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Cortisol stimulates hypo-osmoregulatory ability in Atlantic salmon, *Salmo salar* L.

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Hypo-osmoregulatory ability in juvenile Atlantic salmon, *Salmo salar* L., was improved by cortisol treatment. Implantation of a vegetable shortening pellet containing cortisol (50 mg kg^{-1}) resulted in elevated plasma cortisol titres. Maximum cortisol levels (160–170 ng ml^{-1}) were observed at days 6 and 12 after the implantation and dropped significantly by day 55. Cortisol-implanted fish in fresh water developed a twofold increase in gill Na^+/K^+-ATPase activity at days 6 and 12, and a threefold increase by day 55. Intestinal mucosa Na^+/K^+-ATPase activity was not affected by cortisol. Cortisol-implanted fish exposed to 28 ppt sea water for 48 h tended to show an improved ability to regulate their plasma osmolarity and reduce their ionic load. The osmoregulatory ability attained at days 12 and 55 was further evaluated by exposing fish to 37 ppt sea water for 96 h. While all the control fish died relatively early in these tests, cortisol-implanted fish showed a clear reduction in their mortality rate. These results indicate that cortisol can induce biochemical and organismal changes during winter months that typify preadaptive events normally occurring in the spring.

Key words: osmoregulation; adaptation; salinity tolerance; cortisol; Atlantic salmon.

I. INTRODUCTION

Hypo-osmoregulation is physiologically complex in euryhaline teleosts in general, and in anadromous salmonids in particular. In the case of Atlantic salmon, *Salmo salar* L., these physiological adjustments become critical during the transition from freshwater (FW) parr to seawater (SW) adapted smolts (McCormick & Saunders, 1987; Hoar, 1988). This ontogenetic transformation is called smoltification, and occurs during spring. Despite the environmental shift, Atlantic salmon possess, as do other teleosts, mechanisms to maintain the stability of their internal fluid and electrolyte concentrations (Fontaine, 1975; Foskett *et al.*, 1983; Hoar, 1988).

The osmotic gradients between body and environmental fluids are mainly balanced by the Na^+/K^+-ATPase enzymatic pump located in key osmoregulatory tissues like the intestinal mucosa and the gill epithelium (Epstein *et al.*, 1967; Foskett *et al.*, 1983; Collie & Hirano, 1987). These osmoregulatory mechanisms are under neuroendocrine control (see Hoar, 1988). The pioneering work done in eels (Mayer *et al.*, 1967; Hirano & Utida, 1968; Butler & Carmichael, 1972) revealed the importance of cortisol as a SW adapting hormone in teleost fish.

In anadromous salmonids, both smoltification and entry into SW are accompanied by increases in plasma cortisol and the activity of the Na^+/K^+-ATPase pump (Zaugg & McLain, 1972; Specker & Schreck, 1982; Nichols & Weisbart, 1985; Langhorne & Simpson, 1986; McCormick & Saunders, 1987; Thorpe *et al.*, 1987). These correlative observations suggest a role for cortisol in the development of hypo-osmoregulatory functions in salmonids; however,

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exogenous cortisol has unexpected effects on gill Na^+/K^+-ATPase activity *in vivo*, including inhibition in coho salmon, *Oncorhynchus kisutch* (Walbaum) (Redding *et al.*, 1984) and no effect in Atlantic salmon (Langdon *et al.*, 1984). *In vitro* results recently established a direct stimulatory effect of cortisol on Na^+/K^+-ATPase in coho salmon gill tissue (McCormick & Bern, 1989). Furthermore, in a series of recent reports, gill Na^+/K^+-ATPase activity in non-anadromous rainbow trout, *Oncorhynchus mykiss* (Walbaum) (= *Salmo gairdneri* Richardson), and sea trout, *Salmo trutta* L., has been shown to respond positively to injections of cortisol (Madsen, 1990*a–c*).

The present objectives were to deliver a chronic, physiological level of cortisol to intact Atlantic salmon, to measure the response of Na^+/K^+-ATPase activity in both the gill and the intestinal mucosa, and then to evaluate the functional osmoregulatory status achieved by the fish after transfer to SW. The experiment was conducted at a time of year when Atlantic salmon are not naturally euryhaline. A new procedure for elevating cortisol levels in fishes is described. Cortisol is shown for the first time to improve salmon hypo-osmoregulatory ability and to increase gill Na^+/K^+-ATPase activity at physiological doses.

