

Late migration and seawater entry is physiologically disadvantageous for American shad juveniles

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Juvenile American shad *Alosa sapidissima* were subjected to isothermal transfers into sea water (salinity 24) 'early' (1 September; 24°C) and 'late' (10 November; 10°C) in the autumn migratory season. Early acclimation resulted in a modest osmotic perturbation that recovered rapidly. Haematocrit declined by 14% at 24 h, recovering within 48 h. Plasma osmolality increased by 6% at 4 h, recovering within 8 h. Early acclimation caused a two-fold increase in gill Na⁺, K⁺-ATPase activity by 24 h and a four-fold increase by 4 days. The number of chloride cells on the primary gill filament increased two-fold by 4 days. Chloride cells on the secondary lamellae rapidly decreased from 22 cells mm⁻¹ to <2 cells mm⁻¹ within 4 days. Late acclimation resulted in a severe and protracted osmotic perturbation. Haematocrit levels declined by 23% at 4 days, recovering by 14 days. Plasma osmolality increased by 36% by 48 h, recovering by 4 days. Initial gill Na⁺, K⁺-ATPase activity was two-fold greater than in 'early' fish and did not change during acclimation. Initial numbers of chloride cells on the primary filament were two-fold greater than 'early' fish and did not increase during acclimation. Initial number of chloride cells on the secondary lamellae was five-fold greater than 'early' fish (116 v. 22 cells mm⁻¹) and declined to negligible numbers over 14 days. Differences between initial measures for 'early' and 'late' fish reflect previously described physiological changes associated with migration. These data indicate that late migrants face a greater physiological challenge during seawater acclimation than early migrants. Physiological performance apparently limits the observed duration of autumnal migration. © 2003 The Fisheries Society of the British Isles

Key words: *Alosa sapidissima*; American shad; chloride cells; gill Na⁺, K⁺-ATPase; migration; osmoregulation.

INTRODUCTION

For animals that migrate, the timing of a sustained directional movement is resolved through the integration of physiological and environmental information. These factors impact the neuroendocrine system of an individual, culminating in the release of migratory behaviour. While proximate factors provide an unbiased assessment of external and internal status (e.g. temperature, photoperiod, physiological tolerances and energetic status) it is the interpretation of

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this information that is judged at an individual level. The ultimate factors that stabilize, destabilize or shift the timing of migration include both ecological and physiological factors (Farner & Follett, 1979; Hoar, 1988; Roff, 1988; Taylor, 1990). Ecological factors such as food availability and predation risk can directly impact on an individual's survival. Developmental physiology and environmental tolerances can also impact on an individual's survival by limiting the timing and duration of migration.

For anadromous fishes, seaward migration at the juvenile stage affords a period of high growth in the ocean environment (Gross, 1987). This migration also makes the development and maintenance of appropriate osmotic tolerances necessary for survival. Anadromous salmonids, for example, develop seawater tolerance coincident with migration as part of a complex developmental shift, the parr–smolt transformation. 'Smolting' is a well-characterized suite of behavioural, morphological and physiological changes (Hoar, 1976, 1988; McCormick & Saunders, 1987). Unlike salmonids, American shad *Alosa sapidissima* (Wilson) apparently have no discrete developmental shift associated with downstream migration involving an increase in seawater tolerance.

Adult American shad spawn in fresh water in the spring and young fish develop the ability to enter into full strength sea water at the larval–juvenile transition, months prior to migration (Zydlewski & McCormick, 1997a). This progression is coincident with gill development (Shardo, 1995; Zydlewski & McCormick, 1997a) increased gill Na^+ , K^+ -ATPase activity (Zydlewski & McCormick, 1997a) and differentiation of gill chloride cells (Zydlewski & McCormick, 2001). It is important to note that while American shad are physiologically competent to enter sea water early during their entire freshwater residence as juveniles, most remain in fresh water until autumn.

Seaward migration of juvenile American shad, while largely uncharacterized, is correlated with declining autumnal temperatures. There is evidence for a size-dependent component for the timing of migration (Limberg, 1996) but size does not appear to be a critical factor (Stokesbury & Dadswell, 1989). The peak of migration generally occurs when river temperatures have declined to between 9 and 16°C (Leggett & Whitney, 1972; O'Leary & Kynard, 1986; RMC Environmental Services, pers. comm.). The migration, however, is protracted over several months and over a considerable temperature range. In a prior study (Zydlewski & McCormick, 1997b) actively migrating juvenile American shad were captured in the Connecticut River (river km 198) from early September to late November in 1993 and 1994. River temperatures ranged from 24 to 5°C.

While American shad maintain seawater tolerance through the autumnal period of seaward migration, juveniles gradually lose the ability to regulate their ions in fresh water. This is evidenced by decreased plasma chloride, decreased plasma osmolality and increased muscle moisture if prevented from entering sea water (Zydlewski & McCormick, 1997b; unpubl. data). Associated with impaired hyperosmoregulatory ability is an increase in gill Na^+ , K^+ -ATPase activity which is the result of an intense proliferation and enlargement of Na^+ , K^+ -ATPase-rich chloride cells on both the primary filament and secondary lamellae of the gill (Zydlewski & McCormick, 2001).

There are no data to suggest that this proliferation is associated with increased seawater tolerance. The disappearance of chloride cells on the secondary lamellae upon seawater entry indicates that these cells probably function in

ion uptake. Therefore, contrary to preparing these fish to enter sea water, the proliferation of chloride cells in fresh water may in fact be an impediment to osmoregulating in sea water. The nature of this developmental loss of hyperosmoregulatory ability is not understood, but the acceleration of this physiological change under declining temperature (Zydlewski & McCormick, 1997b, 2001) implies a direct relationship between osmoregulatory ability in fresh water and the parallel expression of downstream migratory behaviour (Fontaine, 1975).

