THE UNIVERSITY OF MANITOBA

THE EFFECTS OF LOW AMBIENT pH AND ALUMINUM ON INTERRENAL AND THYROIDAL FUNCTIONS AND THEIR INTERRELATIONSHIP IN RAINBOW TROUT (*Oncorhynchus mykiss*)

by

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ABSTRACT

Exposure to low pH (H_2SO_4, pH 6.0-4.2) and 0, 3, 5, 10 or 20 µmol/L labile aluminum fluoride (AlF) in both hard and soft water increased plasma cortisol and glucose while depressing plasma ions (Na^+, Cl^-) in rainbow trout, *Oncorhynchus mykiss* (or *Salmo gairdneri*). Depending upon the experiment, low pH induced either a decrease in plasma 3,5,3'-triiodo-L-thyronine (T_3) or an elevation in plasma L-thyroxine (T_4). High AlF (>5 µmol/L) in low pH water exacerbated increases in plasma cortisol and glucose and losses of plasma ions. This combination consistently lowered plasma T_3 and tended to elevate plasma T_4. In contrast, AlF (≤5 µmol/L) in low pH water returned plasma values towards control levels.

Factors (cannulation, diurnal change, serial sampling and vehicle infusion) which might modify hormone kinetics were evaluated. A protocol was established to assess cortisol plasma clearance rate (PCR) or plasma degradation rate (PDR) by infusion of labelled cortisol to constant plasma specific activity in cannulated fish exposed to low ambient pH and/or Al.

Sublethal pH (4.7-5.0) increased the cortisol PDR but did not change the PCR; it did not modify T_3 and T_4 kinetics. Addition of Al intensified the cortisol PCR and PDR, increased the T_4 PDR, but lowered the T_3 PCR and T_3 plasma appearance rate (PAR). This depressed T_3 production may contribute to the poor growth reported for Al-exposed fish.

To examine whether the effects of low pH/Al were due to a hypothesized antagonistic interaction between interrenal and thyroid functions, T_3 metabolism was studied in trout implanted with cortisol. Chronic physiologic cortisol treatment enhanced T_3 PCR, resulting in a decline in T_3 level in both plasma and muscle compartments as measured

by HPLC.

In summary, the responses of the interrenal and thyroid systems to low ambient pH and aluminum were quantified in terms of plasma concentrations and kinetics of cortisol, T_3 and T_4. Differences between the response of T_3 to low pH/Al and its response to cortisol implants suggest that increased cortisol levels are not responsible for depressed T_3 activity.

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Finally, I dedicate this work to my wife, Katherine, and to my sons, Geoffrey, Kyle and Tyler.

ABBREVIATIONS

AlF = total aluminum fluoride species

C = the steady-state concentration of labelled plasma hormone

CR50% = coefficient of reactivity at 50% displacement

HPI = hypothalamo-pituiutary-interrenal axis

HPT = hypothalamo-pituiutary-thyroidal axis

I = rate of infusion of labelled hormone

5'-MD = 5'-mondeiodinase

PAR = plasma appearance rate

PC = plasma concentration

PCR = plasma clearance rate

PCV = packed cell volume

PDR = plasma degradation rate

T_3 = 3,5,3'-triiodo-L-thyronine

T_4 = L-thyroxine

TMS = tricaine methanesulphonate

UOF = unidentified osmotic fraction

V_p = plasma volume

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GENERAL INTRODUCTION

Among their many effects on functions in vertebrates, corticosteroids increase synthesis of a number of key enzymes in the gluconeogenic pathway and have a basic anabolic action in the liver (Hadley 1984). Corticosteroid actions on skeletal muscle and adipose tissue are catabolic and glucose uptake is inhibited. Furthermore, cortisol exhibits known hydromineral effects in teleost fish (Fortner and Pickford 1982) and has been linked to chloride cell proliferation in gills in low Ca^{2+} water (Perry and Wood 1985) and to enhanced whole body Ca^{2+} uptake (Flik and Perry, 1989).

Thyroid hormones together with other hormones promote growth and development, and are generally associated with protein anabolism in fish (Eales and MacLatchy 1989). Thus metabolic effects of these two endocrine systems are opposite and potentially antagonistic.

Under stressful conditions in vertebrates there is increased secretion of corticosteroids and a shift of metabolism from an anabolic to a catabolic state (Bentley 1982). Therefore it reasonably follows that there may be a significant modulatory effect of the interrenal system on the thyroid system. It is probably not advantageous to shunt energy and substrates towards growth and development when large amounts are required to maintain homeostasis and tissue repair. To some extent the antagonistic effects of interrenal activity on aspects of thyroid activity have been demonstrated in birds (Williamson and Davison 1987) and mammals (Cavalieri et al. 1984). However, this interaction has received only limited attention in fish (Eales 1979a; Redding et al. 1984, 1986).

Furthermore there has been no study that has extensively investigated the two endocrine systems simultaneously while fish are exposed to one or more environmental stressors.

Anthropogenic emissions which deposit mineral acids and reduce the pH of our surface waters represent a continuing problem of current concern (Sandén et al. 1987; Watt 1987). Depletion of fish populations from natural waters due to acidity has been attributed mainly to sublethal effects involving recruitment failure (Mills et al. 1987) and mortality of eggs and fry (Peterson et al. 1982). Nevertheless, fish kills have been reported following periods of heavy rainfall and during snowmelt (Farmer et al. 1980; Hendriksen et al. 1984; Hesthagen 1986). Therefore, the ability of fish to withstand and adapt to acute and chronic pH reductions is necessary for their survival.

A secondary consequence of low pH involves mobilization of aluminum from the catchment soils (Cronan and Schofield 1979; LaZerte 1984). Speciation of aqueous aluminum is controlled by environmental factors which include temperature and the availability of various ligands. Organically complexed aluminum and polymeric aluminum appear essentially nontoxic whereas inorganic aluminum (Al) represents the most toxic form (Driscoll et al. 1980). Of the inorganic ligands (OH^-, SO_4^{2-} and F^-) fluoride species represent the major form of labile aluminum found in nature (Driscoll 1984; LaZerte 1984; Harvey and McArdle 1986). However, the toxic effects of aluminum fluoride (AlF) has received only cursory attention (Baker and Schofield 1982; Neville and Campbell 1988).

If sufficiently severe, low ambient pH and Al cause electrolyte loss and acid-base disturbances in fish which can be fatal (Neville 1985; Wood

and McDonald 1987). The sublethal effects of acid and Al are less obvious but compromise ionoregulatory ability (Leivestad 1982; Harvey and McArdle 1986; Neville and Campbell 1988) and impair other energy-dependent processes such as growth (Sadler and Lynam 1987; Tam et al. 1988; Mount et al. 1988) and reproduction (Freeman and Sangalang 1985; Weiner et al. 1986; Mount et al. 1988).

Studies examining factors that may provide connections between the increased energy required for ion balance and impaired fish performance in terms of growth and reproduction are few and have been stimulated largely by the work presented here. Before publication of PART 1 (Brown et al. 1984), it remained to be demonstrated that chronic acid stress activated the hypothalamo-pituitary-interrenal (HPI) axis of fish. In one of the few studies conducted, serum cortisol of rainbow trout, *Oncorhynchus mykiss* (or *Salmo gairdneri*), exposed to low pH (4.2) did not differ from that of controls (Lee et al. 1983). Earlier studies in brook trout, *Salvelinus fontinalis*, documented only transitory (<3 d) increases in cortisol following acute exposure to near-lethal pH reductions (4.0) (Mudge et al. 1977; Ashcom 1979).

The effects of stress, in any form, on other endocrine systems of fish have received far less attention (Pickering 1981, Pickering 1989). However, there are indications that the thyroidal system of salmonids may respond to both acute and chronic stress (Osborn and Simpson 1974; Simpson 1976; Brown et al. 1978; Leatherland and Sonstegard 1978; Eales 1979a). Because adequate thyroidal status seems to be required for normal growth (Eales 1979a; Leatherland 1982; Higgs et al. 1982) and in the early stages of female reproduction (Sage 1973; Cyr et al. 1988), low

ambient pH and/or Al could impair these performance factors in fish by altering thyroid function.

Thus, the general objective of this study was to examine the effects of low ambient pH in the presence and absence of aluminum on the interrenal and thyroid systems of rainbow trout. I asked two specific questions:

i) To what extent are the interrenal and thyroid systems influenced by exposure to low pH and aluminum?

ii) Are the changes in thyroid function due to increased cortisol levels, or due to other effects of low pH and aluminum?

In studying the above questions the activity status of the interrenal and thyroid system were evaluated by measuring both plasma levels and kinetics of plasma hormone turnover. I measured plasma cortisol, 3,5,3'-triiodo-L-thyronine (T_3) and L-thyroxine (T_4) levels because they could provide an easy, rapid and economical means of assessing endocrine responsiveness under the various test conditions (PARTS 1 and 2). Because plasma levels represent a static measurement, kinetic studies were undertaken to more accurately describe hormone utilization. Additionally, the combination of plasma levels and clearance rate provides an estimate of overall hormone production or appearance in plasma under steady state conditions. However, because the HPI and hypothalamo-pituitary-thyroid axes are sensitive to the effects of handling and manipulation (Brown et al. 1978; Pickering 1981; Schreck 1981) development of methodologies suitable to assess cortisol and thyroid hormone kinetics were necessary (PARTS 3 and 4). To address the second question, I focused on T_3 metabolism (PART 5) because it was altered by

exposure to low pH and Al (PART 4) and T_3 represents the hormone form active at the receptor level (Bres and Eales 1986).

In conjunction with the endocrine responses, I monitored some "secondary" stress indicators (Mazeaud et al. 1977). Plasma ions were measured to place the endocrine studies in context with biochemical responses generally considered indicative of acid and/or aluminum induced stress in salmonids (Wood and McDonald 1987). Glucose was measured because corticosteroids may be involved in its metabolism. Previous studies have shown that glucose elevation was a dependable response in cortisol-injected teleosts (Chan and Woo 1978; Leach and Taylor 1982). Furthermore, increases in plasma glucose also represent a consistent response to numerous other environmental stressors (Wedemeyer and McLeay 1981).

In brief, I wished to evaluate the influence that the primarily catabolic interrenal hormones may exert on the primarily anabolic thyroid hormones under stress conditions (low pH and Al). This was achieved, in part, by observing the responses of both endocrine systems to acid and aluminum and, in part, by observing the response of the thyroid to experimentally elevated plasma cortisol levels comparable to those created under stressful conditions. These data will aid in understanding the roles these two endocrine systems play in salmonids subjected to chronically stressed conditions. I used rainbow trout for the following reasons: (i) it represents a species which is known to be sensitive to the effects of low pH; (ii) the effect of low pH on its ionoregulatory capability has been partly described; and (iii) fish of similar size and the strain (Sundalasora) were always available.

PART 1
INTERRENAL, THYROIDAL, AND CARBOHYDRATE RESPONSES TO ENVIRONMENTAL ACIDIFICATION [1]

1.1 SYNOPSIS

Exposure to acid-treated water (H_2SO_4, pH 6.0-4.2) for 21 d altered interrenal and thyroid function in trout. Below pH 5.2 plasma cortisol increased, implying sustained interrenal cortisol release. Eight days of acid exposure (pH 4.7) were required to raise plasma cortisol significantly. Below pH 4.7 the ratio of plasma T_4 to T_3 tended to increase in relation to the controls. Depending upon the experiment this was due to either a significant elevation in plasma T_4 or a decrease in plasma T_3. Eight days of acid exposure (pH 4.7) were required to depress plasma T_3. Preceding higher interrenal activity, plasma glucose was elevated in acid-treated fish (pH < 5.2) after 4 d of exposure. The possible relationships of endocrine changes to accompanying acid-induced elevations in plasma glucose and protein are considered and their possible value as indices of acid stress discussed.

[1] Previously published (Brown et al. 1984).