II. MATERIALS AND METHODS

EXPERIMENTAL FISH AND MAINTENANCE

The animals used in this study were 21-month-old juvenile Atlantic salmon (*Salmo salar*) provided by North Attleboro National Fish Hatchery, U.S. Fish and Wildlife Service, Massachusetts. The parental line of these fish belonged to Union River (Maine) breeders, originally descending from the Penobscot River (Maine) stock. After transportation, they were held in indoor facilities at the Department of Zoology, University of Rhode Island (URI), for more than 6 months. While at URI the fish were kept in a circular tank (1·20 m in diameter, total volume = 600 l) provided with flow-through aerated well water (7·1 l min^{-1}) drained from a central outflow at the tank bottom. Fish were fed Zeigler ASD2-30 commercial salmon food once daily (by hand or with an automatic feeder) at a rate of 4% body weight day^{-1}. Water temperature was not controlled, and fluctuated between 5·75 and 11·5° C throughout the entire experiment. Daily temperature variation was less than 1·5° C. Illumination by white fluorescent tubes (approximately 260 lx) was controlled by a timer adjusted weekly to the natural photoperiod.

EXPERIMENTAL DESIGN

The experiment was conducted between November 1989 and February 1990. On 10 December 1989 (day 0) fish weighing an average of 160 g (range, 97–243 g) were divided into two groups. The first group received a cortisol implant (50 mg kg^{-1}) and the second served as a control, implanted with the vehicle alone. Intraperitoneal implants of vegetable shortening (with or without cortisol, volume, 0·5 ml) were performed in the same way as the cocoa butter implant technique previously described by Pickering & Duston (1983). Fish were starved for 36 h before the implantation in order to avoid accidental punctures of their digestive tracts. Pelvic fin clips were used for later identification. Implantation and fin clipping were preceded by anaesthetizing the animals to the point of equilibrium loss (0·14% 2-phenoxyethanol). After implantation, fish were returned to their original tank where they recovered their swimming abilities and resumed feeding within approximately 20 min.

Untreated fish were sampled on days 14 and 7 prior to implantation, as well as on day 0 (before the implantation), to establish the basal levels of the several parameters measured. Animals in the control and cortisol groups were sampled at days 6, 12 and 55 after implantation. Fish were deprived of food 24 h before each sampling to allow gut evacuation in order to reduce faecal accumulation in the SW exposure tanks, and to obtain cleaner

intestinal mucosa samples. After anaesthesia with 2-phenoxyethanol (0·30%), eight randomly selected fish from each group were killed by a blow to the head. The location of the implant was routinely examined in every animal. Fork length and weight were recorded for each fish and blood was drawn into heparinized capillary tubes from severed caudal vasculature. Plasma was immediately separated by centrifugation. After osmolality determinations using a vapor pressure osmometer (Wescor 5500, Logan, UT), plasma samples were stored at −80° C until later analyses of cortisol levels.

Gill filaments were trimmed from the first branchial arch pair of each fish and kept frozen at −80° C in SEI buffer (Zaugg, 1982) for 1 to 2 weeks. On the same animals, the section of the intestine most actively involved in salmonid osmoregulation (from the annulospiral septum to the anus, Collie & Bern, 1982) was opened by a longitudinal incision and rinsed with a strong jet of chilled distilled water to clear any faecal debris. The entire mucosa was then scraped with a glass slide and stored in SEI medium in the same way as gill samples, until assayed for Na^+/K^+-ATPase activity.

A seawater challenge test (Blackburn & Clarke, 1987) was performed at every sampling date after day −7. In this procedure, eight to 10 fish from the same implant group were transferred from FW to 28 ppt SW (Narragansett Bay filtered SW). Temperature in the 190-l test tank was maintained at 9–13° C and the SW was recirculated through an external activated charcoal filter and continuously aerated and filtered by two bottom filters. After 48 h of exposure to these conditions all the fish were killed as described above. Weights and lengths were recorded, and plasma was obtained for osmolality and cortisol determinations and measurement of total sodium and potassium ions on a flame photometer (Perkin Elmer, Coleman 51-Ca, Oak Brook, IL).

