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NOTES

The Stress Response of Juvenile American Shad to Handling and Confinement Is Greater during Migration in Freshwater than in Seawater

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Abstract.—The physiological responses of juvenile American shad *Alosa sapidissima* were evaluated during the period of downstream migration in freshwater (FW) and after seawater (SW) acclimation associated with postmigration. Fish were subjected to a standardized, acute handling and confinement stress (3 h). Changes in plasma cortisol, plasma chloride, and hematocrit were monitored for 24 h. Basal levels of plasma cortisol were 5 times as great in FW as in SW fish (34 and 7 ng/mL, respectively). Within 0.5 h, both groups exhibited significant increases in plasma cortisol. The increase in FW fish was 4.5 times as great as that in SW fish. While the cortisol levels in SW fish returned to their basal values within 24 h, those of FW fish remained more than 25 times as high as basal values. Changes in plasma chloride occurred in fish after the initial stress, decreasing in FW fish and increasing in SW fish. This perturbation was overcome within 24 h in SW fish but not in FW fish. Hematocrit increased in FW fish 3 h after the initial stress and returned to normal within 24 h; fish stressed in SW exhibited no change in hematocrit. Significant mortalities were observed in the FW group but not in the SW group. During the period of downstream migration in FW, fish exhibited a heightened sensitivity to acute stress compared with that of SW-acclimated postmigrants. Handling and confinement associated with fish transport and fish passage structures are therefore likely to impact the performance scope and survival of juvenile American shad.

American shad *Alosa sapidissima* are anadromous teleosts indigenous to the East Coast of North America. These fish have also been introduced into the Columbia and Sacramento rivers on the West Coast. Adults migrate into coastal rivers and spawn during the spring (Leggett and Whitney 1972). Young generally remain in freshwater (FW) until the fall, when they migrate downstream. The timing of seaward migration is correlated with decreasing water temperature. In general, the peak migration occurs when the temperature falls to between 16 and 9°C (Leggett and Whitney 1972; O'Leary and Kynard 1986).

Associated with downstream migration in FW are increased gill Na^+,K^+-ATPase, a loss of ion uptake capacity, and decreased hematocrit. These and other physiological changes related to migration are increased and hastened by declining fall water temperature (Zydlewski and McCormick 1997a). American shad maintained in FW past the period of migration (unsuccessful migrants) exhibit behavioral perturbations, including the cessation of feeding and impaired swimming ability (Zydlewski and McCormick 1997a). They also experience high mortality (Howey 1985), which is related to impaired hyperosmoregulatory ability (Zydlewski and McCormick 1997a).

On both coasts of North America, populations of American shad are the focus of restoration and mitigation efforts. These efforts may include transport with multiple handling episodes in FW. In addition, downstream passage facilities may entail confinement, handling, and other physiological stressors for migrating fish. While American shad are notoriously sensitive to transport as juveniles in FW, the physiological response to handing and confinement has not been explored. Interestingly, juveniles are more easily handled and show no substantial behavioral perturbations or mortality when acclimatized to seawater (SW; Zydlewski and McCormick 1997a; J. D. Zydlewski, personal observations). It is this apparent difference in sensitivity that is of interest here.

Physiological responses to stress are well documented in many species of fish (reviewed by Bar-

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ton and Iwama 1991) but have not been characterized for American shad. The primary response to a stressor is the release of corticosteroids and catecholamines, which in turn induce secondary responses such as increases in plasma glucose and osmotic perturbations (Mazeaud et al. 1977). Responsiveness to stress has also been shown to change with development. In anadromous salmonids, an increase in responsiveness to stress occurs during the parr–smolt transformation (Barton et al. 1985; Carey and McCormick 1998) as juvenile salmonids prepare for migration into SW.

The objective of this study was to compare the stress responses of juvenile American shad in FW and SW, which correspond to those of (1) migrating juveniles and (2) postmigrants that have fully acclimatized to SW. Fish were exposed to a standardized handling and confinement stress and monitored for 24 h. Changes in plasma cortisol, plasma chloride, and hematocrit are reported.