Because there is a shift in osmoregulatory physiology that progresses through the autumn migratory period, the relative physiological costs of migrating early or late in the season are unclear. Evidence suggests that conditions experienced by late migrants in fresh water (including low temperatures) impair performance. Chittenden (1972) has demonstrated avoidance of temperatures at $<8^{\circ}\text{C}$ as well as other sub-lethal and lethal effects at lower temperatures in fresh water. Zydlewski & McCormick (1997b) have observed a cessation of feeding below 10°C , and high mortality in juvenile American shad held in fresh water beyond the period of migration (at both constant 24°C and declining temperatures). During the period of migration, juvenile American shad also exhibit a heightened responsiveness to acute handling and confinement stress (Shrimpton *et al.*, 2001). Consistent with these findings is the reduced swimming performance observed in American shad in fresh water at the end of migration (J. Zydlewski & S.D. McCormick, pers. obs.). It is unknown if these behavioural and physiological shifts may result in disparate physiological performance during the seawater acclimation of migrants.

It was the goal of this study to introduce juvenile American shad to sea water 'early' and 'late' during the period of migration to determine if the timing of acclimation affects physiological performance. The 'late' fish used in this study are meant to represent cohorts of juveniles that have either 'chosen' to postpone migration or have been delayed at an impediment to migration. Such information has implications for both the ecology and management of this species.

METHODS

EXPERIMENTAL ANIMALS AND DESIGN

Wild American shad juveniles were captured on 23 August 1998 by beach seining in the Connecticut River at a site 2 km north of the dam at Turners Falls ($42^{\circ}59'\text{N}$; $72^{\circ}54'\text{W}$). Fish were transported to the Conte Anadromous Fish Research Center (Turners Falls, MA, U.S.A.) and divided (*c.* 100 fish per tank) into two 1.5 m diameter 1100 l tanks (tanks I and II) maintained as flow through systems with unfiltered river water (151min^{-1}). Both groups were maintained under a natural photoperiod regime throughout the experiment. Juveniles began feeding within 2 days after introduction to the laboratory ('salmon feed', Zeigler Bros., Gardners, PA, U.S.A.) and were fed to apparent satiation twice daily throughout the study (unless otherwise noted).

Fish from tank I were used to investigate the time course of seawater acclimation of 'early' migrant juvenile American shad. On 1 September 1998 at 0800 hours EST, eight fish were removed from each of tanks I and II for sampling. At this time 80 fish from tank I were crowded into buckets and subjected to an isothermal transfer (without de-watering) to an identical 1.5 m diameter 1100 l tank maintained in sea water at a salinity of 24 (Forty Fathoms Marine Mix, Marine Enterprises International Inc., Baltimore, MD, U.S.A.). This was a closed system with biological and particle filtration

and temperature control and a flow rate of 151 min^{-1} . Temperature was maintained at 23°C (within 1°C ; Fig. 1). Fish from this tank were sequentially removed and sampled from this tank at 1, 2, 4, 8 and 24 h after transfer. Juveniles were not fed during this first 24 h period of time. Additionally, juveniles were fasted for 20 h prior to subsequent sampling at 2, 4, 7 and 14 days after transfer. On day 14 (15 September) eight fish maintained in fresh water from tank I were sampled as a reference.

Fish from tank II were used to investigate the time course of seawater acclimation of 'late' migrant juvenile American shad. These fish were maintained in the freshwater (river) conditions until temperature declined to 10°C (Fig. 1). Transfer and sampling was carried out in parallel with the first group of 'early' fish. On 10 November 1998 at 0800 hours EST, eight fish were sampled and 80 American shad juveniles were subjected to an isothermal transfer without de-watering to an identical 1.5 m diameter 1100 l tank maintained at a salinity of 24. Temperature was maintained at 10°C (within 0.5°C ; Fig. 1) and fish were sampled over the next 14 days as described previously.

SAMPLING

All sampled fish were anaesthetized with 100 mg l^{-1} MS-222 (buffered with 12 mM NaHCO_3 , $\text{pH} = 7.0$) and fork length (L_F , cm) mass (M , g) were measured and condition factor (K) calculated ($K = 1000M L_F^{-3}$; see Table I). Gill tissue was removed for measurement of Na^+ , K^+ -ATPase activity and histological analysis. Blood was obtained by severing the caudal peduncle and collecting it in heparinized microhaematocrit capillary tubes. Tubes were kept on ice for 30–45 min prior to spinning in a haematocrit centrifuge for 5 min at $13\,500 \text{ g}$ and haematocrit was measured. The plasma layer was removed into $250 \mu\text{l}$ micro-centrifuge tubes and stored at -80°C prior to analysis.

DETERMINATION OF GILL Na^+ , K^+ -ATPASE ACTIVITY

Gill Na^+ , K^+ -ATPase activity was determined using the microplate method described by McCormick (1993) as validated for American shad by Zydlewski & McCormick (1997a). Briefly, gill tissue was removed and immersed in $100 \mu\text{l}$ of ice cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, $\text{pH} = 7.3$) and stored at -80°C . Gill samples were thawed immediately prior to assay and homogenized in $125 \mu\text{l}$ of 0.1%

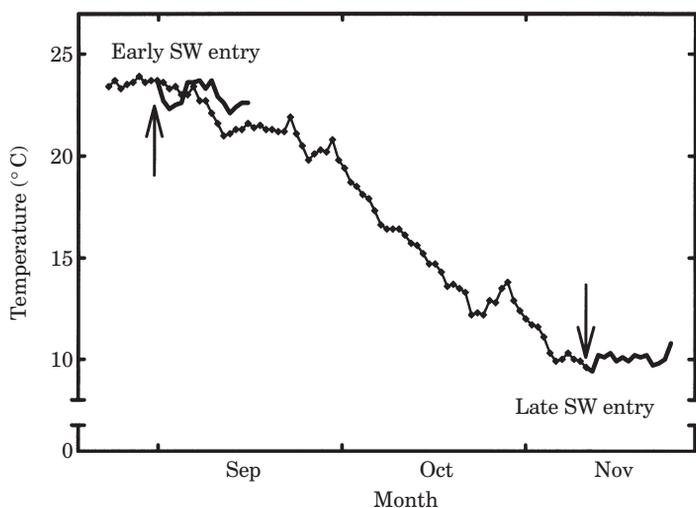


FIG. 1. Temperature profile for juvenile American shad reared in fresh water (\diamond - \diamond), \rightarrow time of transfer into sea water either on 1 September or 10 November, — temperatures experienced by fish in sea water and monitored for 14 days.