At days 12 and 55, 10 cortisol-implanted and 10 control fish were also exposed to a salinity tolerance test (Saunders *et al.*, 1985). The purpose of these tests was to evaluate the ability of the fish to survive in SW by examining the total mortality and mortality rates during a 96 h period after abrupt transfer from FW to 37 ppt SW. The test tank configuration was identical to that in the SW challenge test. Narragansett Bay filtered SW was adjusted to the desired salinity by addition of Instant Ocean salt (Aquarium Systems, Mentor, OH) and monitored with a temperature compensated refractometer (Reichert Scientific Instruments, Buffalo, NY).

CORTISOL RADIOIMMUNOASSAY

Circulating cortisol was measured in unextracted plasma by a radioimmunoassay combining procedures described by Foster & Dunn (1974), Redding *et al.* (1984) and Young (1986). Maximum binding ranged between 42 and 54% when corrected for non-specific binding (NSB). Sensitivity was 0·76 ng ml^{-1}, and NSB was 2·8 to 3·8% of the total activity. Intra- and interassay precision was 6 and 7%, respectively. Recoveries of 7·8, 15·6, 31·3 and 62·5 ng of cortisol added to unextracted plasma were 100, 92, 94 and 83%, respectively. Results from dilution series of Atlantic salmon plasma (5, 10, 20 and 30 µl) defined a line which paralleled the standard curve for the assay.

Na^+/K^+-ATPase ACTIVITY ASSAYS

Differences in tissue characteristics between gill and mucosa samples required slightly different procedures in order to obtain semi-purified enzyme preparations. In the case of the gills, the methodology followed that of Zaugg (1982). Due to the adhesive and gelatinous properties of the mucosa samples, the volume for the first homogenization was tripled (by adding proportional quantities of SEI and distilled water) and 60 strokes during the homogenization applied so that the viscosity of the resulting homogenate was reduced. The pellet obtained from the first centrifugation was resuspended in 400 µl SEID (Zaugg, 1982), and 60 strokes applied again before the second centrifugation.

Ouabain-sensitive Na^+/K^+-ATPase activities in both the gill and intestinal mucosa samples were determined by adjusting the method of Zaugg (1982) as modified by Bradley *et al.* (1989). Inorganic phosphate liberated by enzymatic hydrolysis of 100 µl of 3×10^{-2} M Na_2ATP was determined by the method of Peterson (1978). Absorbances of 200 µl aliquots were read at a wavelength of 700 nm using a microplate reader (MR-300, Dynatech Laboratories Inc., Chantilly, VA). Total protein determination was assessed by a dye binding

TABLE I. Mean body weight, fork length and condition factor [100 (weight length^{-3})] of Atlantic salmon in fresh water

Day	Group	Body weight (g)	Fork length (cm)	Condition factor
−14	Untreated	162·1 ± 17·4	25·3 ± 1·0	0·99 ± 0·05
−7	Untreated	149·3 ± 8·2	24·8 ± 0·6	0·98 ± 0·03
0	Untreated	153·1 ± 13·2	25·0 ± 0·8	0·97 ± 0·03
6	Control	127·9 ± 6·5	23·1 ± 0·6	1·04 ± 0·03
	Cortisol	127·6 ± 8·1	23·5 ± 0·5	0·98 ± 0·03
12	Control	140·3 ± 6·9	23·8 ± 0·4	1·04 ± 0·02
	Cortisol	129·8 ± 9·3	23·5 ± 0·7	0·99 ± 0·03
55	Control	157·5 ± 13·6	24·6 ± 0·6	1·05 ± 0·01
	Cortisol	138·5 ± 9·2	24·0 ± 0·7	1·00 ± 0·03

The sampling schedule is relative to the implantation day (day 0). Values are means of eight individuals (± S.E.M.) in every case.

assay in which BSA was used as reference standard (Bradley *et al.*, 1989). Absorbances were read at 600 nm in the microplate reader. For the gill ATPase assay intra- and interassay coefficients of variation were 6 and 8%, respectively. For the mucosa ATPase assay, both coefficients of variation reached 14%.