Methods

Fish maintenance and experimental design.—In August 1995, nonmigratory juvenile American shad were captured in a cove area 2 km north of the Turners Falls Dam on the Connecticut River (temperature, 18°C) and transported to the S. O. Conte Anadromous Fish Research Center in Turners Falls, Massachusetts. Fish were acclimatized to laboratory conditions in flow-through circular tanks (1.5 m in diameter) supplied with river water at a flow rate of 4 L/min and maintained under natural photoperiod. Fish began feeding 2 d after transport to the laboratory and were fed to apparent satiation twice daily with commercial salmon feed (Zeigler Bros., Gardeners, Pennsylvania).

After acclimation for a minimum of 1 week, fish were evenly divided between two pairs of circular tanks (1.5 m in diameter). In one pair of tanks a flow-through system was maintained, with river water flowing through at a rate of 4 L/min. Fish in these tanks experienced a natural decline in water temperature from August (18°C) to October (14°C). The second pair of tanks contained recirculating SW with biological and particle filtration and temperature control. Fish were isothermally transferred to SW maintained at 18 ± 1°C throughout the study. Salinity was maintained at 32‰ (Forty Fathoms Marine Mix, Marine Enterprises International, Inc., Baltimore, Maryland).

Fish in FW were reared until October 12, which is the date of peak downstream migration observed in the Connecticut River. At that time, fish in one of the two tanks were subjected to a standardized handling and confinement stress (see below) and sampled over 24 h. Fish in the second tank (the reference tank) did not receive acute stress but were sampled in parallel to determine the effect of repeatedly opening the tank to net and remove fish. Sampling procedures are described below. Variations of this experimental design have been successfully used to describe the stress response of fish in a number of studies (e.g., Strange and Schreck 1978; Biron and Benfey 1994; Pottinger et al. 1996). Fish in the FW tanks had a mean (±SE) length of 8.5 ± 0.06 cm and a mean weight of 5.6 ± 0.13 g.

The fish in SW were reared until March 1996, thereby representing postmigrants that had completed their seaward journey and were fully acclimatized to SW. On March 4, fish in one of the two tanks were subjected to a standardized handling and confinement stress and sampled over 24 h. Fish in the other (reference) tank did not receive acute stress but were sampled in parallel. Fish in the SW tanks had a mean length of 9.7 ± 0.05 cm and a mean weight of 7.6 ± 0.16 g.

Standardized stress and sampling.—Fish were not fed the morning of sampling. Immediately prior to being subjected to the standardized stressor, 8–10 fish were removed from each of the stressed and reference tanks and sampled as described below. These fish were used to measure basal (time = 0) levels of physiological parameters. The remaining fish in the stressed tanks were captured and held in a net out of water for 30 s and then crowded (at an approximate density of 100 g/L) into a plastic mesh cage in their original tank for 3 h. This is a standard protocol used in our laboratory to assess stress responses (Carey and McCormick 1998). Fish in the reference tanks were not disturbed except for being removed. Fish from each of the stressed and reference tanks were sampled at 0.5, 1.5, 3, 8, and 24 h after initiation of the stress.

Sampled fish were rapidly captured in a net and transferred to a bucket containing 200 mg/L of tricaine methanesulfonate (neutralized and buffered with 0.3 mM of sodium bicarbonate, pH 7.0). Once fish were anaesthetized, fork length and body weight were measured, the caudal peduncle was severed, and blood was collected in ammonium heparinized capillary tubes. The collection of blood was completed within 5 min of first disturbing the fish, ensuring that a capture-associated rise in plasma cortisol did not occur (Sumpter et al. 1986). Tubes were kept on ice for less than 1 h prior to centrifugation (5 min at 13,500 × grav-

ity). Hematocrit was measured and plasma separated and stored at −80°C prior to analysis.