TABLE I. Mean \pm S.E. fork length, mass and condition factor of juvenile American shad transferred to sea water (SW). Statistical significance is indicated only between samples within the 'early' and 'late' cohorts, respectively. Freshwater (FW) fish sampled on day 14 are included in the 'early' cohort comparison. Data were analysed using a one-way ANOVA and where significant differences were found, was followed by Sheffé multiple comparison test. Different superscript letters indicate statistical differences between groups. Where there are no superscript letters, no differences were found

	Day	<i>n</i>	L_F (cm)	M (g)	K
Early transfer into SW					
(1 September)	0	16	7.0 ± 0.1	2.8 ± 0.1^a	0.83 ± 0.02^a
	7	8	7.4 ± 0.2	3.9 ± 0.2^b	$0.96 \pm 0.03^{b,c}$
(15 September)	14	8	7.1 ± 0.1	4.2 ± 0.4^b	1.06 ± 0.03^b
Reference fish held in FW					
(15 September)	14	8	7.2 ± 0.2	$3.2 \pm 0.2^{a,b}$	$0.89 \pm 0.02^{a,c}$
Late transfer into SW					
(10 November)	0	8	8.8 ± 0.2	6.2 ± 0.5	0.91 ± 0.02
	7	7	9.4 ± 0.3	8.1 ± 0.9	0.94 ± 0.02
(24 November)	14	7	9.1 ± 0.3	7.0 ± 0.6	0.93 ± 0.02

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 mM ouabain in a solution containing 4 U ml^{-1} lactate dehydrogenase, 5 U ml^{-1} 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 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.*, 1985; BCA Protein kit, Pierce, Rockford, IL, U.S.A.) using bovine serum albumen as standard. Activity of gill Na^+ , K^+ -ATPase is expressed as $\mu\text{mol ADP mg protein}^{-1} \text{ h}^{-1}$.

ANALYSIS OF CHLORIDE CELL NUMBER, SIZE AND SHAPE

For both 'early' and 'late' groups of American shad, gill samples from fish at day 0, 2, 4 and 14 were taken. At day 14 for the 'early' group, reference fish maintained in fresh water were also sampled. The first gill arch of the left side was excised immediately and placed into freshly mixed Champy-Maillet's fixative for 16 h (Avella *et al.*, 1987). This method stains phospholipids, and because of the 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 de-ionized 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, U.S.A.). Seven-micron ribbons were placed on warmed gelatin subbed slides. Paraffin was cleared with toluene and slides were mounted with Permount (Fisher Scientific) for later analysis.

Cell number was determined by observing 10 frames ($630 \times 470 \mu\text{m}$) of saggital sections through gill tissue from each individual fish using a microscope equipped with a CCD video camera at a resolution of 640×480 . A stage micrometer was used for calibration. The number of positively stained cells (chloride cells) and lamellar spaces that fit completely in the screen was then counted and the length of the analysed region recorded.

A 'lamellar space' was 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.

For an individual fish, a minimum of 2 mm (range of 2–12 mm) of gill filament was surveyed, and a minimum of 151 cells counted (range of 151–1346 cells). Abundance of positively stained cells on the primary filament and secondary lamellae were 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 mm (of one side of a primary filament, dorsal or ventral). Chloride cell number was also calculated per lamellar space. Trends in cell number were qualitatively similar when expressed as cells per lamellar space and cells mm^{-1} , therefore only cells mm^{-1} are reported here.

Individual chloride cell size and shape were analysed by capturing 10 fields ($220 \times 145 \mu\text{m}$) at a resolution of 1760×1168 using a microscope equipped with a digital camera. Using the criteria described for cell number above, individual cells were identified as being on the primary filament or the secondary lamellae prior to analysis. Images were analysed using 'Image J' software (National Institutes of Health, U.S.A.). The borders of positively stained cells were traced by eye and the area (μm^2) and the perimeter (in μm) were measured using a stage micrometer. Perimeter measurements were used solely for calculating an index of cell shape. 'Shape factor' (defined as $4\pi A P^{-2}$, where A is area and P is perimeter) was calculated for each cell. Values approaching one indicate a near circular shape and lower values indicate a more elongate shape. For all samples, a minimum of 100 cells on the primary filament and a minimum of 75 cells on the secondary lamellae were measured. When it was not possible to measure this minimum for cells on the secondary lamellae, mean values are not reported.

BODY MOISTURE AND PLASMA OSMOLALITY

In order to measure whole body moisture, the fish body (having been bled and two gill arches removed) was weighed to the nearest 0.00001 g. The body was then dried at 60°C for 72 h, weighed, and the per cent difference calculated. Plasma osmolality was measured on a Wescor 5500 Vapor Pressure Osmometer (Wescor Inc., Logan, UT, U.S.A.).

STATISTICS

Significance of statistical analysis is reported at the $P < 0.05$ level. A two-way ANOVA was used to analyse seawater acclimation data using group ('early' and 'late') and time as factors. Significance of factors or of interactions was followed by analysis within each factor. One-way ANOVAs were used for comparison of each of the two seawater acclimation groups, and all freshwater samples. An inclusive one-way ANOVA was also run for comparisons of the 'early' group including the day 14 freshwater group (reference fish). In all analyses, significance with a one-way ANOVA analysis was followed by a Sheffé *post-hoc* test. Means are given with \pm s.e.

