16)	24.5 ± 0.8	—	—	—
Oct 4 (n = 10)	26.4 ± 0.9	27.3 ± 0.4	25.9 ± 0.7	26.4 ± 0.6
Oct 27 (n = 10)	25.7 ± 0.4	25.9 ± 0.7	27.6 ± 0.4[a]	26.5 ± 0.6
Dec 1 (n = 10)	26.2 ± 0.7	26.0 ± 1.0	25.4 ± 0.5	24.8 ± 1.0

Note:Values are given as the mean ± SE. Means followed by different superscript letters are significantly different over time (one-way ANOVA and Newman–Keuls test, $P < 0.05$). Where no letters follow the means, there are no differences among groups.
[1]Lamellar interval length is measured from the proximate side of a secondary lamella to the beginning of the next most distal along the primary filament in microns.

DISCUSSION

Changes in chloride cells through the larval–juvenile transition

The ability of American shad to osmoregulate in full-strength SW develops at the larval–juvenile transition (Zydlewski and McCormick, '97a). The present study has shown a significant 3.5-fold increase in total branchial chloride cell density during this period (Fig. 1). One important feature of this transition is the development of secondary lamellae on the gill filaments (Shardo, '95; Zydlewski and McCormick, '97a). During this period, the proportion of chloride cells found on the secondary lamellae increased from 21% to 38%. Changing gill morphology likely allows for this increase in the proportion of chloride cells on the secondary lamellae by increasing space for cell differentiation.

TABLE 3. Gill Na^+, K^+-ATPase activity (μmol ADP·mg $prot^{-1}·h^{-1}$) and temperature (°C) at sampling for shad (in italics); corresponds to chloride cell data in Figs. 2 and 3

	Gill Na^+, K^+-ATPase activity (*temperature at sampling*)			
Date	FW SNT	FW 24°C	SW SNT	SW 24°C
Aug 31 (n = 16)	2.4 ± 0.2 (21.5)	—	—	—
Oct 4 (n = 10)	5.3 ± 0.4[a] (16.0)	4.4 ± 0.6[a] (23.5)	7.5 ± 0.4[b] (15.5)	7.0 ± 0.7[b] (23.3)
Oct 27 (n = 10)	7.0 ± 0.5[a] (13.3)	4.9 ± 0.6[b] (23.2)	8.0 ± 0.5[a] (13.5)	8.0 ± 0.5[a] (23.3)
Dec 1 (n = 10)	6.2 ± 0.6[ab] (6.1)	5.7 ± 0.6[a] (23.6)	7.8 ± 0.5[b] (5.8)	7.0 ± 1.3[ab] (23.4)

Note:Values are given as the mean ± SE. Means followed by different superscript letters are significantly different within a time point (one-way ANOVA and Newman–Keuls test, $P < 0.05$).

The distinction between chloride cells on the primary filament and secondary lamellae in larval shad (18–36 days post-hatch) is limited because the secondary lamellae were not fully developed. Cell position was easier to define later in development (after 36 days post-hatch), and morphological differences between the cells were more obvious. The proportion of chloride cells on the secondary lamellae did not change through the summer FW residence of shad. The number of cells on the secondary lamellae in 45-days post-hatch juveniles did not differ from older (estimated 70–80 days post-hatch) juveniles (Figs. 1 and 3).

The differentiation of chloride cells follows gill tissue proliferation during the larval–juvenile transition. Since chloride cells were shown to be rich in Na^+,K^+-ATPase through immunolocalization (Fig. 2) chloride cell differentiation is likely to be causal to the observed increase in gill Na^+,K^+-ATPase activity (Table 1). The data suggest that three interdependent processes (gill formation, increased chloride cell density, and increased gill Na^+,K^+-ATPase activity) are necessary for the development of the gills as an osmoregulatory organ. The maintenance of increased chloride cell abundance (along with increased gill index and Na^+,K^+-ATPase activity) is likely to be responsible for sustained seawater tolerance in juveniles through to the period of autumnal migration (Zydlewski and McCormick, '97a).

Prior to the formation of gills during the larval–juvenile transition, other organs must allow shad to osmoregulate over a range of salinities. Larval shad have the ability to develop and grow in salinities from FW to 22.5 ppt SW (Milner, 1876; Leim, '24; Limburg and Ross, '95). The integument of larvae is likely to function in both ion uptake and excretion during these developmental stages. Chloride cells have been observed in the integument and yolk sac of a large number of teleost larvae (see Hwang, '89, for review) including American shad (J. Shardo, University of South Alabama, personal communication).