1.2 INTRODUCTION

The physiological problems encountered by freshwater fish in acidic waters have been the subject of numerous recent investigations. Disturbances in acid-base balance and ionic-osmotic regulation have been implicated as key toxic mechanisms (McWilliams and Potts 1978; Neville 1979 a,b; Packer 1979; McWilliams 1980; Graham and Wood 1981; McDonald and Wood 1981; Booth et al. 1982). Limited research has been conducted on other physiological factors affected by acidification.

Stressful stimuli rapidly activate the HPI axis and adrenergic system which mediate changes in carbohydrate and hydromineral balance (Mazeaud et al. 1977), but it has not been demonstrated that long-term acid stress activates the HPI axis. The effects of stress on other endocrine systems of fish have received far less attention (Pickering 1981). However, there are indications that the thyroidal system may respond to both acute and chronic stress (see GENERAL INTRODUCTION). Low ambient pH could influence fish detrimentally by altering thyroid function. Adequate thyroidal status seems to be required in fish for normal growth and reproduction (Eales 1979a) and for maintenance of normal blood Na^+ concentration and osmotic balance (Knoeppel et al. 1982).

The objective has been to define changes in plasma cortisol and thyroid hormones during 21 d of acid treatment (pH range 4.2-6.0). Plasma glucose levels were monitored also because elevated blood glucose in *Fundulus heteroclitus* appears to be mediated, in part, by increases in plasma cortisol (Leach and Taylor 1980, 1982) and glucose is a frequently used indicator of stress. Further understanding of biochemical stress

responses in fish exposed to acid and the degree to which they adapt could provide important biological indicators of acidification. These indicators are relevant when considering the limits of change to the environment that can be allowed.

1.3 MATERIALS AND METHODS

1.3.1 Experimental Animals

Rainbow trout (2+ yrs; weight, 198 ± 3 g; fork length 25.3 ± 0.1 cm, $\hat{x}$ ± SE, N = 330) were obtained from the Rockwood Hatchery, Freshwater Institute. Fish were held in 500-L fibreglass tanks with flowing, aerated, dechlorinated Winnipeg city water which contained the following major ion concentrations: 0.51 mmol Ca^{2+}/L, 0.24 mmol Mg^{2+}/L, 0.14 mmol Cl^-/L, 0.08 mmol Na^+/L, and 0.04 mmol K^+/L. Hardness was equivalent to 0.75 mmol $CaCO_3$/L (75 mg/L). The average pH of the water was 7.7 (95% confidence interval, 7.64-7.80). Dissolved inorganic carbon measured by gas chromatography (Stainton et al. 1974) was 1.43 ± 0.04 mmol/L ($\hat{x}$ ± SE).

1.3.2 Toxicity Testing

Fibreglass tanks (125 L) or glass aquaria (70 L) were used to expose the fish to H_2SO_4 in groups of 7-12 per tank. All tanks had translucent white plexiglass tops and the sides of the aquaria were covered with black polyethylene. Each tank received at least 2 L of aerated water per gram of fish each day. Test water was obtained by an acidification-decarbonation system consisting of a Radiometer titrator (model TTT2 or TTT80) and a Bach-Simpson six-channel automatic switch.

In addition, the pH of the test tanks was continuously monitored by a titrator or a titrator and a six-channel switch. Instrument calibrations were checked 3 times daily against a standardized Radiometer pH meter. Radiometer magnetic valves or Masterflex pumps with Radiometer magnetic relays were used to add acid. No metal components were in contact with the test water. The CO_2 produced by acidification was removed by an air stripping procedure so that CO_2 averaged 44.2 ± 2.2 µmol/L ($\hat{x}$ ± SE, N = 20) in the test tanks containing fish. Trout were acclimated to the test tanks at pH 7.7 for 10 to 14 d prior to testing. To avoid a large CO_2 surge at the onset of each experiment, acidified water (pH 5.0), stripped of excess CO_2 and titrated to pH 7.7 with NaOH, was supplied to each test tank until 99% replacement of the water volume was achieved. Then the tank was acidified to its nominal pH. The entire process was complete in 6 to 8 h. During this time the free CO_2 was monitored at 60-min intervals. As the water in the tank was replaced with CO_2 stripped water at pH 7.7, the mean free CO_2 decreased from 57 µmol/L to 19 µmol/L (N = 20). When the water was reacidified the average free CO_2 increased to 44 µmol/L. Temperature ranged seasonally from 11.5 to 14.1 °C but during any one experiment varied less than 1 °C. Experiments were conducted between April and September and the photoperiod was adjusted to 14 h light and 10 h darkness. Fish were fed Martin Feeds trout food (Elmira, Ontario) at a ration of 1% wet body weight every second day. All fish were starved for 4 d prior to each sampling.

The effects of acid on trout were investigated in 3 experiments. In Expt 1, fish were exposed to 5 different pH levels (7.7, 6.0, 5.2, 4.7 and 4.2) for 21 d prior to sampling. The experiment was repeated and the

combined results are presented. The time-course of the response was examined in Expt 2 at pH 4.7 and in Expt 3 at pH 5.2, with sampling at 1, 2, 4, 8, 16 and 21 d of exposure. For Expts 1 and 2, trout from a control tank (pH 7.7) and a tank containing acidified water which had been titrated to pH 7.7 with NaOH were compared at 0 and 21 d. Because no difference could be found for any parameter examined, the data from fish held in acidified water titrated to pH 7.7 are not given.

1.3.3 Blood Sampling and Analysis

To avoid possible netting and sampling effects on plasma cortisol levels (Pickering et al. 1982), trout were anesthetized directly in the test tanks by adding tricaine methanesulphonate (TMS, 0.76 mmol/L) solution neutralized to tank pH with ammonium hydroxide (Barton et al. 1980). Immobilization was complete within 30 s. Blood was removed from the caudal vessels in 1 to 2 min using preheparinized 3- or 5-mL syringes and 18 gauge needles. Plasma was immediately separated by centrifugation and stored at -20 °C in polyethylene vials. To minimize potential diurnal effects, samples were taken between 1300 and 1500 h each day. Samples were usually analyzed within 10 d.

Radioimmunoassays for T_3 and T_4 followed the procedures of Brown and Eales (1977). The cortisol radioimmunoassay was done with commercially available kits (Micromedic Systems, Inc., Horsham, PA). Although there was a parallelism between cortisol standards and dilutions of trout plasma to 15 nmol/L, lower dilutions diverged from the standard curve. In addition, the antibody bound fraction from several trout samples contained greater cortisol-3-(*O*-carboxymethyl)oximino-(2-

[^{125}I]iodohistamine) counts than the 0 level standard. These problems were corrected by preparing standards with rainbow trout plasma which had the endogenous steroids removed by incubation with charcoal (Mitsuma et al. 1972; Abraham et al. 1977). The assay protocol used 30 µL of a 1:10 dilution of plasma for measurement. This volume could be increased to 100 µL, giving a five-fold increase in sensitivity without altering other assay characteristics. Furthermore, assays were more reproducible and sensitive if the antibody equilibrium step was carried out at 4 °C for 16 h. The coefficient of reactivity at 50% displacement (CR50%) of labelled cortisol was determined for each of 14 steroids (Abraham 1975). Steroids giving greater than 0.1 CR% with the cortisol antibody were: prednisolone (31.0), 11-deoxycortisol (28.0), 11-deoxy-7-hydroxycorticosterone (22.0), 17-hydroxyprogestrone (4.6), cortisone (0.8), prednisone (0.6), corticosterone (0.2) and 17-hydroxypregnenolone (0.1). Intraassay coefficient of variation (CV), from 10 duplicate analysis of the same sample was 5.4%. Interassay CV of duplicate analysis from 10 assays was 9.1%. Recoveries of cortisol (3-15 nmol/L) added to rainbow trout plasma ranged from 93.5 to 112.0%. The minimum level of sensitivity, defined as that dose level 2 standard deviations away from the 0 dose measurement, averaged 3 nmol/L over 10 assays.

Plasma glucose concentrations were determined employing a Sigma test kit (No. 510) which is based on use of glucose oxidase and peroxidase with o-dianisidine as chromogen. The intraassay CV equalled 5.0% from 10 duplicate measurements on the same sample and interassay CV in duplicate analyses from 10 assays was 7.9%. The minimum detectable level of glucose was 0.1 mmol/L and recovery of added glucose (0.14-5.55 mmol/L)

ranged from 92 to 96%. Plasma protein was determined according to the method of Lowry et al. (1951) using crystalline bovine serum albumin as standard. Intraassay and interassay CV were 7.1 and 8.4% respectively.

1.3.4 Data Analysis

Differences between groups of fish and experiments for any given parameter were tested by one-way or two-way analysis of variance (ANOVA) computed using the Statistical Analysis System (SAS Institute Inc. 1982). Where appropriate, Duncan's multiple range test was used to distinguish differences between means from more than two groups of fish. A probability level of <0.05 was considered significant. Bartlett's test (Snedecor and Cochran 1971) was applied to test for homogeneity of variance and, where necessary, data were log transformed to obtain more uniform variances. However, for clarity of presentation arithmetic means with standard errors have been used in tables and figures. The Pearson product moment correlation was used to test the relationships between cortisol, T_4, T_3 and glucose to different pH levels or times of exposure to a particular pH.

1.4 RESULTS AND DISCUSSION

1.4.1 Mortality

Loss of equilibrium and deaths began after 7 d exposure to pH 4.2 and after 18 d exposure to pH 4.7 or 5.2. In agreement with Giles et al. (1984), 50% mortality occurred after 21 d at pH 4.2 (Table 1.1) in moderately hard water. No trout died at pH 6.0 or 7.7. The most notable

behavioral alteration of fish held at pH 4.2 or 4.7 was their reluctance to feed after 7 to 10 d.

1.4.2 Cortisol

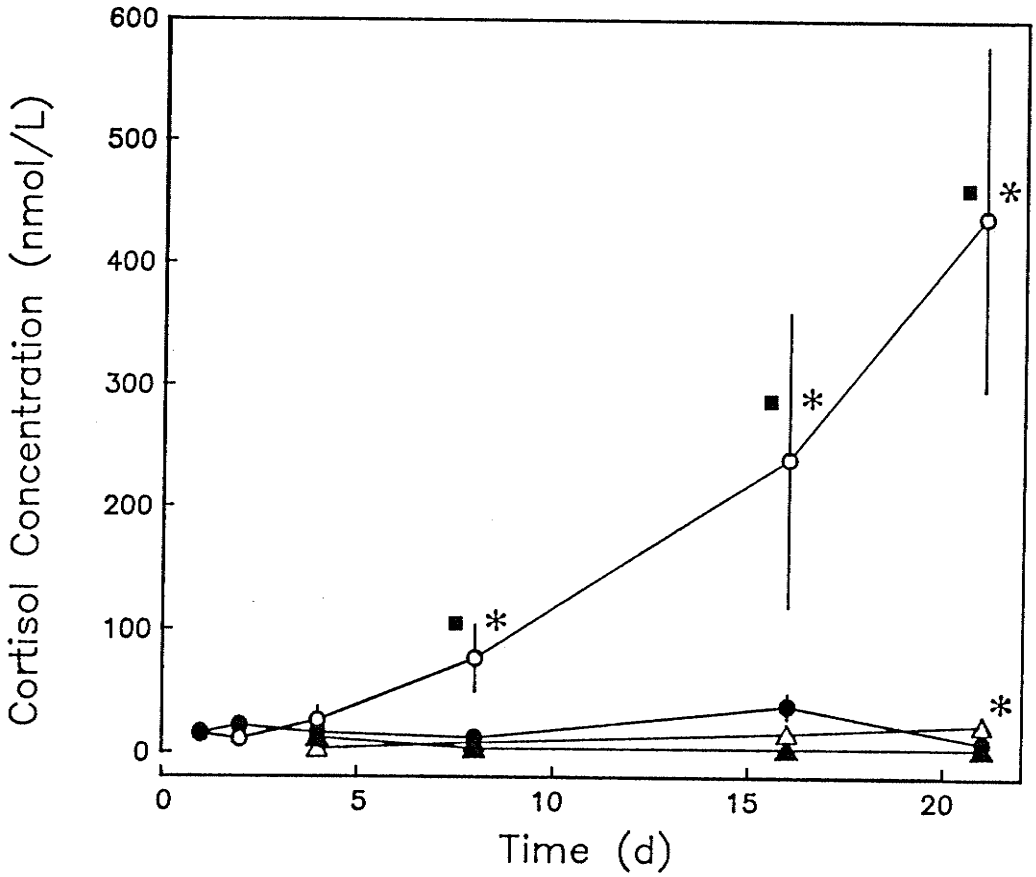
Plasma cortisol in undisturbed control fish was generally less than 30 nmol/L, a result in agreement with those described for rainbow trout (McBride et al. 1975, 1979; Barton et al. 1980; Swift 1981 a,b, 1982; Barton and Peter 1982). This value was lower than reported from earlier studies on rainbow trout (Hill and Fromm 1968; Grant and Mehrle 1973; Sivarajah et al. 1978) and on brook trout (Mudge et al. 1977).