RESULTS

EARLY ACCLIMATION TO SEA WATER

Early seawater acclimation (September) resulted in an osmotic perturbation that recovered within 2 days after seawater entry (Fig. 2). Haematocrit levels declined within 2 h, were still 14% lower than initial levels at 24 h, but recovered within 48 h. Plasma osmolality was perturbed (6%) at 4 h, but recovered rapidly within 8 h. While body moisture declined over time there was no significant depression in this variable. Reference fish in fresh water sampled at day 14 did

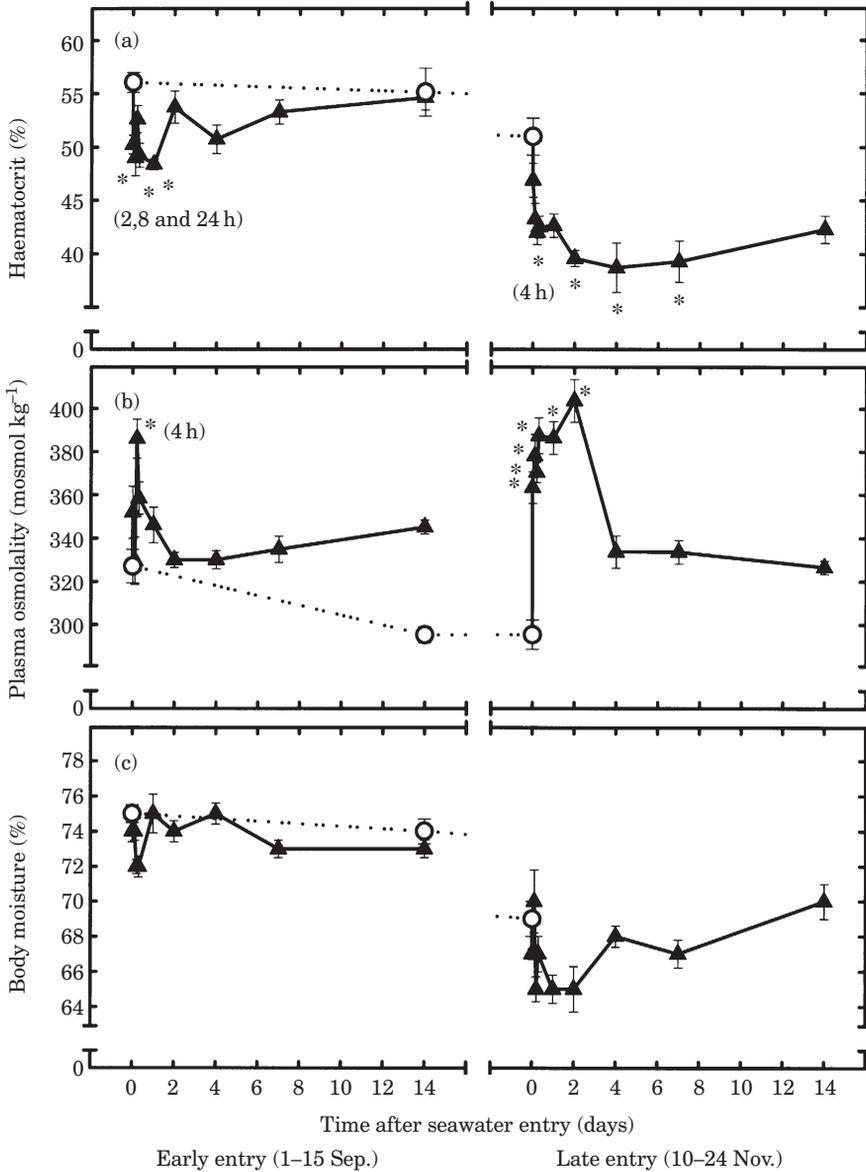


FIG. 2. Changes in mean \pm S.E. (a) haematocrit, (b) plasma osmolality and (c) body moisture of juvenile American shad transferred to sea water (▲) on 1 September and 10 November (corresponding to 'early' and 'late' migration, respectively). *, difference from fish sampled prior to transfer (○). Where points overlap, significant times are identified on the figure in parentheses.

not differ from initial levels in haematocrit, plasma osmolality or body moisture.

Early acclimation to sea water resulted in a two-fold increase in gill Na^+ , K^+ -ATPase within 24 h (2.8 to 4.9 $\mu\text{mol ADP mg protein}^{-1} \text{ h}^{-1}$; Fig. 3). By 4 days this enzyme increased by an additional factor of two (to 9.7). No further