Role of chloride cells on the secondary lamellae in fresh water

Chloride cells on the secondary lamellae are likely to function in ion uptake in FW-acclimated shad. The high concentration of Na^+,K^+-ATPase in chloride cells on the secondary lamellae is evidence for the involvement of these cells in ion transport (Fig. 2). This enzyme is a critical component of current models for both sodium and chloride uptake and excretion (Evans et al., '82; Evans, '93). Increases in gill Na^+,K^+-ATPase activity are coincident with increased size and abundance of chloride cells on the secondary lamellae (Fig. 3). Gill Na^+,K^+-ATPase activity is correlated with both cell size and number. Chloride cells on the secondary lamellae are also mitochondria-rich as evidenced by staining with Rhodamine 1-2-3 (Zydlewski, unpublished data). This suggests a high energetic capacity for these cells, presumably for ion transport.

Chloride cells on the secondary lamellae have been linked to ion uptake demands in other species. Increases in the abundance and size of chloride cells on the secondary lamellae occur under low external ion concentrations in eel (*Anguilla anguilla*; Olivereau, '71) and rainbow trout (*Onchorhyncus mykiss*; Laurent and Dunel, '80; Laurent and Hebibi, '89; Perry and Laurent, '89). Cells on the secondary lamellae of the gills have also been implicated in calcium uptake in fresh water acclimated rainbow trout (Laurent et al., '85; Perry and Wood, '85), American eel (*Anguilla rostrata*), and brown bullhead catfish (*Ictalurus nebulosus*; Perry et al., '92). The increase in chloride cell size and abundance in shad, however, occurs in the absence of a change in the ion composition of the environment.

Patterns in proliferation of chloride cells are tightly correlated with changes in the osmoregulatory physiology of migratory juvenile shad. During the migratory period, juvenile shad lose the ability to hyperosmoregulate. This is evidenced by decreased plasma chloride, decreased plasma osmolality, increased muscle moisture, and high mortality if prevented from entering SW (Zydlewski and McCormick, '97b; Zydlewski, unpublished data). Also associated with this loss is an increase in gill Na^+,K^+-ATPase activity, likely due to the proliferation of chloride cells (Table 3; Fig. 3). These changes are presumably developmental processes related to downstream migration and/or changing environmental conditions. The parallel influence of temperature regime on chloride cell proliferation, enlargement, and these physiological measures (Zydlewski and McCormick, '97b) further implies a direct relationship.

If, in fact, chloride cells on the secondary lamellae function in ion uptake, the increases (in both size and number) of these cells and gill Na^+,K^+-ATPase activity are likely to be compensation for the decline in ion regulatory ability. It has been hypothesized that changes in ion and water balance could be causal to the migratory behavior of some diadromous fish (Fontaine, '75; Zydlewski

and McCormick, '97b). The shift in this suite of characters may be necessary for the full expression of migration.

Changes in chloride cells during seawater acclimation

During SW acclimation, chloride cells on the secondary lamellae disappear while chloride cells on the primary filament remain without an increase in abundance. These cells do, however, increase in size while gill Na^+,K^+-ATPase activity increases. Na^+,K^+-ATPase was localized to chloride cells on both the primary filament and the secondary lamellae in this study (Fig. 2), hence the total number of chloride cells rich in this enzyme decreases during SW acclimation. Because gill Na^+,K^+-ATPase activity is the same or higher in SW-acclimated shad as in FW-acclimated shad, it can be inferred that enlargement of chloride cells on the primary filament includes basolateral surface proliferation for Na^+,K^+-ATPase pump insertion, increasing the capacity of the cell for ion excretion. Enrichment of chloride cells with Na^+,K^+-ATPase during SW acclimation is consistent with observations in other teleosts (Karnaky et al., '76; McCormick, '90).

The shift in chloride cell position during SW acclimation in shad is likely due to differential turnover rates of chloride cells in FW and SW. Uchida and Kaneko ('96) investigated cell differentiation and renewal in the gills of chum salmon fry (*Onchorhynchus keta*) using immunolocalization of the thymidine analog 5-bromo-2′-deoxyuridine (BrdU) as a marker. During SW acclimation, chloride cell turnover rates increased to levels 3.5-fold higher than in FW. Newly differentiated cells were largely restricted to the primary filament. The result was a rapid decrease of cells on the secondary lamellae in SW-acclimated fish.