Plasma analyses.—Plasma cortisol was quantified using a competitive, solid-phase microtiter enzyme immunoassay following a protocol similar to that of Munro and Stabenfeldt (1984), as outlined by Carey and McCormick (1998) and validated for American shad (our unpublished data). Rabbit anticortisol antibodies (Cat F3–314, lot 345-10-22-80, Endocrine Science Products, Calabasas Hills, California) were coated to microtiter plates. Cortisol-horseradish peroxidase conjugate (Coralee Munro, University of California, Davis) was used as the label. Color development was carried out with 3,3′,5,5′ tetramethylbenzidine containing 0.01% hydrogen peroxide. The reaction was terminated with 0.5 M HCl and absorbance read at 450 nm. All samples were run in duplicate and measured within the standard curve. High values initially beyond the standard curve were measured through serial dilutions (producing linear measurements within the standard curve). Validity was also confirmed by supplementary experiments in which we extended the standard curve.

Plasma chloride was measured on a Labconco model 442–5000 digital chloridometer using NaCl solutions as external standards.

Statistics.—Two-way analysis of variance was used to determine differences between the timing and treatment factors. Although fish were housed communally in the tanks, the stress response measured in this study is an individual response. Due to the short-term manipulation of the fish, it is unlikely that tank effects would be manifested. Each fish, therefore, was treated as an individual data point. Cortisol data was $\log_{10}$-transformed prior to analysis to meet the assumption of normality. When factors (group or time) were found to be statistically significant, a Tukey's test was used to determine differences between sampling points. Statistical significance is reported at a level of $\alpha < 0.05$. All values are expressed as means ± 1 SE.

Results

Stress in Freshwater ''Migrants''

American shad held in FW had a basal plasma cortisol concentration of 34 ng/mL. After initiation of the stress, plasma cortisol increased nearly 13-fold (to 434 ng/mL) within 0.5 h and peaked at a level about 40-fold higher than the basal level (nearly 1,400 ng/mL) at the completion of the 3-h stress (Figure 1). While plasma cortisol dropped to approximately 700 ng/mL after 8 h, it remained nearly 20 times as high as the basal level at 24 h. A more than eightfold rise in cortisol was observed in the reference fish at 0.5 and 1 h. The plasma cortisol levels of reference fish did not differ from the basal levels at 3, 8, or 24 h. The plasma cortisol levels of stressed fish were significantly ($P < 0.005$) higher than those of reference fish at 3, 8, and 24 h.

Plasma chloride concentrations were lower in the stressed group than in the reference group. Although these concentrations did not differ significantly from the basal levels in either group, the stressed fish had significantly different levels at 24 h (Figure 1). Hematocrit levels were higher in the stressed group than in the reference group (Figure 1). The hematocrit of stressed fish increased 20% to a maximum level after 3 h, returning to basal levels by 24 h. There was no change in the hematocrit of the reference fish over time.

High mortality was observed in the stressed group; 17 mortalities (12%) occurred by 8 h and 20 mortalities (14%) by 24 h after initiation of the stress. In contrast, one fish in the reference group died within this time. Stressed fish showed considerable hemorrhaging on the rostrum and around the eyes, though there were no signs that abrasion was the cause and no other obvious differences from the reference group were seen.

Stress in Saltwater ''Postmigrants''

Fish acclimatized to SW had a basal plasma cortisol level of 7 ng/mL, nearly 20% of basal levels of plasma cortisol measured in FW fish. Initiation of the standardized stressor resulted in an increase in plasma cortisol by 0.5 h that peaked at a level 37 times as high as the basal level at 1.5 h (262 ng/mL; Figure 2). This peak value was less than 20% of the peak value observed in fish stressed in FW. Cortisol began to decline after 3 h, before the stress event had been completed. By 24 h, it had returned to basal levels. There was a significant ($P < 0.001$) 20-fold increase in plasma cortisol in reference fish at 0.5 h and a 17-fold increase at 1 h. Plasma cortisol in reference fish did not differ from basal levels at 3, 8, and 24 h. Plasma cortisol levels of stressed fish were significantly higher than those of reference fish at 3 and 8 h but did not differ at 24 h.