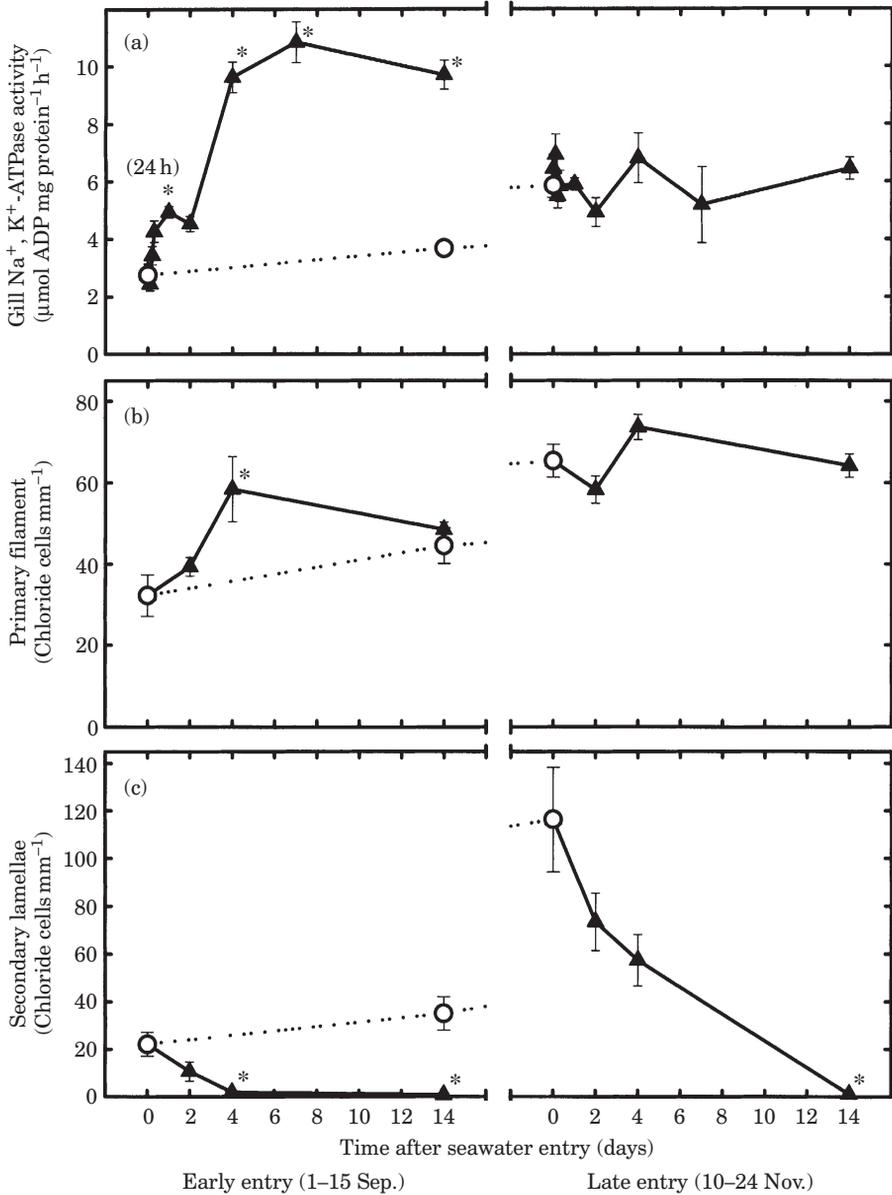


FIG. 3. Changes in mean \pm S.E. (a) gill Na⁺, K⁺-ATPase activity and number of chloride cells found on the (b) primary filament and (c) secondary lamellae of the gills of juvenile American shad transferred to sea water (▲) on 1 September and 10 November (corresponding to 'early' and 'late' migration). *, difference from fish sampled prior to transfer (○). Where points overlap, significant times are identified on the figure in parentheses.

increase occurred between days 4 and 14. These rapid increases were paralleled by rapid changes in gill chloride cell numbers (Figs 3 and 4). Chloride cells found on the primary gill filament increased nearly two-fold (32 to 58 cells mm⁻¹) by 4 days, but did not differ from initial freshwater numbers

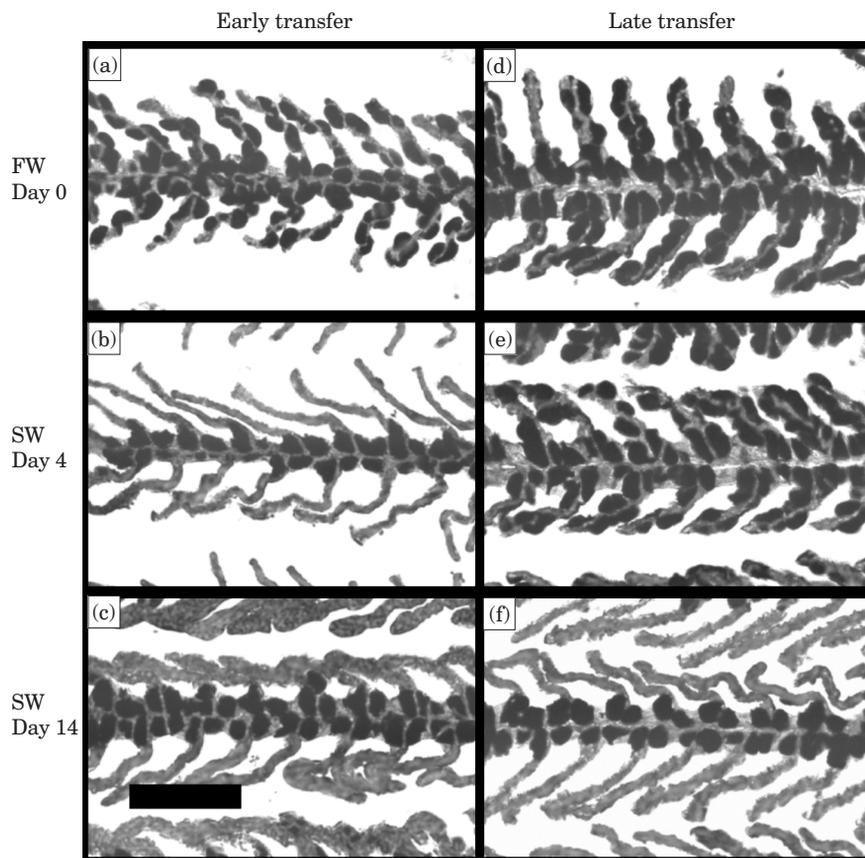


FIG. 4. Histological preparations of gill filaments of American shad juveniles stained in Champy–Maillet fixative. Gills from fish in (a) and (d) fresh water, (b) and (e) sea water for 4 days, and (c) and (f) sea water for 14 days. Individuals from the left column (a), (b) and (c) were transferred into sea water 'early' (1 September); individuals from (d), (e), and (f) were transferred to sea water 'late' (10 November). The bar represents 50 μm .

by 14 days after seawater entry. By day 14, cells on the primary filament had become enlarged (36 to 49 μm^2) and more round (increase in shape factor of 0.81–0.84) (Table II). In sharp contrast, chloride cells on the secondary lamellae declined in number from 22 to <2 cells mm^{-1} over the course of 4 days (Fig. 3). Chloride cells on the secondary lamellae were all but absent by day 14. There was no change in size or shape of these cells from day 0 and 2 (Table II). There were not adequate numbers of cells to analyse after day 2. Reference fish in fresh water sampled at day 14 did not differ from initial levels gill Na^+ , K^+ -ATPase, chloride cell size, number or shape factor.