STATISTICAL PROCEDURES

Comparisons between control and cortisol group parameters at each sampling date (days 6, 12 and 55), and the temporal progression of those parameters were examined by performing a 2 × 3 factorial analysis of variance (two-way ANOVA). Significance in main effects was followed by a Tukey test, and the significant interactions between sampling date and treatment over any parameter were analysed by a simple effects test, followed by a Tukey test where appropriate (Keppel, 1982). In every statistical test the significance level was set at $\alpha = 0{\cdot}05$. Results of the sampling at days −14, −7 and 0 were not considered in the statistical analyses, yet they were reported due to their importance as indicators of basal conditions prior to experimental manipulations. All values were expressed as arithmetic means and their standard errors.

III. RESULTS

Throughout the entire experiment, no mortalities were observed in FW animals or those exposed to SW challenge tests. Both control and cortisol fish ate avidly, exhibited no jumping behaviour, and appeared healthy at all times. The condition factor was not affected by treatment and did not change over time (Table I).

The vegetable shortening implants resulted in elevated circulating cortisol titres within the physiological range. Recovery following the implantation procedure was complete after a few minutes, and no irritation or haemorrhages resulting from mechanical damage by the implant were observed on the internal organs. Mean plasma cortisol levels in the cortisol-implanted group showed a significant, eight fold increase with respect to control values at days 6 ($F_{(1,42)} = 29{\cdot}89$, $P < 0{\cdot}05$) and 12 ($F_{(1,42)} = 30{\cdot}28$, $P < 0{\cdot}05$) and dropped significantly ($P < 0{\cdot}05$, Tukey test) at day 55 [Fig. 1(a)]. Control values of circulating cortisol, however, did not change with time. It is noteworthy that although the mean cortisol titre of the cortisol-

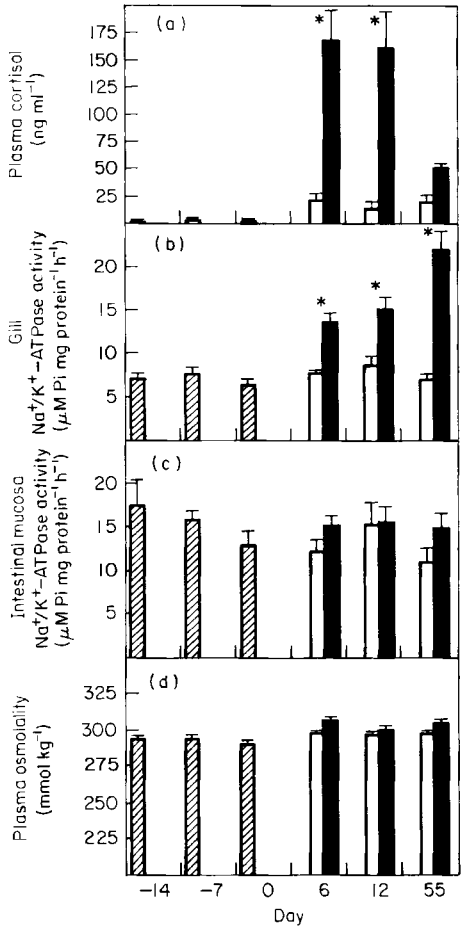

FIG. 1. Plasma parameters and enzymatic activity in untreated (▨), control (□) and cortisol-implanted (■) Atlantic salmon in fresh water. (a) Circulating cortisol; (b) gill Na^+/K^+-ATPase activity; (c) intestinal mucosa Na^+/K^+-ATPase activity; (d) plasma osmolality. The sampling schedule is relative to the implantation day (day 0). Bars represent the arithmetic mean of samples of eight animals ± S.E.M. Asterisks indicate a statistical difference ($P<0.05$) between the control and the cortisol-implanted fish groups at a particular sampling date.

implanted group at day 55 was apparently higher than the control value, this difference did not reach statistical significance [Fig. 1(a)].