Plasma chloride increased 15% in the stressed group, reaching peak levels at 3 and 8 h. Plasma chloride returned to the basal level by 24 h (Figure 2). Plasma chloride concentration did not change in the reference group over the course of the ex-

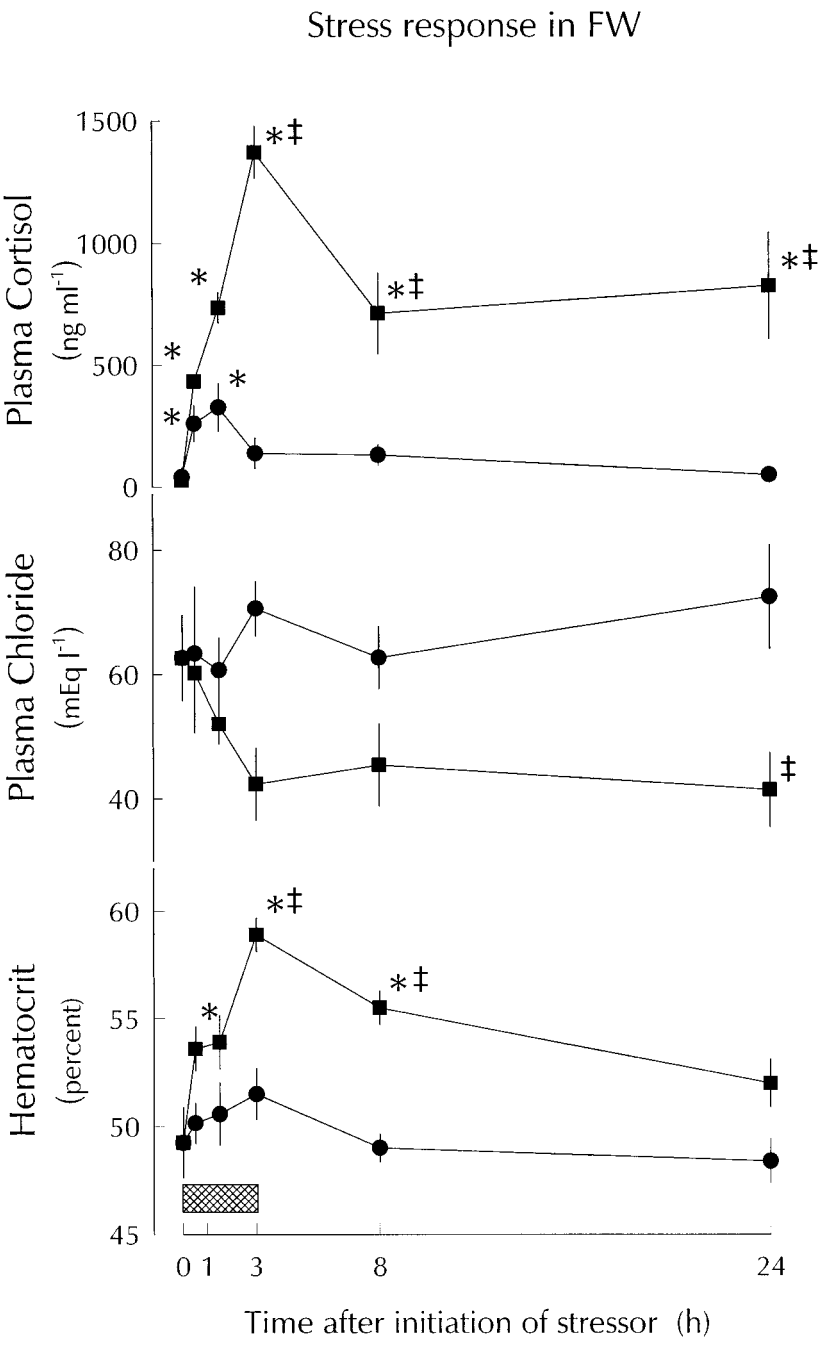

FIGURE 1.—Concentrations of plasma cortisol, plasma chloride, and hematocrit in juvenile American shad in freshwater during and after exposure to a 3-h standardized handling and confinement stress. The duration of the stress is shown by the crosshatched box. Circles apply to reference fish (which were exposed only to sampling disturbance), squares to stressed fish. An asterisk indicates that the value is significantly ($P < 0.05$) different from the basal (initial) value, a double plus sign that the value is significantly different from that of the reference fish. Sample size is 8–10 for each point; error bars are ± 1 SE.