The growth of American shad transferred to sea water in September was comparable to that of fish remaining in fresh water (Table I). Mass increased 50% over 14 days and was not different than the growth observed in fresh water over the same period. The value of K also increased in sea water from 0.83 to 1.06, a greater increase than was observed over the same time period in fresh water.

TABLE II. Mean \pm s.e. chloride cell size and shape factor ($4\pi AP^{-2}$) of juvenile American shad transferred into sea water (SW). Statistical significance is indicated only between samples within the 'early' and 'late' cohorts, respectively. Freshwater (FW) fish sampled on day 14 are included in the 'early' cohort comparison. Data were analysed using a one-way ANOVA and where significant differences were found, was followed by Sheffé multiple comparison test. Different superscript letters indicate statistical differences between groups. Where there are no superscript letters, no differences were found

	Day	n	Cells on the primary filament		Cells on the secondary lamellae	
			Cell size (μm^2)	Shape factor	Cell size (μm^2)	Shape factor
Early transfer into SW (1 September)	0	5	35.7 ± 2.5^a	0.812 ± 0.004^a	37.7 ± 2.4	0.788 ± 0.005
	2	4	$44.2 \pm 1.5^{a,b}$	$0.822 \pm 0.007^{a,b}$	31.1 ± 2.5	0.794 ± 0.002
	4	4	$46.0 \pm 3.7^{a,b}$	0.833 ± 0.003^b	—	—
	14	5	48.8 ± 2.1^b	0.839 ± 0.003^b	—	—
(15 September) Reference fish held in FW	14	5	37.1 ± 1.6^a	0.814 ± 0.007^a	32.9 ± 1.2	0.763 ± 0.009
Late transfer into SW (10 November)	0	5	67.6 ± 2.2^a	0.777 ± 0.013^a	56.2 ± 2.1	0.806 ± 0.011
	2	5	67.0 ± 3.8^a	$0.788 \pm 0.013^{a,b}$	57.9 ± 2.3	0.829 ± 0.006
	4	5	$75.3 \pm 3.4^{a,b}$	$0.785 \pm 0.011^{a,b}$	58.1 ± 4.1	0.815 ± 0.010
(24 November)	14	5	81.4 ± 1.6^b	0.835 ± 0.009^b	—	—

LATE SEAWATER ACCLIMATION

Acclimation of American shad to sea water in November resulted in an osmotic perturbation that persisted beyond 2 days after seawater entry (Fig. 2). In contrast to the 'early' fish, recovery was largely accomplished only after 7 days. Haematocrit levels declined within 4 h and were 23% lower than initial levels at 2, 4 and 7 days after transfer. Haematocrit levels at day 14 were not different from initial values. Plasma osmolality was greatly perturbed (increasing 23% from 295 to 363 mosmol kg⁻¹) within 1 h. Plasma osmolality further increased, reaching peak levels (403 mosmol kg⁻¹) 48 h after transfer (an increase of 36%). Plasma osmolality recovered to initial levels by 4 days. While there was a significant change in body moisture during seawater acclimation individual means did not differ from initial values.

In sharp contrast to 'early' acclimation to sea water, gill Na⁺, K⁺-ATPase did not increase over the 14 day acclimation period starting in November (Fig. 3). Initial gill Na⁺, K⁺-ATPase activity levels in fresh water, however, were more than two-fold greater in November than in September (5.8 v. 2.8 μmol ADP mg protein⁻¹ h⁻¹).

Unlike 'early' fish, chloride cells found on the primary gill filament did not increase through the acclimation period (Figs 3 and 4), however, initial cell number was two-fold higher than initial numbers in September (65 v. 32 cells mm⁻¹). As with fish acclimated in September, cell size increased 19% (to 81 μm²) and cells became more round (shape factor increased from 0.78 to 0.84) in 'late' acclimated fish (Table II). Initial cell size was nearly two-fold greater in 'late' fish (68 μm²) than 'early' fish (36 μm²), though initial cell shapes were comparable (Table II).

Like the 'early' fish, abundance of chloride cells on the secondary lamellae sharply declined to negligible numbers. The time course and initial abundance of chloride cells, however, were different from fish in the 'early' acclimation. While cell number sharply declined, a significant reduction was observed only on day 14 (Fig. 3). Initial cell numbers in November were more than five-fold greater than initial numbers in September (116 v. 22 cells mm⁻¹). There was no change in size or shape of chloride cells on the secondary lamellae between days 0 and 4 (Table II). There were not adequate numbers of chloride cells on the secondary lamellae to analyse after day 4.

American shad transferred to sea water in November did not increase in size or mass in sea water over the period of 14 days (Table I) and *K* did not change.

DISCUSSION

American shad juveniles acclimated to sea water in September at (23° C) exhibited a greater tolerance to sea water than did fish transferred in November (10° C). Both groups of fish exhibited two clear phases of acclimation, an 'acute adaptive phase' marked by a rapid osmotic perturbation and partial recovery followed by a 'chronic regulatory phase' during which a new ionic homeostasis was established (Holmes & Donaldson, 1969). Similar patterns of ionic perturbation

and subsequent recovery have also been described for other teleosts (Jacob & Taylor, 1983; Evans, 1984). The severity and duration of these phases, however, were quite dissimilar between 'early' and 'late' fish. It should be noted that complete acclimation (the chronic regulatory phase) may extend beyond the 14 day time course of this study.