The response of SW acclimation is not identical in all species. Unlike shad, chloride cells of teleosts generally both proliferate and enlarge upon acclimation to increased salinity. This is observed in killifish (*Fundulus heteroclitus*), tilapia (*Oreochromis niloticus* and *O. mossambicus*), and Atlantic salmon (*Salmo salar*; Doyle and Gorecki, '61; Karnaky et al., '76; Bartles and Potter, '91; Cioni et al., '91; King and Hossler, '91). Other patterns, however, are observed. Chloride cells of SW acclimated striped bass (*Morone saxatilis*) are found only on the primary filament and do not differ from FW-acclimated fish in abundance, but they are slightly larger (Madsen et al., '94). Similarly, in the long-jawed mudsucker (*Gillichthys mirabilis*), chloride cell density in branchial epithelium does not change when acclimated to salinities between 1.5 ppt to full-strength SW (Yoshikawa et al., '93). These results suggest that some fish maintain high densities of excretory chloride cells regardless of the external salinity.

The size increase of chloride cells on the primary filament during SW acclimation of shad implies an ion excretory role. However, chloride cells are also found on the primary filament in FW-acclimated shad. These cells, like those on the secondary lamellae, proliferate and enlarge during autumn when shad experience impaired hyperosmoregulatory ability. There are at least two possible explanations for these circumstances. Chloride cells on the primary filament may function exclusively in ion excretion, and their increase in the number and size might be causal to the autumnal decrease in hyperosmoregulatory ability. A more likely scenario is that chloride cells on the primary filament are bifunctional and appropriately shift their role upon SW entry. Explicitly differentiating between these two scenarios is not possible on the basis of these data.

Temperature effects

Temperature regime significantly influenced chloride cell size in both FW and SW and cell abundance in FW. In SW, there was no difference in chloride cell number between temperature treatments, but chloride cell size was greater when temperature decreased (Fig. 3). Cells were more round (as measured by SF) in shad acclimated to SW at 6°C versus 16°C (Fig. 4). Interestingly, there was no difference in gill Na^+,K^+-ATPase activity between temperature treatments (Table 3). Indeed, Na^+,K^+-ATPase activity was not correlated with cell number in SW acclimated fish. While gill Na^+,K^+-ATPase activity was correlated with cell size, this relationship explained less than 1% of the variation.

It should be noted that measured gill Na^+,K^+-ATPase activities reflect in vitro V_{max} values measured at 25°C (see MATERIALS AND METHODS). Temperature affects the kinetics of enzyme action, theoretically decreasing enzyme activity by a factor of approximately 2 with a 10°C decrease in temperature (the Q_{10} effect). The linear relationship between enzyme activity and temperature has been demonstrated for gill Na^+,K^+-ATPase in coho salmon (McCormick and Bern, '89). Thus while in vitro Na^+,K^+-ATPase activities are similar for SW acclimated shad at 6°C and 16°C, the effective activity at 6°C would be 2-fold lower in vivo.

This effective decrease in activity may be offset by changes in passive permeability rates and/or reduced gill perfusion rates at low temperatures. Acclimation temperature has been shown to change gill permeability (Kieffer and Tufts, '96; Gonzales and McDonald, '94), membrane fluidity, and ion permeability (Schwartzbaum et al., '92).

In FW-acclimated shad, temperature effects on chloride cells are likely to be complicated by the interaction of migratory physiology with chloride cell regulation. Chloride cell size and abundance increased in both FW groups but was delayed and of a lesser magnitude in the 24°C group (vs. SNT). This closely parallels the thermal effect on impaired hyperosmoregulatory ability and the increase in gill Na^+,K^+-ATPase activity (Table 3; Zydlewski and McCormick, '97b). Gill Na^+,K^+-ATPase activity increased in both FW groups (Table 3) with the greater increase being observed in the SNT group. Other factors beyond compensation for thermal effects on enzyme kinetics (environmental and/or developmental) must also influence these changes. Though significantly delayed, proliferation and enlargement of chloride cells (and decreased plasma chloride and increased gill Na^+,K^+-ATPase activity) occur in shad maintained at a constant temperature (24°C) in FW (Fig. 3).