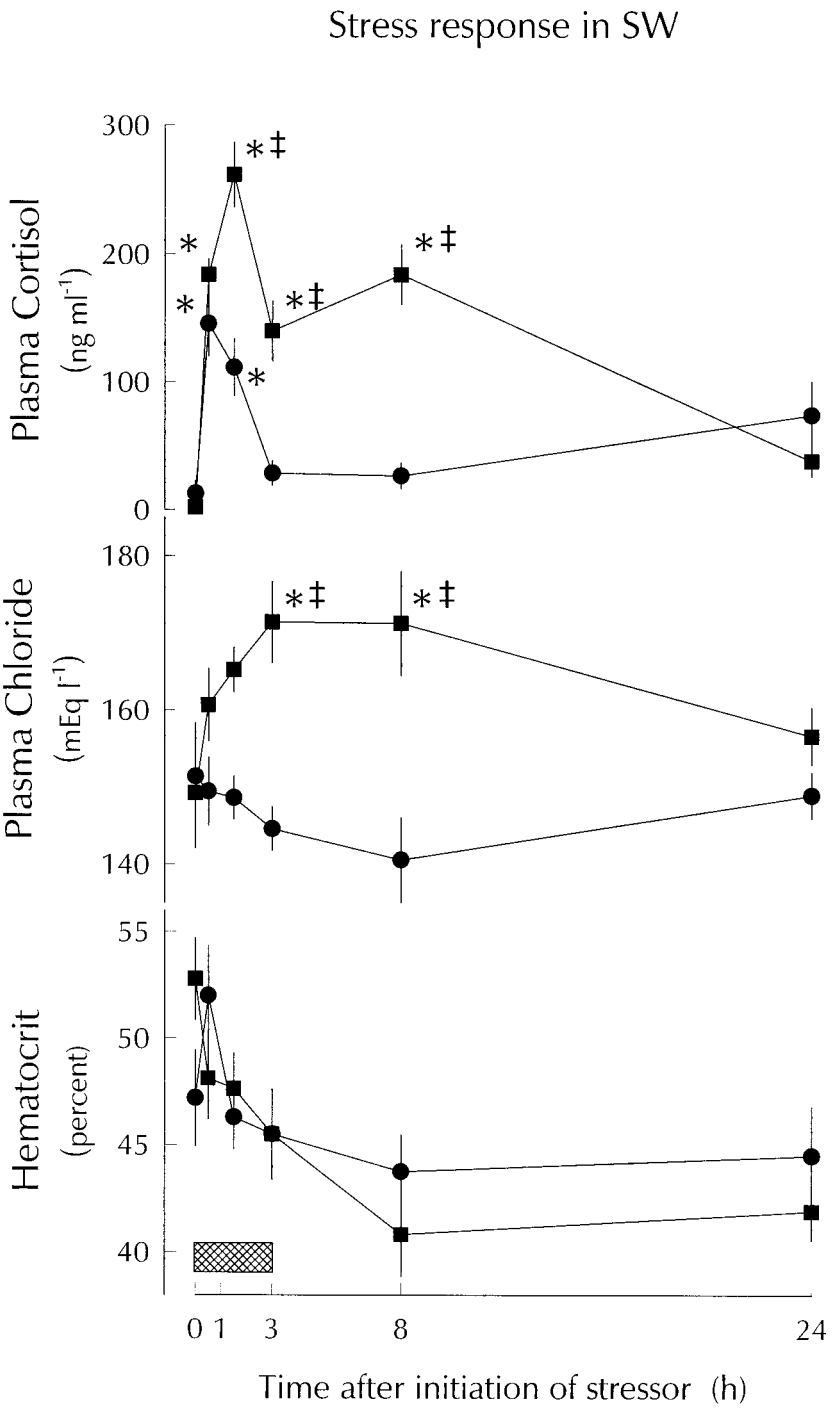

FIGURE 2.—Concentrations of plasma cortisol, plasma chloride, and hematocrit in juvenile American shad previously acclimatized to seawater during and after exposure to a 3-h standardized handling and confinement stress. The duration of the stress is shown by the crosshatched box. Circles apply to reference fish (which were exposed only to sampling disturbance), squares to stressed fish. An asterisk indicates that the value is significantly ($P < 0.05$) different from the basal (initial) value, a double plus sign that the value is significantly different from that of the reference fish. Sample size is 8–10 for each point; error bars are ± 1 SE.