When trout were exposed to different pH levels for 21 d cortisol levels increased in a dose-dependent fashion (Table 1.1). The threshold occurred between pH 6.0 and 5.2. At pH 4.7 cortisol concentrations became significantly higher at 8 d, and continued to rise over the course of the experiment (21 d) (Fig. 1.1). When trout were exposed to pH 5.2 blood cortisol levels were significantly elevated over control values only after 21 d. The magnitude of the response was approximately 10% of that found in trout at pH 4.7. In contrast, plasma cortisol in acid-stressed rainbow trout (14-21 d, pH 7.0-4.2) from a previous study (Lee et al. 1983) did not differ significantly from controls. Also Lockhart and Lutz (1977) found no difference in circulating cortisol levels between white sucker, *Catostomus commersoni*, captured from acidified (pH 4.8-5.3) George Lake and those captured from a non-acidified control area. However, it must be recognized that long-term adaptation or selection may occur in natural populations.

TABLE 1.1 Effects of pH (95% confidence interval) on mortality and plasma cortisol, glucose, protein, T_4, T_3 and T_4/T_3 in rainbow trout exposed for 21 d. Values represent mean (SE) of 9-18 fish. Asterisk indicates significant difference from the corresponding value at pH 7.6-7.8 (P <0.05).

pH interval	Mortality (%)	Cortisol (nmol/L)	Glucose (mmol/L)	Protein (g/L)	T_4 (nmol/L)	T_3 (nmol/L)	T_4/T_3
4.19-4.23	50	228 * (57)	13.5 * (2.7)	32.7 * (3.3)	2.5 * (0.6)	1.6 (0.3)	2.4 * (0.7)
4.65-4.74	33	168 * (63)	12.1 * (2.6)	29.0 * (2.6)	1.7 (0.4)	1.8 (0.2)	1.6 * (0.4)
5.17-5.25	11	21 * (9)	9.2 * (2.3)	26.7 (2.8)	0.8 (0.1)	2.1 (0.3)	0.6 (0.1)
5.98-6.04	0	8 (5)	4.7 (0.2)	24.8 (1.6)	0.9 (0.1)	1.8 (0.3)	0.8 (0.2)
7.64-7.84	0	4 (5)	4.0 (0.2)	25.4 (1.9)	1.4 (0.2)	2.3 (0.3)	0.9 (0.2)

FIGURE 1.1. Temporal changes of plasma cortisol concentrations in trout exposed to pH 4.7 (○) and pH 5.2 (△). Control fish at pH 7.7 were examined at each time (●,▲). Points represent mean ± SE of 7-12 fish. Means significantly different from control are indicated (*). Values significantly different from those at day 1 are indicated (■).


600
500
400
300
200
100
0
Cortisol Concentration (nmol/L)
0
5
10
15
20
Time (d)
*
*
*

Blood cortisol levels in brook trout acutely exposed to acid (pH 4.0) for 3 to 24 h showed transitory increases (Mudge et al. 1977). In the present study cortisol may have been acutely elevated upon initial exposure to the acid, but returned to a basal values by the first sampling time at 24 h. The possibility that starvation stress may have contributed to the increased plasma cortisol caused by low pH seems unlikely. Milne et al. (1979) did not observe differences in plasma cortisol levels even after 65 d starvation. In addition, cannulated trout which were starved for 21 d after recovery from cannulation procedures showed no change in cortisol levels (PART 3).

A physiological stress response such as plasma cortisol is difficult to interpret because it may represent a general response to several stressors (Thomas et al. 1981). However, plasma cortisol levels may provide a useful indicator of the general well-being of fish (Tomasso et al. 1981). In support of this, unusually high cortisol levels have been observed in dying fish (Fagerlund 1967; Strange et al. 1977). The use of plasma cortisol levels may be particularly appropriate where fish are exposed to two or more sublethal stressors (e.g. acid and heavy metals or elevated CO_2) as the response represents the integrated effect of the components (Donaldson 1981).

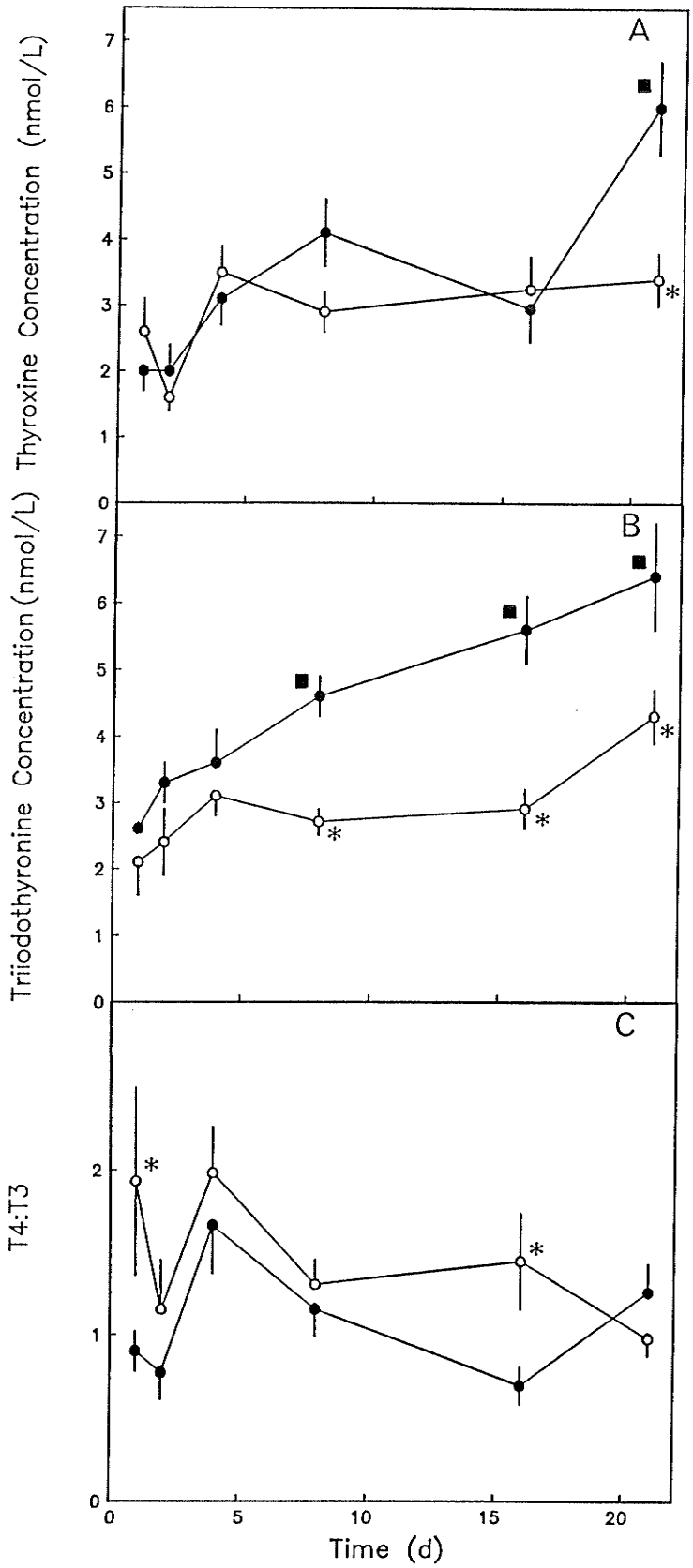
1.4.3 T_3 and T_4

Plasma T_3 levels were not significantly altered by pH in Expt 1. Three weeks exposure to pH 4.2 and 4.7 elevated plasma T_4 levels and T_4/T_3 ratio (Table 1.1). Plasma T_4 levels (Expt 1) were positively correlated with plasma cortisol levels ($r = 0.467$, $N = 47$). In Expt 2.

plasma T_3 levels rose steadily, from 2.6 to 6.4 nmol/L at pH 7.7 (Fig. 1.2B), while acid-exposed fish failed to show such an increase and had significantly lower T_3 levels which did not rise significantly until 21 d of exposure. The time-exposure at pH 4.7 (Expt 2) showed contrasting findings to Expt 1, because acid exposed trout did not demonstrate significant elevations in plasma T_4 (Fig. 1.2A), control fish had increased T_4 levels at 21 d, and T_4 levels were not correlated with cortisol levels. No changes were evident for either plasma T_3 or T_4 or their ratios at pH 5.2 (Expt 3, data not shown). For all experiments, plasma T_3 and T_4 were significantly correlated with plasma protein in both acid-exposed (T_3, r = 0.260, N = 151; T_4, r = 0.441, N = 152) and control (T_3, r = 0.486, N = 124; T_4, r = 0.584, N = 124) trout.

The reason for the differences in plasma thyroid hormone responses between Expt 1 and 2 was not clear but may be related to a seasonal or maturational effect. However, as in Expt 1 exposure to pH 4.7 (Expt 2) tended to increase the T_4/T_3 ratio (Fig. 1.2C). Because no changes in thyroid histology (see Brown et al. 1984) were noted, it is possible that the acid effects were directed primarily at the peripheral metabolism of thyroid hormones. An increase in T_4/T_3 could result from reduced T_4 to T_3 conversion as described in starvation stress (Higgs and Eales 1977, 1978). Because experimental fish ate less than control fish after 7-10 d of acid exposure, it is possible that reduced food intake contributed to the increase in T_4/T_3. An additional complication is that T_3 and T_4 exist in trout blood in both free and protein-bound forms (Falkner and Eales 1973) and during acid stress plasma protein increases. Increases in plasma

FIGURE 1.2. Temporal changes of plasma T_4 (A) and T_3 (B) concentrations, and their ratios (C) in trout exposed to pH 4.7 (○) and pH 7.7 (●). Points represent mean ± SE of 10-12 fish. Means significantly different from control are indicated (*). Values significantly different from those at day 1 are indicated (■).


A
B
C
Thyroxine Concentration (nmol/L)
Triiodothyronine Concentration (nmol/L)
T4:T3
Time (d)
0
1
2
3
4
5
6
7
10
15
20
*

protein(s) responsible for binding T_3 and/or T_4 would tend to elevate plasma levels of T_3 and/or T_4. The difference in plasma protein levels between Expts 1 and 2 may have contributed to the differential T_4 response. This could also explain the tendency of plasma T_4 to increase in acid stress, the reverse of the response reported for other chronic stress conditions where suppression of thyroid activity has been observed (Sage 1971; Osborn and Simpson 1972, 1974; Lewis and Dodd 1976; Pickering 1976; Simpson 1976; Leatherland and Sonstegard 1978; Eales 1979a). However, increases in plasma protein levels cannot explain why plasma T_3 was depressed in Expt 2 relative to controls. Further work on the peripheral metabolism and binding of thyroid hormones to plasma proteins is required to define acid effects on the trout thyroid.