Temperature had a different effect on the morphology of chloride cells in FW acclimated fish. Chloride cells in the SNT group became gradually more round as they proliferated and enlarged (Figs. 3 and 4) whereas the 24°C group showed no change in SF during the study. Differences in the roundness of cells may reflect contrasting allocation of space for transport proteins. Alternatively, roundness of chloride cells may reflect changes in the morphology and size of the apical surface. The absence of an increase in SF in the FW 24°C group suggests that other structures and/or transport proteins are being mobilized in FW as temperatures decline.

A number of factors are likely to contribute to the different effects of temperature on chloride cells in FW and SW. Temperature effects on chloride cells vary among species and may be influenced by the range of salinities and temperatures to which a species is normally exposed. Under low acclimation temperatures, chloride cell number increases in eel (*Anguilla anguilla*; Dunel-Erb et al., '96) but does not change in the long-jawed mudsucker (*G. mirabilis*; Kültz and Somero, '95, '96). Temperature also influences chloride cell distribution in a species-specific manner. Low acclimation temperature increases the number of chloride cells on the secondary lamellae in flounder (*Platichthys americanus*; Boyd et al., '80) but not Arctic cod (*Trematomus borchgrevinki*; Boyd et al., '80).

For shad, thermal effects on chloride cells may perturb other facets of performance in shad. Temperature influences both behavior and physiology of juvenile shad during their FW migration. Thermal avoidance occurs below 8°C, and other sublethal and lethal effects occur at low temperatures in FW (Chittenden, '72). Zydlewski and McCormick ('97b) reported the cessation of feeding at 13°C and a lower thermal tolerance of 4°C.

Impact of chloride cells on migratory biology

The proliferation and enlargement of chloride cells during seaward migration may present a considerable energetic challenge to late migrant shad. The relative costs of active transport versus chloride cell proliferation and enlargement are poorly understood. Theoretical models estimate the cost of osmoregulation to be as low as 0.5% and 1.0% of resting metabolic rate in SW or FW, respectively (Eddy, '82), but direct calculations vary greatly (Farmer and Beamish, '69; Eddy, '75). The cost of SW acclimation in tilapia (*Oreochromis mossambicus*) has been calculated to be as high as 20% of total resting metabolic rate (Morgan et al., '97). Chloride cells are metabolically active in FW and SW, indicated by richness in mitochondria and Na^+,K^+-ATPase (Kültz and Jurss, '93). For shad in FW, cell numbers increased 1.5- and 2-fold for cells on the primary filament and secondary lamellae, respectively, during the migratory period. After the larval–juvenile transition, shad appear to maintain SW readiness throughout their residence in FW (Zydlewski and McCormick, '97a).

When these physiological expenses are coupled with some of the possible behavioral effects of declining temperatures (increased migratory activity and reduced feeding) the energetic costs of osmoregulation in FW may be significant. Osmotic stress has been demonstrated to affect schooling behavior and reduce escape success from predators, significantly reducing the survival of Atlantic salmon (*S. salar*) during downstream migration (Handeland et al., '96). Consistent with this finding is the reduced swimming performance observed in shad in FW at the end of migration (J. Zydlewski and S.D. McCormick, personal observations).

Another potentially important cost of the proliferation of chloride cells in FW is direct interfer-

ence with respiration and other gill functions. Shad maintained in FW under declining temperatures experience an elevated incidence of mortality associated with decreased plasma chloride (Zydlewski and McCormick, '97b). As chloride cells on the secondary lamellae proliferate during this period, they begin to cover an increasing proportion of the respiratory surface. Proliferation of chloride cells has been shown to reduce the efficiency of gas transfer in rainbow trout (*O. mykiss*) (Bindon et al., '94a,b). Reduced respiratory efficiency may be partly offset by the greater oxygen capacity of water at cooler temperatures, but chloride cells become more numerous and round, further increasing diffusion distance. The osmorespiratory compromise would then require greater perfusion of blood through the gills to maintain gas exchange at a cost of increased ion loss. If this is the case, the very mechanism to augment ion uptake further handicaps the process by reducing the efficiency of respiration. These circumstances may contribute to poor migratory performance of late migrants and likely provide a strong stabilizing effect on the timing of downstream migration.

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