This study was designed to emulate the conditions and timing of seawater entry that juvenile American shad experience in nature as 'early' and 'late' migrants. As a result, there are a number of factors that are likely to have contributed to the differences in the seawater acclimation responses for these treatments. These groups were separated chronologically by nearly 10 weeks. While American shad do not have a distinct developmental shift analogous to the salmonid parr-smolt transformation, changes in morphology and osmoregulatory physiology associated with the period of migration have been described (Zydlewski & McCormick, 1997*a, b*, 2001). The temperature differences experienced by 'early' and 'late' fish are also likely to contribute to the disparity in physiological responses by directly affecting biochemical processes as well as through physiological shifts associated with thermal acclimation. In addition, 'late' fish were afforded an extended period for growth in advance of seawater entry. As a result, 'late' fish were more than twice as large as 'early' fish (Table I). The relative contribution of these factors is of interest here.

Dissimilarities in the freshwater osmoregulatory physiology of 'early' and 'late' fish prior to seawater transfer are expected and are consistent with previously described changes over a similar temperature range (23 to 10° C) and time course (September to November) in fresh water. During the period of migration juvenile American shad gradually lose the ability to regulate their ions in fresh water through the autumnal period of seaward migration (Zydlewski & McCormick, 1997*a*). Associated with impaired hyperosmoregulatory ability is an increase in gill Na⁺, K⁺-ATPase activity (Zydlewski & McCormick, 1997*b*) which is the result of an intense proliferation and enlargement of Na⁺, K⁺-ATPase-rich chloride cells on both the primary filament and secondary lamellae of the gill (Zydlewski & McCormick, 2001). Decreasing temperature accelerates these changes, linking this physiological change to downstream migratory behaviour.

Evidence suggests that these changes are not associated with preparation for seawater entry (as occurs in salmonids during the parr-smolt transformation). American shad develop the ability to successfully osmoregulate in full strength sea water at the larval-juvenile transition, months in advance of migration for most juveniles (Zydlewski & McCormick, 1997*a*). Additionally, there is no evidence for any increase in seawater tolerance through the period of migration (Zydlewski & McCormick, 1997*a*). Rather the increase in chloride cells (in fresh water) is probably a physiological response to increased ion uptake demands (Zydlewski & McCormick, 2001).

In accord with these previous findings 'late' fish in this study had higher gill Na⁺, K⁺-ATPase activity and a greater abundance and average size of chloride cells (on both the primary filament and secondary lamellae) than 'early' fish prior to transfer (Figs 3 and 4). These marked differences in chloride cells and gill Na⁺, K⁺-ATPase activity prior to acclimation are probably responsible, in part, for the observed disparity in acclimation responses.

During seawater acclimation of juvenile American shad there is a radical rearrangement of chloride cell number and distribution. This rearrangement includes the disappearance of cells found on the secondary lamellae (Zydlewski & McCormick, 2001). Chloride cells on the secondary lamellae of freshwater acclimated fishes have been implicated in the uptake of sodium, chloride and calcium as well as acid-base regulation (Perry, 1997). Similarly, the pavement cells of some fishes have also been implicated in sodium uptake (Wilson *et al.*, 2000). The process by which these chloride cells disappear probably reflects the shift from a 'freshwater' function of the gill to that of ion excretion.

It follows then, that the swiftness of reduction of these cells would reflect the progress of physiological acclimation to sea water. In this study, 'early' fish (initially with few small chloride cells on the secondary lamellae) exhibited a rapid decline in chloride cells on the secondary lamellae paralleled by a rapid recovery of osmotic perturbation (Figs 2 and 3). In contrast, the abundance of large chloride cells on the secondary lamellae of 'late' fish declined only by day 14, matched by a protracted recovery from osmotic perturbation.

Although chloride cells on the secondary lamellae declined in number during seawater acclimation, these cells did not change in size or shape (within the window of time when they were present in sufficient numbers to be analysed; Table II). This suggests that these cells are not individually down-regulated, but rather these cells disappeared through changes in cell turnover rates. A similar decrease of cells on the secondary lamellae occurs during seawater acclimation in chum salmon *Oncorhynchus keta* (Walbaum) fry. Uchida & Kaneko (1996) demonstrated that cell turnover rates in sea water were three and a half-fold higher than in fresh water. The restriction of newly differentiated cells to the primary filament resulted in a rapid decrease of cells on the secondary lamellae.

Because chloride cells found on both the secondary lamellae (in fresh water) and the primary filament (in both fresh and sea water) are rich in Na^+ , K^+ -ATPase (Zydlewski & McCormick, 2001) changes in chloride cells are probably the dominant (if not commanding) influence on measured enzyme activities in gill homogenate. The pattern of chloride cell distribution and abundance is similar between the 'early' and 'late' fish (rapid disappearance of cells on the secondary lamellae), however, patterns of gill Na^+ , K^+ -ATPase activity during seawater acclimation differ markedly (Fig. 2). 'Early' fish exhibit an increase in gill Na^+ , K^+ -ATPase activity while 'late' fish had no increase. While there is a similar pattern of chloride cell rearrangement during acclimation, notable differences also exist.

For 'early' fish, chloride cells on the primary filament increased during seawater acclimation, while cells on the secondary lamellae disappeared. The overall chloride cell number (sum of cells on the primary filament and the secondary lamellae) did not change (Fig. 3). Cells on the primary filament increased in size (Table II) suggesting basolateral surface proliferation for increased Na^+ , K^+ -ATPase pump insertion. Enrichment of chloride cells with Na^+ , K^+ -ATPase during seawater acclimation is consistent with observations in other teleosts (Karnaky *et al.*, 1976; McCormick, 1990) and are presumably responsible for the observed increase in gill Na^+ , K^+ -ATPase activity.

In 'late' fish, however, the number of chloride cells on the primary filament did not change while chloride cells on the secondary lamellae disappeared,

resulting in a net decline in gill chloride cells. In spite of this, 'late' fish had more cells on the primary filament prior to seawater acclimation than did 'early' fish after 14 days of seawater acclimation. As chloride cells on the primary filament did enlarge (Table II), it is reasonable to suggest that a decrease in gill Na^+ , K^+ -ATPase activity due to the decline in total chloride cell number was offset but not overcome by the enrichment of chloride cells on the primary filament with the enzyme.