Gill Na^+/K^+-ATPase activity was significantly affected by cortisol treatment [Fig. 1(b)]. At days 6 and 12, this enzyme's activity in the cortisol-implanted group approximately doubled over the control values ($F_{(1,42)}=11.24$; $F_{(1,42)}=12.74$, respectively, $P<0.05$). At day 55, the mean gill Na^+/K^+-ATPase activity of the cortisol-implanted group was more than three times that of the control group ($F_{(1,42)}=76.56$, $P<0.05$) and was significantly elevated compared to the previous measurements at days 6 and 12 ($P<0.05$, Tukey test). Gill Na^+/K^+-ATPase activity did not change in the control group and was not different from basal levels prior to experimentation [Fig 1(b)]. Intestinal mucosa Na^+/K^+-ATPase activity [Fig. 1(c)] was unaffected by cortisol treatment.

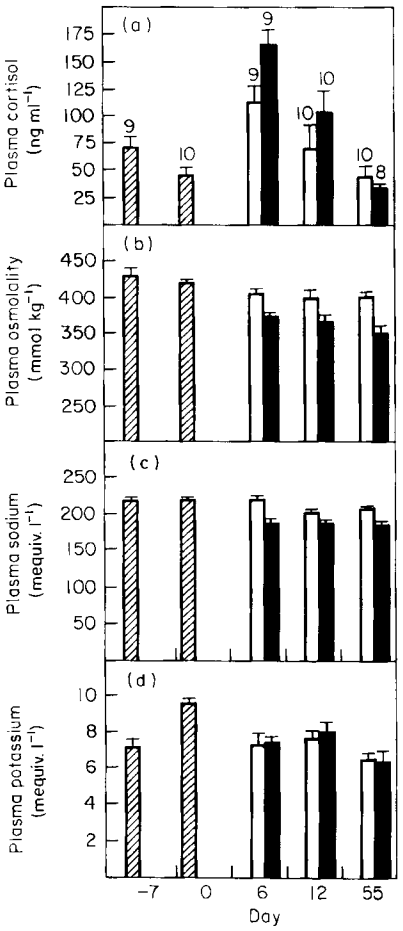

FIG. 2. Plasma parameters in untreated (▨), control (□) and cortisol-implanted (■) fish after exposure to the SW challenge test (28 ppt SW, 48 h). (a) Circulating cortisol; (b) osmolality; (c) sodium content; (d) potassium content. The sampling schedule is relative to the implantation day (day 0). Bars represent the arithmetic mean of sample sizes indicated above each bar ± S.E.M.

The main effect of cortisol administration resulted in a significant elevation in the combined plasma osmolality value for this treatment group ($F_{(1,42)} = 18{\cdot}55$, $P < 0{\cdot}05$) [Fig. 1(d)]. Mean plasma osmolality of fish in FW was maintained at approximately 300 mmol kg^{-1}, regardless of the treatment or the sampling date.

Mean plasma cortisol concentrations in fish exposed to 28 ppt SW for 48 h are presented in Fig. 2(a). Plasma cortisol levels did not show statistical differences between both groups at any single sampling date, although a significant main effect of sampling time resulted from the analysis ($F_{(1,50)} = 20{\cdot}65$, $P < 0{\cdot}05$). A significant difference ($F_{(1,50)} = 25{\cdot}40$, $P < 0{\cdot}05$) existed between the mean plasma osmolalities of both fish groups after SW challenge [Fig. 2(b)], as the result of the main effect of the treatment (i.e. ignoring the time axis). Control plasma osmolalities oscillated around 400 mmol kg^{-1}, whereas a slight decrease from approximately 370 mmol kg^{-1} at day 6 to 350 mmol kg^{-1} at day 55 was observed in the cortisol-implanted group. Despite this apparent tendency of the cortisol-implanted fish to