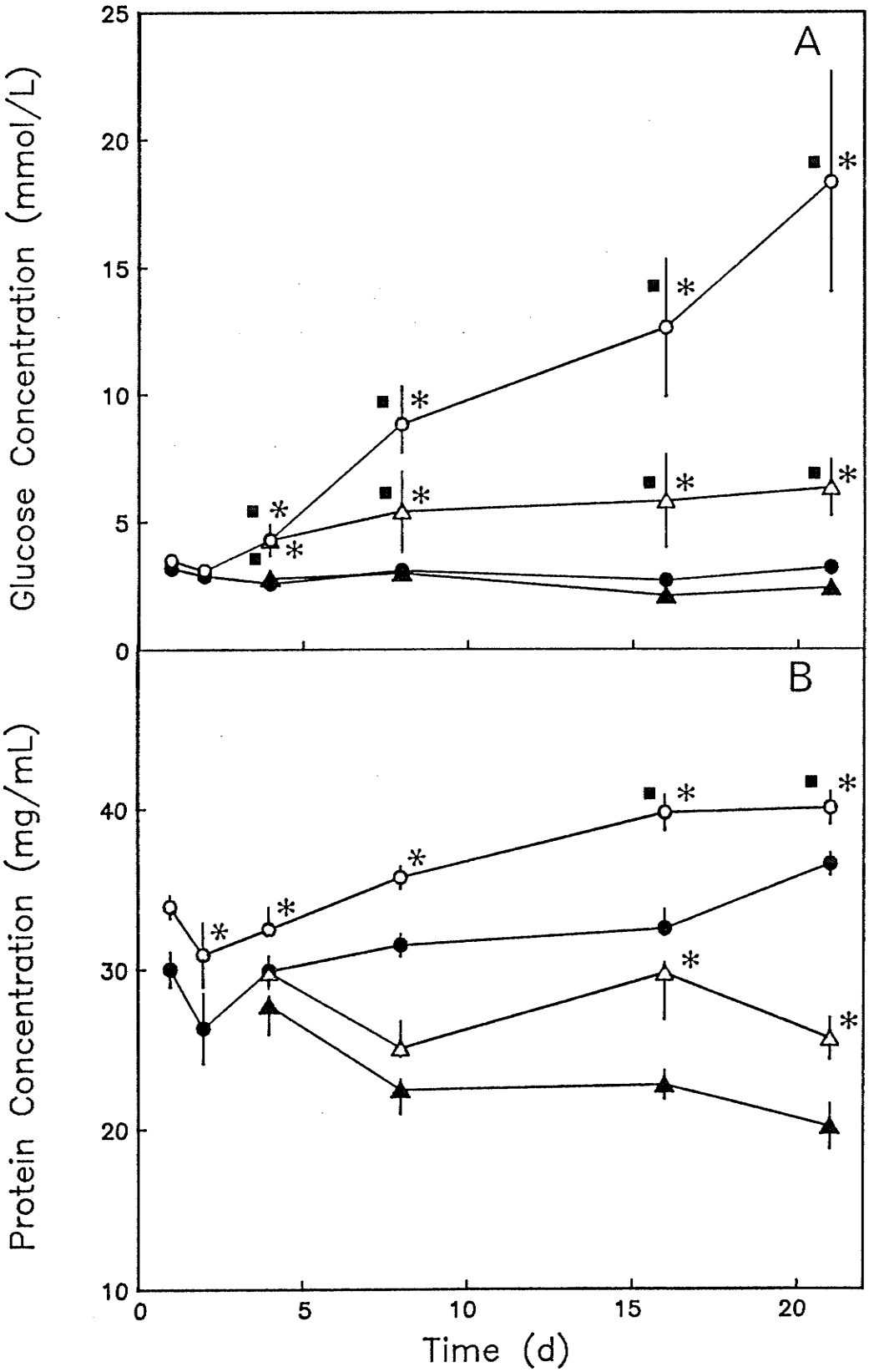
1.4.4 Non-hormonal Plasma Parameters

Blood glucose concentration is a frequently used indicator of stress in teleost fish (Wedemeyer and McLeay 1981), responding to a variety of stressors including pollutants (Silbergeld 1974; Hattingh 1976; McLeay 1977; Wedemeyer and Yasutake 1977; Thomas et al. 1980, 1981). Glucose levels increased with increasing H^+ concentration in Expt 1 (Table 1.1). The threshold was between pH 5.2 and 6.0. Significant elevations in plasma glucose were detected after 4 d exposure to both pH 4.7 and pH 5.2 (Fig. 1.3A). At pH 4.7 glucose continued to rise throughout the experiment while at pH 5.2, glucose increased until 8 d and then remained constant. In fish, the hyperglycemic stress response is apparently mediated by increases in plasma cortisol and catecholamine (Nakano and Tomlinson 1967; Mazeaud et al. 1977; Leach and Taylor 1980, 1982). In the present study

plasma glucose levels were significantly (P <0.01) correlated with plasma cortisol in acid-exposed fish from all three experiments (r = 0.485, N = 150). Furthermore, in the assessment of chronic acid stress on rainbow trout, plasma glucose may be more useful as an indicator than plasma cortisol. Significant and sustained elevations of glucose were evident after 4 d exposure to pH 5.2, while elevation of circulating cortisol was not evident until 21 d. Cortisol could still be at least partially responsible for the hyperglycemia because plasma hormone measurements at a given time do not necessarily reflect the rate of utilization. Recent studies by Lee et al. (1983) in unfed rainbow trout and by Nieminen et al. (1982) in whitefish (*Coregonus peled*) and brown trout (*Salmo trutta*) exposed to acidic conditions also showed that low pH can increase plasma glucose levels. In addition to providing a ready energy source during acid stress, elevations in plasma glucose may be important in maintaining plasma osmolality despite declining blood ion levels (Bergstrom 1971; Bashamohideen and Parvatheswararao 1972; Giles et al. 1984). The plasma glucose response depends on strain, nutritional status and temperature (Nakano and Tomlinson 1967; Chavin and Young 1970; Wendt and Saunders 1973; Wydoski et al. 1976; Wedemeyer and McLeay 1981). Further study involving these factors and acid stress will determine the general applicability of the blood sugar assay to evaluate acid stress.

Plasma protein values were generally greater in trout exposed to H^+ than in control fish (Table 1.1). The effect was significant after

FIGURE 1.3. Temporal changes of plasma glucose (A) and plasma protein (B) in trout exposed to pH 4.7 (○) and pH 5.2 (△). Control fish at pH 7.7 were examined at each time (●,▲). Points represent mean ± SE of 7-12 fish. Means significantly different from control are indicated (*). Values significantly different from those at day 1 are indicated (■).


A
B
Glucose Concentration (mmol/L)
Protein Concentration (mg/mL)
Time (d)
25
20
15
10
5
0
40
30
20
10
0
5
10
15
20

21 d at pH 4.2 and 4.7. In Expt 2 the elevation was apparent at 2, 4, 8, 16 and 21 d at pH 4.7, and at 16 and 21 d at pH 5.2 (Fig. 1.3B). The increase was probably due to plasma volume changes associated with acid stress (Spry et al. 1981). Milligan and Wood (1982) found that 3-d acid exposure (pH 4.0-4.5) reduced the plasma volume by 27% and increased protein concentration by 90% in rainbow trout. Lockhart and Lutz (1977) found no effect of acidic (pH 4.8-5.3) George Lake waters on plasma protein of white suckers. As with plasma cortisol, long-term adaptation or selection may occur in natural populations. The increase in plasma protein (25%), in the present study, is not of sufficient magnitude to explain the 20- to 50-fold increase in plasma glucose and cortisol levels.

1.5 CONCLUSIONS

Exposure to low pH stimulated interrenal activity as inferred from plasma cortisol concentrations. Depending upon the experiment, altered plasma thyroid hormone levels were due to either a significant elevation in plasma T_4 or a decrease in plasma T_3. Preceding the higher interrenal activity, plasma glucose was elevated in acid-treated trout suggesting that factors other than cortisol were involved in its regulation. The acid-induced elevation in plasma protein implies compromised fluid balance.

PART 2

EFFECTS OF LOW AMBIENT pH AND ALUMINUM ON PLASMA LEVELS OF CORTISOL, T_3, T_4, ELECTROLYTES AND GLUCOSE

2.1 SYNOPSIS

Rainbow trout were exposed for up to 14 d to pH 4.7, 5.2 or 7.7 plus 0, 5, 10 or 20 µmol/L labile AlF in moderately hard water (520 µmol Ca^{2+}/L) and to pH 5.0 or 6.6 plus 0, 3 or 5 µmol/L labile AlF in soft water (72 µmol Ca^{2+}/L). Plasma cortisol, T_3, T_4, Na^+, Cl^-, osmolality and glucose were measured. Exposure to low pH in both hard and soft water increased plasma cortisol and glucose while reducing plasma Na^+, Cl^- and osmolality. High concentrations of AlF in hard (20 µmol Al/L) and soft (5 µmol Al/L) low pH water further increased plasma cortisol and glucose as well as reducing plasma ions. AlF at low pH reduced plasma T_3 and tended to elevate plasma T_4. However low concentrations of Al in hard (5-10 µmol Al/L) and soft (3 µmol Al/L) low pH water generally returned all plasma values towards control levels. In summary, low pH and Al exposure increased interrenal activity and lowered plasma levels of T_3, the presumed active thyroid hormone at the tissue level, in addition to impairing ion and carbohydrate balance.

2.2 INTRODUCTION

In addition to its known effects on ionoregulation in fish (Wood and McDonald 1982), low ambient pH activates the pituitary-interrenal system thereby increasing circulating levels of cortisol (PART 1; Adams et al. 1985; Barton et al. 1985; Brown et al. 1986a; Tam et al. 1987, 1988). The role of cortisol in adaptation to low pH remains to be defined but it alters both hydromineral and carbohydrate metabolism (Leach and Taylor 1980, 1982; Perry and Wood 1985; Flik and Perry 1989). Recent studies have shown that acute (3 d) exposure to low pH and Al elevated plasma cortisol in rainbow trout (Goss and Wood 1988) and brown trout (Whitehead and Brown 1989). Wood et al. (1988a) also demonstrated that cortisol was increased in brook trout after 70 d exposure to low pH and Al. However, the effects of Al treatment between 3 and 70 d require documentation.

The effects of environmental stressors on the thyroid system have received little study in fish (Eales 1979a). Thyroid hormones promote growth, development and possibly early phases of reproduction in fish (Eales 1979a; Leatherland 1982), processes which are also impaired during low pH exposure. Recently, Tam et al. (1988) showed that pituitary thyrotrophs were less active in acid-exposed fish. Although the effects of low pH on plasma thyroid hormone levels have been described (PART 1; Brown et al. 1986a; Edwards et al. 1987), the results were equivocal. To date, the effects of low pH combined with Al exposure on thyroid functon have received only limited attention (Brown et al. 1986c; Whitehead and Brown 1989).

My primary objectives were to determine the extent to which interrenal and thyroid functions are influenced by low ambient pH and Al and to define conditions to be used in subsequent hormone kinetic studies (see PARTS 4 and 5). Plasma hormone concentrations were used to assess endocrine activity. Plasma ions, osmotic properties and glucose were measured to substantiate previously described effects of low pH and Al (Neville 1985; Wood and McDonald 1987) and to determine their potential relationships with the endocrine changes. Finally to place the laboratory study, conducted in moderately hard water, into a more environmentally relevant context, part of the study was repeated using soft lake water.