periment. There was no difference in hematocrit between the stressed and reference groups at any time. No mortalities were observed in either the stressed or reference groups in SW over the course of this experiment.

Discussion

Handling and confinement resulted in a rapid rise in plasma cortisol levels in both FW fish and those acclimatized to SW. In FW fish, however, both the basal and peak levels of cortisol were markedly higher than in SW-acclimatized fish. The basal levels of plasma cortisol were five times as high in FW fish as in SW fish (34 versus 7 ng/mL), and the magnitude of the cortisol response in FW fish was more than five times as great as that in fish acclimatized to SW (mean peak values of 1,371 versus 262 ng/mL; Figures 1, 2). The cortisol response in American shad held in FW is much greater than that reported for most other fish. Similar levels of cortisol, however, are not without precedent. For example, a 5-h transport stress resulted in an increase in plasma cortisol to 1,700 ng/mL in striped bass (Mazik et al. 1991). The peak level of plasma cortisol in SW-acclimatized fish (262 ng/mL) is more typical of the general stress response of teleosts (see review by Barton and Iwama 1991). The time required for cortisol recovery in FW fish was protracted beyond that in SW-acclimatized shad. While the SW fish exhibited a more typical recovery pattern (reaching basal levels within 24 h), the FW fish did not recover within that time frame. After 24 h, plasma cortisol levels in FW fish remained nearly 25 times as great and were not different from cortisol levels at 8 h. At 24 h, plasma cortisol levels in FW fish were more than three times as high as those observed in SW fish.

Reference fish in both experiments were exposed to the relatively mild but repeated disturbance of sampling. In both FW and SW reference fish, plasma cortisol levels were higher than basal levels at 0.5 and 1 h (Figures 1, 2). However, the absence of elevated levels at 3, 8, and 24 h indicates that recovery from the mild disturbance of sampling is complete within 2 h. This is consistent with the findings of Biron and Benfey (1994), who reported that cortisol concentrations increased and remained elevated for 1 h following tank disturbance. For the stressed group, American shad sampled at 8 and 24 h were undisturbed for 5 and 16 h, respectively. The sampling procedure, therefore, likely did not affect cortisol levels. Additionally, as the responses of reference fish in SW and FW were similar, it is likely that the marked difference between the stressed FW and SW groups can be fully attributed to differences in response to the standardized stress.

Fish held at cooler temperatures generally have a lower stress response than fish at higher temperatures (Strange 1980; Davis and Parker 1990). Additionally, resting levels of cortisol are inversely proportional to temperature (Davis et al. 1984). Temperature is not likely to be responsible for the differences observed between the FW and SW groups. SW American shad (at 18°C) would be expected to have a greater stress response and a lower basal cortisol level than fish in FW (at 14°C). Our results show just the opposite for the stress response, suggesting that if the two experiments were conducted at the same temperature, the differences would have been even greater.

Differences in responsiveness to stress between FW and SW American shad are likely due to developmental and physiological changes associated with downstream migration and SW acclimation. The stress response of salmonids has been demonstrated to be sensitive to developmental stage, particularly during the parr–smolt transformation. Smolts are more responsive to confinement stress than parr (Barton et al. 1985; Carey and McCormick 1998). This sensitivity to stress in smolts has been linked to increased responsiveness of the interrenal to adrenocorticotrophic hormone (ACTH; Young 1986).