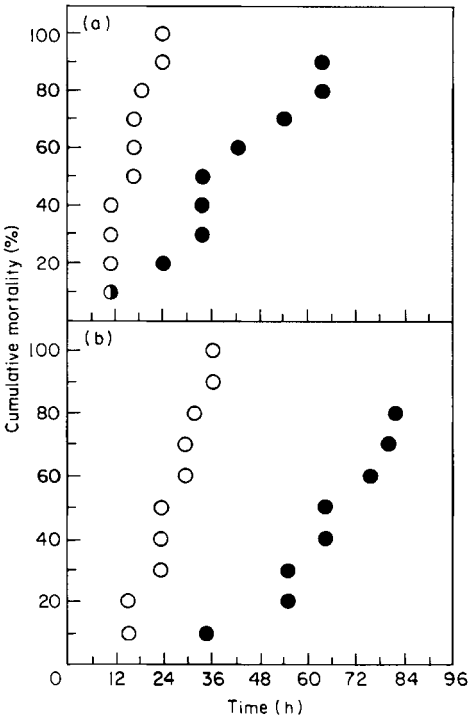

FIG. 3. Cumulative mortality in 10 control (○) and 10 cortisol-implanted (●) Atlantic salmon during exposure to the salinity tolerance test on (a) day 12 and (b) day 55 after implantation.

reduce their plasma osmolality, the mean osmolality of both groups at any particular sampling day was not statistically different. A main effect of the cortisol treatment also resulted in a statistical difference ($F_{(1,50)} = 30{\cdot}23$, $P < 0{\cdot}05$) between the mean plasma sodium levels [Fig. 2(c)] of the control and cortisol-implanted fish after SW challenge. Lower sodium ion concentrations in the cortisol-implanted group resembled the pattern observed in the plasma osmolality of these fish. A main effect of time ($F_{(1,50)} = 4{\cdot}34$, $P < 0{\cdot}05$) was also reflected in the mean plasma potassium concentrations [Fig. 2(d)]. In any case, neither sodium nor potassium levels were affected by a significant interaction between treatment and sampling date.

The salinity tolerance test initiated on day 12 resulted in 100% mortality in the control group during the first 24 h of exposure [Fig. 3(a)]. Cortisol-implanted fish, however, showed a different pattern with mortalities widely distributed over time, reaching 90% upon completion of the test. Similar results were observed on day 55 [Fig. 3(b)], although an overall shift toward increased tolerance times was observed in both groups. In this case, all the control fish died in the first 36 h of the test, whereas the first death for the cortisol-implanted group was not recorded until 34 h. Cumulative mortality of the cortisol-implanted group reached 80% at termination.

IV. DISCUSSION

Implantation of a cortisol-containing pellet in juvenile Atlantic salmon resulted in elevated circulating cortisol over an extended period of time. Previous literature

supports the physiological character of the present cortisol titres (Langhorne & Simpson, 1981; Virtanen & Soivio, 1985). The high titres observed at days 6 and 12 were followed by reduced cortisol levels on day 55. This decrease was probably due to a reduction in the potency of the implant or physiological changes in the cortisol metabolism of the fish. Additionally, cortisol levels of control fish were constant, though higher than in untreated fish. The different titres between control and untreated fish were most likely related to physico-chemical disturbances associated with the presence of the implant.

The present study establishes that cortisol stimulates gill Na^+/K^+-ATPase activity *in vivo* in juvenile Atlantic salmon. This finding is similar to those previously described in eels, *Anguilla* spp. (Epstein *et al.*, 1971; Forrest *et al.*, 1973), in killifish, *Fundulus heteroclitus* L. (Pickford *et al.*, 1970), in rainbow trout, *Oncorhynchus mykiss* (Madsen, 1990*a*, *c*), in sea trout parr, *Salmo trutta* (Madsen, 1990*b*), and in pre-smolting coho salmon, *Oncorhynchus kisutch* (Richman *et al.*, 1985; Richman & Zaugg, 1987).

Abundant information exists concerning the natural activation of gill Na^+/K^+-ATPase during Atlantic salmon smoltification. In general, the reported magnitude for the activity peak ranges between three and ten times the basal (parr) enzyme activities (Boeuf & Prunet, 1985; Boeuf *et al.*, 1985; Virtanen & Soivio, 1985; Langhorne & Simpson, 1986; McCormick *et al.*, 1987; Bradley *et al.*, 1989). In the present study, the gill Na^+/K^+-ATPase activation fell within the lower end of this range. This fact, together with the results from the salinity tolerance tests and the additional osmotic and ionic evidence, indicated that the gill Na^+/K^+-ATPase activity was stimulated, but not maximally, by the cortisol treatment. Moreover, cortisol treatment triggered the activation of gill Na^+/K^+-ATPase, but did not seem to be required for the long term maintenance of this activation. Despite the decline of cortisol titres noted at day 55, gill Na^+/K^+-ATPase activity continued to increase. The same pattern had been previously observed in Atlantic salmon undergoing smoltification (Langhorne & Simpson, 1986).