2.3 MATERIALS AND METHODS

2.3.1 Experimental Animals

Rainbow trout (2+ yrs; weight 211.4 ± 47.4 g; fork length 24.7 ± 1.8 cm; $\hat{x}$ ± SE, N = 216) were held as described in PART 1. The Winnipeg city water contained the following major ions: 520 µmol Ca^{2+}/L, 221 µmol Mg^{2+}/L, 112 µmol Cl^-/L, 73 µmol Na^+/L, 51 µmol F^-/L, 34 µmol K^+/L and 22 µmol SO_4^{2-}/L. Hardness was equivalent to 741 µmol $CaCO_3$/L. Average water pH was 7.7 (range 7.5-7.9). Dissolved inorganic carbon (DIC) measured by gas chromatography was 1340 ± 45 µmol/L. Total dissolved organic carbon (DOC) was 560 ± 29 µmol/L. Fish were fed trout food (Martin Feeds, Elmira, Ontario) at a ration of 1.5% wet body weight per day and were acclimated to these conditions for at least 3 weeks prior to their use in experiments. Further details of holding conditions are given with the protocol for each experiment.

2.3.2 Toxicity Testing and Experimental Protocol

Test water was obtained by an acidification-decarbonation system consisting of titrators, six-channel switches and magnetic valves to monitor pH and add diluted H_2SO_4 (see PART 1). Water temperature was 13.5 ± 0.8 °C and free CO_2 concentration equalled 46 ± 5 µmol/L. Instrument calibrations were checked daily against a standardized pH meter. Aluminum ($AlKSO_4$) stock solutions were prepared in distilled deionized water and acidified with 1 mL concentrated HNO_3 per liter. The Al was perfused into the incoming water line of each test tank using multichannel peristaltic pumps. Each tank received at least 2 L of aerated water per gram of fish each day and the time for 99% water replacement was <5 h. Replicate tanks were run at each exposure.

The effects of low pH and AlF on trout were investigated in 4 experiments. Fish were acclimated to experimental tanks for 14 d before beginning experiments. The experiments were conducted between April and September. In Expt 1, fish were exposed in the laboratory for 14 d prior to sampling to 8 acid/Al combinations and to water at pH 7.7 (controls). Water was acidified to a nominal pH of 5.2 or 4.7 and Al was added to provide nominal concentrations of 0, 5, 10 and 20 µmol/L. In Expt 2, fish were exposed to acidified (pH 5.2) water plus Al (nominal concentrations 0, 10 and 20 µmol/L). Control trout were held at pH 7.7 (0.3 µmol Al/L). Fish were sampled on days 3, 6 and 10. Expt 3 was similar to Expt 2 except that the water was maintained at pH 4.7. For Expt 4, aquaria were set up in a field laboratory by Lake 239 at the Department of Fisheries and Oceans' Experimental Lakes Area, northwestern Ontario. This provided natural soft water (pH range 6.5-6.8; Temp. 16.6 ± 0.2 °C;

DOC 530 µmol/L; DIC 190 µmol/L; alkalinity 137 µEq/L; hardness 104 µmol $CaCO_3$/L; 72 µmol Ca^{2+}/L; 57 µmol SO_4^{2-}/L; 48 µmol Na^+/L; 33 µmol Mg^{2+}/L; 17 µmol Cl^-/L; 10 µmol K^+/L; 3 µmol F^-/L) which was pumped from Lake 239 into the laboratory. Fish were exposed for 14 d to ambient water (pH 6.6) or to acidified (pH 5.0) lake water at nominal AlF ($AlKSO_4$ and NaF) concentrations of 0, 5, 10 and 20 µmol/L.

2.3.3 Aluminum Measurements

Fractionation of Al was similar to the scheme outlined by Campbell et al. (1983). Total Al was measured in samples acidified to pH 1.4 for 24 h to dissolve polymeric forms. Dissolved Al was measured in samples filtered through 0.2-µm polycarbonate filters that were rinsed in 1N HNO_3 and distilled water before use. Aliquots of the filtered samples were cation desalted using ion-exchange columns containing Amberlite IR-120 resin in the sodium form (Driscoll 1984). All Al concentrations were determined in the various fractions with the lumogallion fluorometric method (Playle et al. 1982) before and after standard additions. The difference between values in filtered water and values obtained following cation exchange were considered labile Al. Fluoride levels were measured using the fluoride ion-selective electrode. Free fluoride was determined by direct potentiometric determination and total fluoride was measured after addition of a total ionic strength adjuster and buffer. The ion-exchange separations were verified with the fluoride computational method (Driscoll 1984) and equilibrium dialysis (LaZerte 1984). Total inorganic SO_4^{2-} levels were measured by ion chromatography. Calculations of Al species in the labile fraction were performed according to Driscoll

(1984). A complete set of Al and speciation measurements was conducted in duplicate on water collected from the outflow of each test tank after 1, 6 and either 9 or 12 d exposure. All measurements were completed on the day of sampling.

2.3.4 Blood Sampling and Analysis

Trout were anesthetized in pH-neutralized TMS (0.76 mmol/L), blood was removed from the caudal vessels using ammonium-heparinized syringes, and plasma was obtained by centrifugation (PART 1). Plasma cortisol, T_3, T_4 and glucose were measured as previously described (PART 1). Plasma osmolality was measured on 50 µL aliquots using a Precision Systems Model 5004 µOsmette. Plasma Na^+ was measured with an Instrumentation Laboratory Model 943 flame photometer, and plasma Cl^- was determined using a Corning Model 925 chloride analyzer. Interassay coefficients of variation from duplicate analyses in at least 10 assays for osmolality, Na^+ and Cl^- were 1.5, 3.8 and 4.0%, respectively. Assuming complete dissociation of Na^+ and Cl^-, the unidentified osmotic fraction (UOF) in plasma was derived from measurements of osmolality, Na^+ and Cl^- using UOF = [1 - ((Na + Cl)/osmolality)] X 100 (Giles et al. 1984).

2.3.5 Statistics

Bartlett's test for homogeneity of variance was applied to data and where required, data were transformed according to Taylor's power law to obtain more uniform variances (Southwood 1978). For clarity, untransformed arithmetic means and standard errors are presented. ANOVA was computed (Snedecor and Cochran 1971). Two-way ANOVA using

pH and Al concentration as independant variables was applied to data from Expt 1, three-way ANOVA using pH, Al, and time was applied to data from Expts 2 and 3, and one-way ANOVA was applied to Expt 4. Tukey's Studentized Range Test was used to distinguish differences between group means. Probability levels of <0.05 were considered significant.

2.4 RESULTS

Ambient pH and total Al levels were close to nominal values (Tables 2.1-2.4). Labile Al as a percentage of total Al was greater in hard Winnipeg city water (79.3-100%) than in soft Lake 239 water (21.4-65.8%). Speciation calculations indicated that labile Al was >98% aluminum fluoride (AlF) species under all conditions.

2.4.1 Experiment 1: Effects of Al (0, 5, 10 and 20 µmol/L) at pH 4.7 and 5.2 for 14 d (Table 2.1)

At pH 5.2 mortality at nominal Al levels below 20 µmol/L was 10% and at 20 µmol Al/L mortality was 20%. At pH 4.7 mortality was 20% without added Al and was 0% at 5 µmol Al/L. Mortality rose to 20% and 30% respectively at 10 and 20 µmol Al/L.

At pH 5.2 endocrine changes involved an increase in plasma cortisol and a decrease in plasma T_3 at 20 µmol Al/L. At pH 4.7 plasma cortisol was increased at the lowest Al concentration (0 µmol Al/L), was not different from pH 7.7 at 5 µmol Al/L, but again increased at the highest Al concentration (20 µmol/L) to a value 20-fold that of controls (pH 7.7). At pH 4.7 both plasma T_3 and T_4 were depressed by 20 µmol Al/L.

TABLE 2.1 Effects of low ambient pH and Al on mortality and plasma cortisol, T_3, T_4, Na^+, Cl^-, osmolality, undefined osmotic fraction (UOF), and glucose in rainbow trout exposed for 14 d to Winnipeg city water (Expt. 1). The number of pH measurements in each range equalled 42. Measurements for Al represent mean (SD), N=12. Plasma values represent mean (SE) of 2 replicate groups each containing 5 fish. Square indicates significantly different from pH 7.7. Triangle indicates significant difference between pH and Al treatment.

pH range	Nominal Al (µmol/L)	Total Al (µmol/L)	Labile Al (µmol/L)	Mortality %	Cortisol (nmol/L)	T_3 (nmol/L)	T_4 (nmol/L)	Na^+ (mmol/L)	Cl^- (mmol/L)	Osmolality (mosmol/kg)	UOF %	Glucose (mmol/L)
7.7 - 7.9	0	0.3 (0.1)	0.3 (0.1)	0	32 (11)	2.9 (0.3)	1.5 (0.2)	150 (1)	131 (1)	295 (2)	4.4 (0.6)	4.2 (0.2)
5.1 - 5.3	0	0.3 (0.1)	0.3 (0.1)	10	37 (11)	1.7 (0.2)	1.6 (0.2)	134■ (4)	118■ (3)	280 (5)	10.1■ (1.1)	11.2■ (1.4)
5.2 - 5.3	5	4.9 (0.7)	3.9 (0.4)	10	27 (20)	2.4 (0.2)	1.9 (0.3)	136 (6)	122 (5)	280 (9)	8.0 (1.6)	9.9 (2.6)
5.1 - 5.3	10	12.5 (1.9)	11.2 (1.6)	10	15 (4)	1.9 (0.5)	1.1 (0.2)	129■ (3)	113■ (3)	279 (3)	13.2■ (1.5)	14.5■ (2.7)
5.2 - 5.3	20	26.3 (1.6)	21.6 (1.0)	20	88■▲ (27)	1.1■ (0.3)	1.8 (0.4)	118■▲ (5)	106■▲ (5)	265■ (9)	15.5■▲ (1.7)	20.5■▲ (2.4)
4.6 - 4.8	0	0.3 (0.1)	0.3 (0.1)	20	132■ (71)	1.9 (0.4)	1.7 (0.4)	111■ (9)	100■ (8)	245■ (20)	13.3■ (1.6)	12.9■ (3.5)
4.6 - 4.8	5	5.6 (0.3)	5.1 (0.1)	0	54 (15)	1.5 (0.4)	1.2 (0.3)	129■ (7)	109■ (6)	271 (11)	12.3■ (1.9)	15.3■ (3.2)
4.6 - 4.8	10	10.5 (0.6)	9.6 (0.4)	20	180■ (99)	1.3 (0.3)	2.3 (0.5)	122■ (10)	106■ (10)	268■ (11)	15.6■ (4.0)	12.3■ (2.6)
4.6 - 4.8	20	21.0 (2.7)	19.8 (1.7)	30	648■▲ (221)	1.2■ (0.3)	0.6■▲ (0.2)	105■▲ (5)	92■▲ (5)	249■ (12)	17.9■▲ (1.8)	21.5■▲ (4.2)

Plasma Na^+ and Cl^- were decreased at pH 5.2 and to a greater extent at pH 4.7. Exposure to 20 µmol Al/L at pH 5.2 and pH 4.7 further reduced plasma Na^+ and Cl^- in addition to lowering plasma osmolality.UOF was elevated at pH 5.2 and pH 4.7. Exposure to 20 µmol Al/L at both pH 5.2 and pH 4.7 further increased UOF. Plasma glucose was elevated at pH 5.2 (0, 10 and 20 µmol Al/L) and at pH 4.7 (all Al concentrations).

2.4.2 Experiment 2: Effects of 3, 6 and 10 d Exposure to Al (0, 10 and 20 µmol/L) at pH 5.2 (Table 2.2)

At 3 d there was no mortality under any condition, an increase in plasma cortisol at 20 µmol Al/L but no other endocrine changes. There were no changes in plasma Na^+ and Cl^- levels, osmolality or glucose. UOF was increased at 10 and 20 µmol Al/L.

At 6 d mortality was 10% at 0 and 10 µmol Al/L. Plasma cortisol was increased at 10 µmol Al/L and 20 µmol Al/L. There were no changes in plasma T_3 or T_4 under any condition. Both plasma Na^+ and osmolality were reduced at the highest Al level.