The high sensitivity of American shad to stress during the period of seaward migration coincides with impaired osmoregulatory ability (Zydlewski and McCormick 1997a). Shad develop the ability to enter into SW at the larval–juvenile metamorphosis months prior to migration (Zydlewski and McCormick 1997b). During migration, the developmentally and environmentally influenced loss of ion regulatory ability in FW may serve as a proximate cue for migration (Zydlewski and McCormick 1997a). This loss is evidenced by declines in plasma chloride, plasma osmolality, and muscle moisture content (Zydlewski and McCormick 1997a, 1997b). Additionally, if fish remain in FW past the period of migration, hyperosmoregulatory ability further declines and is associated with high mortality (Zydlewski and McCormick 1997a).

Mortality was observed among the stressed FW fish, whereas there was no mortality among the fish stressed in SW. It has been suggested that mortality from confinement stress stems from the exhaustion of metabolic reserves and the buildup of waste products (Barton et al. 1980). It is possible

that the ionic disturbance associated with stress further compromised the fish and led to mortality.

While American shad stressed in FW exhibited a far greater cortisol response than SW-acclimatized fish, the changes in plasma chloride were not correspondingly disparate. In FW fish, plasma chloride concentrations were significantly lower (30%) in the stressed group than in the reference group at 24 h (Figure 1). In SW fish, a 15% increase occurred in the stressed group, and recovery occurred within 24 h. Stress-induced perturbations of plasma chloride in rainbow trout *Oncorhynchus mykiss* and Atlantic salmon *Salmo salar* generally abate within 24 h (Postlethwaite and McDonald 1995; Carey and McCormick 1998). Ion perturbations in both FW and SW fish are likely due to increased blood flow, gill permeability, and respiration during the stress event.

In FW fish, the severity of the ion perturbation following stress may have been masked by the developmental loss of osmoregulatory ability. Nonmigrant shad in FW typically have plasma chloride levels of 110–125 mM (Zydlewski and McCormick 1997a, 1997b). Fish in this study already had a low plasma chloride concentration (basal concentration of 63 mM), perhaps limiting the amount of decrease that was possible following stress. Additionally, the high mortality of fish in FW likely resulted in a ''survivor bias.'' That is, fish that died likely experienced the most severe ion perturbations.

The general trends in hematocrit after stress mirrored the changes in plasma chloride; hematocrit increased in FW fish and decreased in SW fish. Changes in hematocrit have been reported in the first hour following acute stress (Biron and Benfey 1994) or grading and transport (Flos et al. 1988). Increases in hematocrit in FW fish reflect decreased plasma osmotic pressure, which leads to the swelling of red blood cells. Decreased hematocrit in SW fish is likely due to red cell dehydration in response to the increased plasma osmolality. In spite of differences in plasma chloride between the stressed and reference SW fish, hematocrit did not differ significantly between the groups or from basal levels.

The seaward migration of American shad is correlated with declining autumnal temperatures. The peak migration occurs when river temperatures fall to between 16 and 9°C (Leggett and Whitney 1972; O'Leary and Kynard 1986). Based on thermal tolerance alone, downstream migration is required for the survival of American shad spawned in a river that cools to temperatures below 4°C in the winter (Zydlewski and McCormick 1997a). Juvenile shad avoid temperatures below 8°C (Chittenden 1972) and likely prefer the 13–18°C isotherms followed by adult shad in the Atlantic Ocean (Leggett and Whitney 1972). Decreased temperature during migration in FW may impose an increased basal level of stress through ionic disturbance. This may account for the high basal cortisol levels in FW fish. Handling (or any other stressor) may exacerbate this second stress in FW fish. This synergy may contribute to the exceptionally high cortisol response in FW fish and result in poor performance scope as well as mortality.

This study demonstrated a high sensitivity of juvenile American shad to acute stress in FW during the period of their downstream migration. This response is much greater in magnitude than the more typical response observed in SW-acclimatized shad (corresponding to postmigrants). Handling and confinement, particularly in conjunction with delays in migration, are likely to reduce the capacity for migration and predator avoidance. The physiological sensitivity of migrant juvenile shad to acute stress needs to be considered in mitigation efforts (including transport and downstream passage) that include confinement, handling, or other physiological stressors.

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