It is also important to note that 'early' fish exhibited a two-fold increase in gill Na^+ , K^+ -ATPase within 24 h of seawater entry. Because of the time frame, it is unlikely to be the result of cellular recruitment; rather this increase is probably the result of the mobilization of intracellular stores or the activation of pumps. This rapid increase, which is correlated with a very rapid osmotic recovery, is absent in the 'late' fish. This suggests that 'late' fish are unable to launch a similar rapid response and may rely heavily on cellular recruitment for osmotic recovery, contributing to a protracted recovery period.

Temperature probably had a considerable influence on the relative differences observed in gill Na^+ , K^+ -ATPase activity and chloride cell number prior to and during seawater acclimation. Prior to seawater acclimation, 'early' fish were acclimated at 23° C, and 'late' fish at 10° C. While measured Na^+ , K^+ -ATPase activities were two-fold greater in the 'late' fish, it should be noted that measured activities reflect *in vitro* V_{max} (maximum kinetic velocity of an enzymatic reaction) values measured at 25° C. Temperature affects the kinetics of enzyme action, theoretically decreasing enzyme activity by a factor of approximately two with a 10° C decrease in temperature (the Q_{10} effect). A linear relationship between enzyme activity and temperature has been demonstrated for gill Na^+ , K^+ -ATPase in coho salmon *Oncorhynchus kisutch* (Walbaum) (McCormick & Bern, 1989). Thus while *in vitro* Na^+ , K^+ -ATPase activities (in fresh water) are greater in the 'late' fish held at 10° C, the relative *in vivo* activities of 'early' and 'late' fish are likely to be comparable. Conversely, after 14 days of acclimation in sea water, the *in vivo* gill Na^+ , K^+ -ATPase activity of 'late' fish is probably lower than that of 'early' fish. More detailed studies on the thermal effects on Na^+ , K^+ -ATPase kinetics (measuring activities at acclimation temperature and standard temperatures) are necessary in order to directly evaluate *in vivo* activities in juvenile American shad.

It follows then, that the greater abundance of chloride cells in 'late' fish is also a result, at least in part, of thermal acclimation. Other factors, however, such as development must also exert an effect on these osmoregulatory variables as increases in gill Na^+ , K^+ -ATPase activity and chloride cell number have been shown to occur in American shad maintained at constant 24° C temperature (Zydlewski & McCormick, 1997a). It should be noted that these increases are delayed and of less magnitude than in fish held under declining temperature. Factors such as gill permeability (Gonzalez & McDonald, 1994; Keiffer & Tufts, 1996) membrane fluidity and ion permeability (Schwartzbaum *et al.*, 1992) are also influenced by temperature and may be included in the execution of thermal acclimation. *In vivo*, differences in enzyme kinetics may be offset by changes in passive permeability rates and reduced gill perfusion rates at low temperatures.

In addition to affecting the initial chloride cell number and distribution, temperature is also likely to be responsible for the dissimilar time course of osmotic recovery after seawater entry. The process by which chloride cell size, number and position are changed during seawater acclimation must include transcription and translation of gene products. The kinetics of these processes are subject to the same effects of temperature described above. Therefore, two thermal effects, the proliferation of 'freshwater' chloride cells at low temperatures and the restricted ability to up-regulate the products necessary for ion excretion at low temperatures, must contribute to the protracted acclimation process in 'late' fish.

The relative size of American shad entering sea water is also likely to have affected the extent and magnitude of osmotic perturbations. Late fish weighed more than twice as much as 'early' fish due to extended growth opportunities in fresh water. Size has been demonstrated to influence osmotic tolerance (McCormick & Naimen, 1984) presumably because of the shift in surface to volume ratio. While this explanation would afford greater osmotic tolerance to the larger fish, the opposite was observed here. Thus if some advantage was afforded to these fish through size, the detrimental aspects of waiting to enter sea water (development and thermal effects) dominated.

Late migrant American shad face a greater physiological challenge than do 'early' fish, both during freshwater residence and (as demonstrated here) during seawater entry. During freshwater residence, American shad gradually lose the ability to hyperosmoregulate with no discernable increase in seawater tolerance (Zydlewski & McCormick, 1997b). The proliferation of chloride cells in late migrants has been implicated in this physiological change and is thought to be responsible for reduced performance and increased mortality in fresh water (Chittenden, 1972; Zydlewski & McCormick, 1997b; 2001). This study has linked these changes with decreased physiological performance during seawater acclimation; late migrants experience a more protracted and severe osmotic perturbation than early migrants. In Atlantic salmon *Salmo salar* L. osmotic perturbations have been demonstrated to reduce escape success from predators, significantly reducing the survival of Atlantic salmon during downstream migration (Handeland *et al.*, 1996). It is not unreasonable to hypothesize that differences in osmoregulatory performance are likely to differentially affect the survival of seaward migrating American shad.

Downstream migration occurs over a number of weeks and over a considerable temperature range. If there is no apparent physiological cost to entering sea water as an early migrant, why is migration protracted late into the autumn? Indeed, fish acclimated to sea water 'early' had comparable growth to those fish remaining in fresh water (Table I) and a rapid recovery of osmotic perturbations. Conversely, the physiological disadvantages of postponing migration until later in the season are clear, in both fresh water and during acclimation to sea water. Osmoregulatory physiology in juvenile American shad delineates the effective conclusion of migration. Individual variation in age, size and osmoregulatory physiology may partly explain the protracted nature of the migration but other factors (together with osmoregulatory physiology) must stabilize the timing of downstream migration. Advantages afforded to juveniles that delay migration (potentially including reduced predation risk and extended growth opportunities) must off set the physiological perils.

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