At 10 d mortality was 10% at 20 µmol Al/L. Plasma cortisol was increased at 10 and 20 µmol Al/L, and plasma T_4 at 20 µmol Al/L. Plasma Na^+, Cl^- and osmolality were decreased at 0 and 20 µmol Al/L. However, only Na^+ was lowered at 10 µmol Al/L. Plasma UOF and glucose were increased at 0 and 20 µmol Al/L but at 10 µmol Al/L only UOF differed from values observed at pH 7.7.

TABLE 2.2 Effects of ambient pH 5.2 and Al on mortality and plasma cortisol, T_3, T_4, Na^+, Cl^-, osmolality, undefined osmotic fraction (UOF) and glucose in rainbow trout exposed 3, 6 and 10 d to Winnipeg city water (Expt 2). Values for Al represent mean (SD) of duplicate analyses from each sampling day. Plasma values represent mean (SE) of 2 replicate groups each containing 5 fish. Square indicates significantly different from pH 7.7. Triangle indicates significant difference between pH and Al treatment.

Day	pH range	Nominal Al (µmol/L)	Total Al (µmol/L)	Labile Al (µmol/L)	Mortality %	Cortisol (nmol/L)	T_3 (nmol/L)	T_4 (nmol/L)	Na^+ (mmol/L)	Cl^- (mmol/L)	Osmolality (mosmol/kg)	UOF %	Glucose (mmol/L)
3	7.7 - 7.9	0	0.3 (0.1)	0.3 (0.1)	0	15 (4)	3.0 (0.4)	2.1 (0.4)	151 (1)	134 (1)	298 (2)	4.3 (1.0)	3.2 (0.1)
	5.3 - 5.4	0	0.3 (0.1)	0.3 (0.1)	0	50 (34)	3.9 (0.2)	1.8 (0.3)	156 (3)	128 (3)	302 (4)	6.2 (0.8)	4.1 (0.4)
	5.3 - 5.4	10	10.3 (1.0)	8.2 (0.8)	0	63 (54)	3.4 (0.7)	2.0 (0.4)	147 (3)	128 (3)	304 (3)	9.7■ (1.3)	6.0 (1.2)
	5.2 - 5.3	20	20.6 (1.9)	16.9 (1.7)	0	177■▲ (88)	3.6 (0.7)	2.9 (0.4)	145 (7)	124 (6)	295 (7)	9.4■ (2.9)	8.3 (2.5)
6	7.6 - 7.8	0	0.3 (0.1)	0.3 (0.1)	0	17 (5)	4.9 (0.4)	3.5 (0.5)	148 (1)	131 (1)	288 (2)	3.2 (0.7)	5.2 (0.9)
	5.1 - 5.4	0	0.3 (0.2)	0.3 (0.2)	10	35 (22)	3.9 (0.3)	4.0 (0.7)	142 (1)	130 (1)	281 (2)	3.4 (0.6)	4.9 (0.3)
	5.3 - 5.4	10	12.1 (0.8)	9.8 (0.6)	10	140■▲ (45)	3.8 (0.5)	4.5 (0.6)	149 (2)	124 (4)	291 (5)	6.4 (1.7)	7.6 (2.9)
	5.1 - 5.3	20	18.0 (1.8)	14.8 (1.4)	0	137■▲ (38)	4.4 (0.5)	4.7 (0.8)	131■▲ (5)	123 (3)	270■▲ (5)	6.2 (1.0)	7.1 (1.1)
10	7.6 - 7.8	0	0.3 (0.1)	0.3 (0.1)	0	28 (12)	5.7 (0.5)	4.3 (0.4)	151 (2)	130 (2)	289 (2)	3.0 (1.0)	5.1 (0.3)
	5.1 - 5.4	0	0.3 (0.1)	0.3 (0.1)	0	92 (81)	3.9 (0.3)	3.0 (0.3)	128■ (5)	117■ (3)	262■ (8)	6.5■ (0.9)	13.6■ (2.7)
	5.1 - 5.3	10	9.2 (0.9)	7.5 (0.6)	0	143■ (62)	4.5 (0.3)	5.0 (0.5)	138■ (2)	123 (3)	277 (5)	5.8■ (0.7)	7.5 (1.4)
	5.1 - 5.2	20	20.5 (1.9)	16.9 (1.6)	10	379■▲ (157)	4.5 (0.5)	7.3■▲ (1.0)	125■ (1)	109■ (6)	264■ (1)	12.1■▲ (2.5)	11.9■ (1.4)

2.4.3 Experiment 3: Effects of 3, 6 or 8, and 10 d Exposure to Al (0, 10 and 20 µmol/L) at pH 4.7 (Table 2.3)

At 3 d there was no mortality under any condition and, apart from an increases in plasma glucose at 20 µmol Al/L, no changes in endocrine or other measured parameters.

At 6 or 8 d plasma cortisol was elevated at 20 µmol Al/L but no changes were evident in plasma T_3 or T_4. At 0 and 20 µmol Al/L, plasma Na^+, Cl^- and osmolality were decreased and plasma UOF and glucose increased. At 10 µmol Al/L only plasma Na^+ differed from controls (pH 7.7, 0 µmol Al/L).

At 10 d mortality was 10% at 0 and 20 µmol Al/L, but there was no mortality at 10 µmol Al/L. Plasma cortisol and plasma T_4 were elevated at the highest Al concentration but plasma T_3 was decreased. At 0 and 20 µmol Al/L, plasma Na^+, Cl^- and osmolality were reduced. Plasma UOF was increased at 0 and 20 µmol Al/L whereas, glucose was increased only at 20 µmol Al/L. At 10 µmol Al/L only plasma Na^+ was decreased.

2.4.4 Experiment 4: Effects of Al at pH 5.0 in Soft Water for 14 d (Table 2.4)

At 1.6 µmol labile Al/L, mortality in pH 5.0 soft water was 30%. The only significant differences from controls (pH 6.5-6.7, 1.4 µmol labile Al/L) were a decrease in plasma Na^+ and an increase in plasma glucose. At 2.9 µmol labile Al/L, mortality was 30% and the only change from control was a small but significant increase in plasma glucose. At 4.8 µmol labile Al/L, mortality was 30%. Plasma cortisol, UOF and glucose were increased and plasma T_3, Na^+ and Cl^- were decreased. At 12.8 µmol labile Al/L, only one

TABLE 2.3 Effects of ambient pH 4.7 and Al on mortality and plasma cortisol, T_3, T_4, Na^+, Cl^-, osmolality, undefined osmotic fraction (UOF) and glucose in rainbow trout exposed 3 - 10 d to Winnipeg city water (Expt. 3). Values for Al represent mean (SD) of duplicate analyses for each sampling day. Plasma values represent mean (SE) of 2 replicate groups each containing 5 fish. Square indicates significantly different from pH 7.7 - 7.9. Triangle indicates significant difference between pH and Al treatment.

Day	pH range	Nominal Al (µmol/L)	Total Al (µmol/L)	Labile Al (µmol/L)	Mortality %	Cortisol (nmol/L)	T_3 (nmol/L)	T_4 (nmol/L)	Na^+ (mmol/L)	Cl^- (mmol/L)	Osmolality (mosmol/kg)	UOF %	Glucose (mmol/L)
3	7.5 - 7.6	0	0.3 (0.1)	0.3 (0.1)	0	15 (7)	3.6 (0.1)	2.4 (0.3)	149 (3)	133 (3)	299 (1)	5.9 (1.7)	4.1 (0.2)
	4.7 - 5.0	0	0.3 (0.1)	0.3 (0.1)	0	19 (5)	4.0 (0.4)	1.6 (0.4)	152 (2)	134 (3)	294 (2)	2.6 (0.4)	4.9 (0.3)
	4.7 - 4.8	10	10.8 (1.0)	9.8 (2.9)	0	6 (2)	2.9 (0.3)	1.4 (0.4)	145 (3)	137 (1)	291 (3)	3.0 (0.8)	4.8 (0.4)
	4.7 - 5.0	20	24.5 (1.9)	22.1 (1.7)	0	39 (23)	3.1 (0.5)	1.7 (0.4)	143 (3)	129 (2)	294 (3)	7.4 (1.6)	7.0 ■▲ (1.5)
6	7.6 - 7.8	0	0.3 (0.1)	0.3 (0.1)	0	15 (4)	3.7 (0.6)	2.2 (0.3)	145 (1)	133 (2)	293 (3)	4.6 (0.9)	4.2 (0.4)
	4.6 - 4.8	0	0.3 (0.1)	0.3 (0.1)	0	46 (22)	2.9 (0.3)	2.0 (0.4)	133■ (5)	116■ (4)	275■ (7)	9.7■ (0.9)	11.4■ (2.0)
	4.6 - 4.9	10	10.4 (0.8)	9.5 (0.5)	0	19 (5)	2.4 (0.3)	2.9 (0.3)	136■ (2)	129 (2)	283 (3)	6.4 (0.3)	6.3 (0.7)
8a	4.7 - 4.8	20	22.6 (0.6)	20.5 (0.5)	0	75■ (43)	2.8 (0.3)	2.3 (0.3)	127■ (8)	120■ (8)	275■ (10)	10.6■ (2.9)	13.8■ (5.3)
10	7.6 - 7.9	0	0.3 (0.1)	0.3 (0.1)	0	22 (2)	3.4 (0.3)	1.9 (0.2)	153 (2)	129 (1)	293 (3)	3.9 (1.1)	5.0 (1.1)
	4.6 - 5.0	0	0.3 (0.1)	0.3 (0.1)	10	36 (12)	3.9 (0.7)	4.2■ (0.9)	131■ (5)	114■ (5)	269■ (6)	9.0■ (1.8)	8.4 (1.1)
	4.6 - 4.9	10	11.8 (1.1)	10.5 (0.9)	0	23 (5)	3.8 (0.4)	2.7 (0.4)	141■ (2)	121 (2)	278 (3)	5.6 (0.6)	6.0 (0.1)
	4.7 - 4.9	20	23.9 (2.4)	21.3 (2.1)	10	159■▲ (43)	2.3■▲ (0.3)	9.2■▲ (1.8)	111■▲ (3)	104■▲ (2)	258■ (4)	15.4■▲ (2.4)	23.3■▲ (5.5)

[a]Due to a failure in acid delivery the 6 d pH 4.7 and 20 µmol/L group was replaced with an 8 d group. Significant differences were determined from the closer 6 d or 10 d pH 7.7 group.

TABLE 2.4 Effect of low pH and Al in softwater on mortality and plasma cortisol, T_3, T_4, Na^+, Cl^-, osmolality, undefined osmotic fraction (UOF) and glucose in rainbow trout exposed for 14 d to water from Lake 239 (Expt 4). The number of pH measurements in each range equalled 42. Measurements for Al represent mean (SD), N=12. Plasma values represent mean (SE) of 2 replicate groups each with 5 fish. Square indicates significantly different from pH 6.5 - 6.7. Triangle indicates significant difference between pH and Al treatment.

pH range	Nominal Al (µmol/L)	Total Al (µmol/L)	Labile Al (µmol/L)	Mortality %	Cortisol (nmol/L)	T_3 (nmol/L)	T_4 (nmol/L)	Na^+ (mmol/L)	Cl^- (mmol/L)	Osmolality (mosmol/kg)	UOF %	Glucose (mmol/L)
6.5 - 6.7	0	1.4 (0.2)	0.3 (0.1)	0	10 (2)	3.2 (0.3)	0.8 (0.2)	145 (1)	133 (2)	294 (2)	5.2 (0.5)	3.9 (0.1)
4.9 - 5.1	0	1.6 (0.2)	0.4 (0.1)	30	28 (10)	2.2 (0.3)	1.6 (0.6)	137■ (3)	128 (2)	282 (5)	6.0 (0.5)	5.7■ (1.0)
4.9 - 5.1	5	5.0 (0.6)	2.9 (0.2)	30	44 (24)	2.4 (0.3)	1.5 (0.6)	141 (2)	130 (3)	286 (4)	5.8 (0.7)	4.6■ (0.3)
4.9 - 5.1	10	7.3 (0.4)	4.8 (0.3)	30	245■▲ (19)	2.0■ (0.1)	1.8 (0.3)	135■ (3)	125■ (3)	280 (3)	8.2■▲ (1.1)	7.3■ (0.2)
4.9 - 5.1	20	19.8 (2.0)	12.8 (1.7)	90	144	1.3	1.2	124	121	280	12.5	11.1

fish survived (mortality 90%) precluding statistical comparisons. This fish had high levels of cortisol, UOF and glucose and low plasma T_3, Na^+, Cl^- and osmolality.