The increase in enzymatic activity at the gills developed in FW, after 6 days of cortisol treatment. This time frame is comparable to that reported in previous investigations (Richman & Zaugg, 1987; McCormick & Bern, 1989; Madsen, 1990*a–c*). It also indicates the independence of the initial activation of the Na^+/K^+-ATPase from external changes in salinity and supports the notion that gill Na^+/K^+-ATPase activation in salmonids occurs as a preparation for—and not a consequence of—life in SW (Langhorne & Simpson, 1986; McCormick & Saunders, 1987).

The whole-animal response to the salinity tolerance test provides a practical measure of the osmoregulatory status resulting from the cortisol treatment. The exposure to this high salinity is regarded as an indicator of effective SW readiness and adaptation to SW (Saunders *et al.*, 1985). It has been demonstrated in previous experiments that fish can show a short-term tolerance to salinities lower than 30 ppt but begin to die in a few weeks when transfered to full-strength SW (McCormick & Saunders, 1987). Higher salinity tolerance was evident in the cortisol-implanted fish. The poor osmoregulatory performance of the control fish when put in SW suggests that a cortisol rise after SW entry is not enough to enable survival in this environment. Although the control and cortisol-implanted groups never showed 100% survival, the mortality rate was clearly reduced by cortisol

treatment. Aside from the treatment effect, a general improvement in salinity tolerance was also observed over time. Both groups of fish tested at day 55 had improved salinity tolerance. The larger size of the fish (McCormick & Saunders, 1987) and the relative time of the year might account for this progress with respect to the ability observed at day 12.

Exposure to the SW challenge conditions resulted in less pronounced cortisol increases as the experiment progressed. This probably resulted from a combination of at least two factors. First, a reduction in the amount of cortisol being released from the implants might have resulted in desaturation, and consequent improvement, of the clearance mechanisms of cortisol-implanted fish. Second, a reduction in the endogenous cortisol production in both fish groups might have occurred after SW transfer. Sampling on day 55 was performed early in February when the fish were not only larger in size, but also closer to the upcoming smoltification season than individuals sampled at the beginning of the experiment and, thus, presumably more tolerant to SW osmotic stresses (McCormick & Saunders, 1987). Plasma osmolality and sodium concentration were highly correlated and their magnitudes in each fish group showed an overall ability of the cortisol-implanted fish to approach the plasma electrolyte and osmotic regulatory ability characteristic of fully SW adapted fish (Stagg *et al.*, 1989). In contrast, observations on plasma osmolality and electrolyte concentrations in the control group indicate the difficulties of these fish in maintaining a homeostatic balance. Plasma osmolality and ionic composition of fish exposed to SW are frequently reported as indicators of organismal adaptability to this medium (Blackburn & Clarke, 1987). Thus, osmolality data further support the suggestion that cortisol-implanted fish had improved hypo-osmoregulatory ability.

Unlike Na^+/K^+-ATPase activity in the branchial tissue, the activity of this enzyme in the intestinal mucosa was unaffected by cortisol treatment. This outcome conflicts with available evidence showing a stimulation of both gill and intestinal mucosa Na^+/K^+-ATPase by cortisol treatment in rainbow trout (Madsen, 1990*a*). Differential responsiveness of the same enzyme, located in separate tissues, to a unique cortisol dose (Richman *et al.*, 1987) is a possible explanation for the present results. Comparisons between Na^+/K^+-ATPase activities at the gills and the gut could not be made since the characteristics of the assay for branchial and intestinal enzyme activity were slightly different.

In summary, cortisol has been demonstrated to elicit a clear stimulation of some physiological mechanisms necessary for life of Atlantic salmon in SW. However, this appeared to be a partial stimulation indicating that the interaction of additional factors, or modifications of the present treatment, should be explored. Future investigations including a dose–response analysis and a time series for treatment duration, will help determine the optimal cortisol dose and treatment duration necessary to enhance SW survival.

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