2.5 DISCUSSION

Low plasma cortisol levels (<50 nmol/L) in undisturbed control trout in both soft and hard water were consistent with our previous findings (PART 1; Brown et al. 1986a,b). Thus it could not be confirmed that holding rainbow trout in soft water elevated plasma cortisol as suggested in other studies (Perry and Wood 1985; Goss and Wood 1988).

Exposure to acid or acid and Al causes early transient (<1 d) increases in plasma cortisol as reported previously in salmonids (Mudge et al. 1978; Lee et al. 1983; Adams et al. 1985; Barton et al. 1985; Goss and Wood 1988). The later (>3 d, pH 5.2, 20 µmol Al/L; >8 d, pH 4.7, 20 µmol Al/L) sustained elevations likely represent the point where initial compensation to acid or acid and Al is lost, and hence additional stress is imposed on the organism. Furthermore, elevated cortisol has been linked to chloride cell proliferation in low Ca^{2+} water (Perry and Wood 1985) and to enhanced whole body Ca^{2+} uptake (Flik and Perry 1989). Both effects may aid in ion balance during adaptation to low pH and high Al environments. Trout exposed to Al had fewer gill chloride cells than control fish (Evans et al. 1988). The markedly elevated cortisol levels may stimulate chloride cell production. Further work is required to resolve the relationship between cortisol and chloride cell proliferation and activity in acid/Al conditions.

Increased plasma cortisol for fish held in hard water at pH 4.7 and for fish held in soft water at pH 5.0 occurred *subsequent* to depressed plasma ions and increased glucose levels. Consequently, changes in the activity of the interrenal cells, as inferred from plasma cortisol levels, followed ionoregulatory disturbances. In contrast, changes in plasma cortisol preceded alterations in ionoregulatory disturbance and plasma glucose at pH 5.2, implying a different profile of response to Al toxicity between pH 4.7 and 5.2. Furthermore, Al also induced changes in gill morphology more rapidly at pH 5.2 than at pH 4.7 (Evans et al. 1988). These temporal differences in response between pH 4.7 and 5.2 may be attributed to the lower solubility of Al near pH 5.5. Toxicity to Al has been observed to shift from ionoregulatory malfunction to respiratory disturbance over a similar pH range (Neville 1985; Goss and Wood 1988; Neville and Campbell 1988; Wood et al. 1988b).

Treatment of teleosts with exogenous cortisol shifts carbohydrate metabolism towards gluconeogensis and elevates plasma glucose (Chan and Woo 1978; Leach and Taylor 1982). Temporal inconsistencies between changes in interrenal activity and plasma glucose exist in this and other studies (PARTS 1 and 3; Nichols and Weisbart 1985; Kuhn et al. 1986). Thus the results show that other factors may be controlling plasma glucose levels during acid or acid and Al exposure. However, cortisol may be necessary for the complete development of the glucose response to stress induced by acid or acid and Al.

Plasma T_3 and T_4 levels in control fish were similar to those reported previously (PART 1; Brown et al. 1986a). Evidence for lower plasma T_3 and increased plasma T_4 levels during acid exposure has been

reported in rainbow trout (PART 1) and brown trout (Edwards et al. 1987). However the plasma T_4 response to acid alone was not consistent. When fish were exposed to high levels of Al, plasma T_4 was either unchanged or increased. On the other hand, with the exception of Expt 2 where exposure conditions may not have been sufficiently severe, plasma T_3 was consistently depressed by exposure to low pH/Al beyond 10 d. These results suggest that tissue production of T_3 is reduced (Eales 1985) when fish are exposed to low pH/Al. Because clear interpretations of physiologic thyroidal status are not possible from plasma hormone concentrations alone, additional studies are necessary. Information on plasma kinetics for both T_3 and T_4, their extent of binding by plasma proteins and estimates of T_4 to T_3 converting activity are required to substantiate the hypothesis that low ambient pH and Al impair T_3 production. Moreover, the low plasma T_3 concentration does not necessarily reflect a direct effect of low pH and Al. Because low plasma T_3 was associated with high plasma cortisol, the reduced T_3 levels may result from antagonistic effects of interrenal activity on thyroid function (Eales 1979a).

Plasma ion losses caused by exposure to low pH Winnipeg city water were consistent with previous findings in rainbow trout (Giles et al. 1984; Brown et al. 1986a). At pH 5.0 the magnitude of plasma ion loss in soft Lake 239 water was comparable to that seen in Winnipeg city water at pH 5.2, but not as extensive as that for exposure to Winnipeg city water at pH 4.7. Ion loss eventually results in severely compromised fluid balance which ultimately causes death in acute acid exposure (Wood and McDonald 1987). Therefore the greater mortality seen in Lake 239 water at pH 5.0

cannot be explained without information on tissue ion pools and rates of whole body ion losses.

Low concentrations of AlF (5-10 µmol/L) partly prevented ion loss and mortality in Winnipeg city water. This beneficial effect was not as evident in Lake 239 water but plasma ions did tend to increase in the group at the lowest level of added Al. Baker and Schofield (1982) and Neville (1985) have described beneficial effects of Al on acid toxicity. The protective effect was attributed to the concentration potential for diffusion of ions between blood and water in conjunction with an increased electrical potential of gill membranes found at higher ionic strength (Hutchinson et al. 1987). Also Wood et al. (1988b) describe Al-induced acclimation which protects fish from subsequent exposure.

High Al levels exacerbated plasma ion loss caused by exposure to low pH in Winnipeg city water. However the effect was not as prominent in soft water. The present effects of Al on ionoregulatory function in soft water were less severe and occurred at higher levels of labile Al than effects described in other studies using artificial soft water (Neville 1985; Witters et al. 1987; Goss and Wood 1988; Audet et al. 1988). Under artificial conditions labile Al largely consists of free Al^{3+} ions and Al hydroxides which appear very toxic. In contrast, labile Al in this study consisted mostly of AlF species. Baker and Schofield (1982) using artificial soft water found that adding fluoride significantly reduced Al toxicity. This probably occurs because fluoride complexes of Al are more soluble than free Al^{3+} ion or Al hydroxides (Hem 1968) and therefore less likely to precipitate on gill membranes whose pH would be similar to blood pH (≈7.4).

The impact of the reductions in plasma ions on plasma osmotic conditions were offset to some extent by increases in UOF. This fraction represents plasma solutes other than Na^+ and Cl^- and consists of glucose, amino acids and other ions which are acquired from intracellular compartments or the diet. These may aid in maintaining extracelluar fluid volume (Fugelli and Vislie 1982; Milligan and Wood 1982; McDonald 1983). The exact contribution of glucose to UOF remains to be fully defined, but they are generally highly correlated with each other during acid exposure (Brown et al. 1986a; Scherer et al. 1986). During conditions of high ion loss, it would be advantageous to use less permeable solutes such as glucose as osmotically active species. Alterations in carbohydrate metabolism favoring high blood glucose are a consistent response of several fish species to acidic conditions (Haya et al. 1985; Tam et al. 1987; Wood et al. 1988c).

The dissolved organic carbon levels of Winnipeg city water and Lake 239 water were similar and should have resulted in a similar speciation profile for Al. The higher level of labile Al in the treated Winnipeg city water is probably due to the UV-photo-oxidation method for dechlorination and the water's high fluoride content. Photo-oxidation considerably reduces organic binding of Al (Campbell et al. 1983). Fluoride dominates Al chemistry at low pH and small free fluoride concentrations will dissolve considerable quantities of Al (Hem 1968).

2.6 CONCLUSIONS

In conclusion, the combination of low ambient pH and Al, in addition to compromising aspects of ionoregulation, also increase interrenal activity and decrease plasma T_3 levels. At pH 4.7 the endocrine changes temporally followed reductions in plasma ionoregulatory properties and probably represent part of the compensatory processes dealing with the stress of ion loss. Moreover, the endocrine responses occurred in both hard and soft water. High cortisol might be beneficial by facilitating glucose production and chloride cell proliferation in the gill. However it can also increase susceptibility to pathogens and impair aspects of reproduction (Pickering 1989). The reduced levels of plasma T_3 in Al-exposed fish are of particular concern because this hormone plays an important role in fish growth (Eales 1979a; Leatherland 1982) and the early stages of female reproduction (Cyr et al. 1988).

PART 3
PROTOCOL FOR ESTIMATING CORTISOL PLASMA CLEARANCE IN ACID-EXPOSED TROUT [1]

3.1 SYNOPSIS

The cortisol plasma clearance rate (PCR) and degradation rate (PDR) were determined in acid (H_2SO_4)-stressed and control rainbow trout cannulated by the dorsal aorta. Recovery from cannulation, as judged by plasma cortisol, glucose, protein and packed cell volume (PCV), was complete by 2 to 6 d. However, serial blood sampling increased plasma cortisol. Furthermore, although no major or consistent diel change in plasma cortisol occurred in terminally sampled free swimming control trout, fluctuations were observed in serially bled cannulated trout. These findings preclude cortisol PCR estimation by any serial sampling method. Although plasma cortisol was temporarily elevated by constant infusion (70 µL/h) of the saline:ethanol vehicle, a satisfactory protocol was established for determining cortisol PCR by infusion of labelled cortisol to constant plasma specific activity. The PCR for control trout in water pH 7.7 for 7 d was 303 ± 42 mL/h per kg which did not differ from that of trout in water pH 5.0. However, the cortisol PDR was greater at pH 5.0 (59 ± 12 pmol/h per kg) than at pH 7.7 (15 ± 3 pmol/h per kg) due primarily to an increased plasma cortisol concentration at pH 5.0. It is concluded that low pH increases cortisol production and secretion by the interrenal.

[1] Previously published (Brown et al. 1986b).

3.2 INTRODUCTION

The plasma cortisol level has been used as an index of the extent to which environmental changes induce stress responses in fish (Schreck and Lorz 1978; Thomas et al. 1980; Pickering et al. 1982; Davis and Parker 1983). Plasma cortisol levels also increase in response to environmental acidification (Ashcom 1979; Brown et al. 1984). However plasma cortisol concentrations only provide a static estimate of interrenal activity and may not necessarily reflect the rate of cortisol use. To evaluate more fully the effect of acid exposure on interrenal activity, it is necessary to also determine the plasma clearance rate (PCR) of cortisol. This represents that volume of plasma completely cleared of cortisol per unit time (mL/h). The product of PCR and plasma cortisol concentration then provides the cortisol plasma degradation rate (PDR) or the mass of cortisol leaving the plasma pool per unit time (nmol/h) (Oppenheimer and Gurpide 1979).

The two most common methods of estimating hormone PCR are either by following plasma washout of pulse-injected labelled hormone or by infusion of labelled hormone at a constant rate until plasma specific activity reaches a steady state (Oppenheimer and Gurpide 1979). Both these procedures involve fish handling, anesthetization, injection and blood sampling and may themselves represent stressors confounding interpretations of plasma hormone levels and kinetics. This is particularly important where the hormone in question, cortisol, is highly responsive to stress. A possible procedure for minimizing experimental stress in these instances is to use fish with "permanently" implanted cannulae which can

provide routes for both labelled cortisol injection and blood removal (Owen and Idler 1972; Henderson et al. 1974; Leloup-Hatey 1976; Bry and Zohr 1980; Nichols and Weisbart 1984a).

The primary objective of this study was to determine the effect of experimental acidification on the cortisol PCR and PDR of rainbow trout. To this end the effects of cannulation and confinement, serial blood sampling, diel change and vehicle infusion on plasma cortisol and other stress-susceptible blood parameters were studied. In the light of these findings the most appropriate method and protocol for estimating the cortisol PCR and PDR were applied to control and acid-treated trout.

3.3 MATERIALS AND METHODS

3.3.1 Fish Maintenance

Rainbow trout (2-3 yrs; weight 261.1 ± 23.2 g; $\hat{x}$ ± SD, N = 127) were held as described in PART 1 and were acclimated for at least 3 weeks prior to use in experiments. They were fed a ration of 1.5% wet body weight per day. The water temperature ranged from 11.0-13.5 °C but during any one experiment varied less than 1 °C. The photoperiod was adjusted to 12 h light and 12 h darkness (light, 0730-1930).

Fish that were cannulated via the dorsal aorta were held in individual holding boxes modified from those described by Swift (1981a). The boxes were made from black acrylic and the dimensions were designed to limit turning but not to restrain the trout. A small clear acrylic window was hinged with a stainless steel screw to allow the cannula to pass through the top. Uncannulated experimental fish were held in glass

aquaria (70 L), which had translucent white Plexiglas tops and sides covered by black polyethylene. Water was supplied to each box or aquarium from aerated head tanks at a flow of 2 L per gram fish weight per day. Fish were not fed during experiments. Further details of holding conditions are given with the protocol for each experiment.

3.3.2 Cannulation of the Dorsal Aorta

The procedure was based on those described previously (Soivio et al. 1972; Soivio et al. 1975; Bry and Zohr 1980). Fish were anesthetized in aerated water at 11 °C containing pH-neutralized TMS (0.38 mmol/L). When inactive, but still displaying opercular movement, the fish were placed ventral side up on a cooled operating table. During the operation, either fresh water or water containing TMS (0.38 mmol/L) was supplied to the gills to maintain slight opercular movement. The cannulae (Clay-Adams PE-50) were filled with heparinized (200 units/mL) saline. Each cannula was implanted into the dorsal aorta using an 18-gauge canine catheter equipped with a 20-gauge needle (Monoject Division of Sherwood Medical, St. Louis, Mo.). The dorsal aorta was pierced between the first and second gill arches and the cannula inserted about 1.5 cm into the vessel through the catheter. After removing the catheter by sliding it over the cannula, 2 sutures in the roof of the mouth and a suture on the edge of the operculum were used to fix the cannula. The procedure required approximately 15 min to complete.

3.3.3 Blood Sampling and Analyses

After elimination of the cannula dead volume, blood samples (100 µL) were collected in 500-µL polyethylene centrifuge tubes. After sampling, the cannula was refilled with heparinized saline and closed. At the end of the experiment both cannulated and non-cannulated trout were bled via the caudal vessels after they had been anesthetized by immersing the holding boxes in TMS (0.76 mmol/L) or by adding TMS directly to the aquaria. Immobilization was complete within 30 s and blood was removed within 3 min using preheparinized syringes. The packed cell volume (PCV) was determined in triplicate employing Clay-Adams heparinized microhematocrit tubes and the plasma separated by centrifugation was stored at -20 °C in 0.5-mL polyethylene vials. Samples were usually analyzed within 10 d.

The cortisol radioimmunoassay was performed on 20 µL of plasma with a commercial kit (Micromedic Systems, Inc., Horsham, PA). Glucose concentrations were measured on 10-µL plasma aliquots using the Sigma test kit (No. 510). Protein was determined from 5 µL of plasma (Lowry et al. 1951) using crystalline bovine serum albumin as standard. Quality control characteristics and modifications for the various assays have been described previously (PART 1).

3.3.4 Data Analysis

Bartlett's test for homogeneity of variance was applied to the data and where required data were transformed according to Taylor's power law (Southwood 1978) to obtain more uniform variances. For clarity of presentation, arithmetic means and standard errors have been used in the

Results and Discussion. A single-factor repeated-measures ANOVA was applied to test for significance in Expts 1 and 3 (Winer 1971). Then differences between group means were determined according to the multiple comparison procedure of Dunnett (Winer 1971). Expt 2 data were analyzed using a mixed model ANOVA which took into account that fish were nested within treatments (cannulated vs non-cannulated), and also that some were repeatedly measured over time. This ANOVA had elements of a split-plot analysis and of a nested design. The significance of the overall treatment effect was tested with the fish-within-treatment term used as the error. This error term is validly obtained only from the cannulated fish, and was obtained by treating all non-cannulated fish as if they were one. The significance of the time effects and the treatment-by-time effect was tested with the residual mean square for error. Data from Expt 4 represent independent samples and were analyzed by two-way ANOVA. Group means were examined using Tukey's Studentized Range Test. Paired *t*-tests were used to distinguish differences in Expt 5. Probability levels of <0.05 were considered significant.

3.4 EXPERIMENTAL PROTOCOL

3.4.1 Experiment 1: Effects of Cannulation

Ten trout were cannulated and held in individual boxes. Five fish were sampled via the cannula at 6 h and 1, 2, 5, 9, 11 and 16 d, post-operatively and the remaining 5 fish sampled twice at 9 and 16 d, post-operatively. Immediately prior to cannulation of each of the above groups of fish and also at 9 and 16 d, an additional 5 (non-cannuated) fish were

removed from the stock tank and terminally sampled from the caudal vessels.

3.4.2 Experiment 2: Diel Changes in Cannulated and Non-cannulated Trout

Ten trout were cannulated and held in individual boxes. At the same time, 50 control trout were removed from the holding tanks and placed in groups of 5 in 10 aquaria. All fish were allowed 10 d to recover from the effects of the transfer and cannulation. The fish were not fed during the experiment. Control trout were sacrificed, one tank at a time, at 0900, 1300, 1700 and 2100 on day 11 and at 0100, 0600, 0900, 1700 and 2100 on day 12. Also, 100 µL of blood was removed from each of 5 cannulated trout in the following order: Fish numbers 1, 3, 5, 7 and 9 at 0900, 1700, 0100, 0900 and 1700 respectively; fish numbers 2, 4, 6, 8 and 10 at 1300, 2100, 0600 1300 and 2100. Thus, each cannulated trout had 500 µL blood removed over 2 d.

3.4.3 Experiment 3: Effects of Serial Blood Sampling

Ten days after cannulation, 5 fish were given a pulse delivery of 20 µL of Cortland saline (without glucose): ethanol (16:1, v/v) via the cannula and then bled (100 µL) 8 times over 4 h. This bleeding regime and saline: ethanol injection procedure were similar to those employed when evaluating cortisol kinetics by pulse-injection techniques in the eel, *Anguilla anguilla* (Leloup-Hatey, 1976). Control cannulated trout were left undisturbed and bled once at the end of the experiment. The experiment was repeated and the combined results are given.

3.4.4 Experiment 4: Effects of Vehicle Infusion

On day 10 after cannulation, saline:ethanol (16:1, v/v) was infused continuously through the cannula using a 4-channel Compact Infusion Pump (Harvard Apparatus Co., Millis, Mass.) at a rate of 70 µL/h. In series 1, each fish was terminally bled from the caudal vessels at 2, 4, 8 and 24 h of infusion. The experiment was repeated 4 times and the combined results are shown. Cannulated trout which had not been infused served as controls. Series 2 was similar to series 1 except that infusion was started 24 h prior to blood sampling.

3.4.5 Experiment 5: Effect of Acid on Cortisol Levels and Kinetics

Nine days after cannulation, 4 trout were infused as described in Expt 4 except that [1,2,6,7-^{3}H(N)]cortisol (114.9 Ci/mmol, New England Nuclear) was added to the saline:ethanol medium after 24 h of vehicle infusion. The pump was momentarily stopped and the syringes exchanged for those containing labelled cortisol. The amount of radioactivity administered averaged 0.185 µCi/h. A priming dose of labelled cortisol (1.03 ± 0.08 µCi, $\hat{x}$ ± SE) was infused in 20 µL of saline vehicle 30 min before exchanging syringes. Prior to use, a sample of labelled cortisol was mixed with authentic cortisol (Sigma) and its purity examined by thin-layer chromatography (TLC) (Quesenberry et al. 1965). If purity was less than 95%, the cortisol was purified using the TLC system.

Infusions of labelled cortisol were routinely conducted for 24 h. An initial blood sample (70 µL) was removed prior to cortisol infusion and then no blood was taken until the terminal sample was withdrawn from the caudal vessels. If the values for PCV, plasma protein, cortisol and glucose

from the initial blood sample were not within 15% of those for the terminal sample, that particular fish was not used. Plasma cortisol could be measured in fish infused with [^{3}H]cortisol because the Micromedic cortisol assay employs cortisol-3-(*O*-carboxymethyl)oximino-(2-[^{125}I]iodohistamine) as tracer. The [^{3}H]cortisol does not interfere with detection of the ^{125}I-tagged cortisol which was counted in a Beckman Gamma 8000 spectrometer.

Plasma radioactivity was measured in three ways: (1) Total plasma radioactivity was determined following digestion of 100 µL of plasma in NCS solubilizer (Amersham). (2) Extractable radioactivity was determined as follows: Duplicate plasma aliquots (300 µL) were transferred to glass culture tubes and then closed with teflon caps. These extraction tubes contained standard reference steroids (28 nmol each of cortisol and cortisone) and 1000 to 1500 dpm of [^{14}C]cortisol (55 mCi/mmol) which had been dried on the bottom of the tube. Dichloromethane extracts were prepared by shaking the plasma mixture with water (200 µL) and dichloromethane (5 mL). After low speed centrifugation, distilled water (3 mL) was added and the aqueous layer aspirated. An aliquot (200 µL) of the extract was dried in a scintillation vial. (3) The TLC fraction was obtained by drying the remaining extract under nitrogen at 37 °C. Small washes of methanol were added to concentrate the residue in the tip. The residue was redissolved in 50 µL dichloromethane: methanol (4:1, v/v) and applied to Whatman LK6DF precoated thin-layer plates. After chromatography (Quesenberry et al. 1965), the area containing cortisol was visualized under UV (254 nm), removed by suction and eluted (Idler and Horne 1968) into scintillation vials. The eluate was dried and counted in Scintiverse (Fisher) cocktail with a Beckman LS7500 liquid scintillation

spectrometer calibrated for dual isotope counting. Corrections for losses of [^{3}H]cortisol were based on recovery of added [^{14}C]tracer for each sample. Although the measured radioactivity was co-eluted with the added authentic cortisol, the possibility exists that radioactive components other than cortisol could have contributed to this fraction. I refer to the radioactive component co-eluting with the added cortisol as the TLC fraction because recrystallization to constant specific activity was not done.

PCR was calculated from the known rate of infusion of [^{3}H]cortisol (I, dpm/h) and the steady-state value of [^{3}H]cortisol in plasma (C, dpm/mL) as outlined by Oppenheimer and Gurpide (1979); PCR = I/C. From this, the cortisol PDR in nmol/h adjusted to a body weight of 1 kg was determined as PDR = PCR (mL/h per kg body weight) X PC, where PC represents the plasma cortisol concentration (nmol/mL).

Nine days after cannulation, trout were held for 5 d in acid water (pH 5.0) or in control water (pH 7.7). On day 6, infusion with saline vehicle began in 2 acid-treated and 2 control fish for 24 h. The procedure was repeated until 5 fish from each group had been examined over successive 1-d intervals (d7-d8, d8-d9, d9-d10). Thus, in the last pair, 1 fish was exposed to acid for a total of 10 d. This experimental series was repeated 3 times. Details of the acid delivery system and water chemistry were described in PART 1.

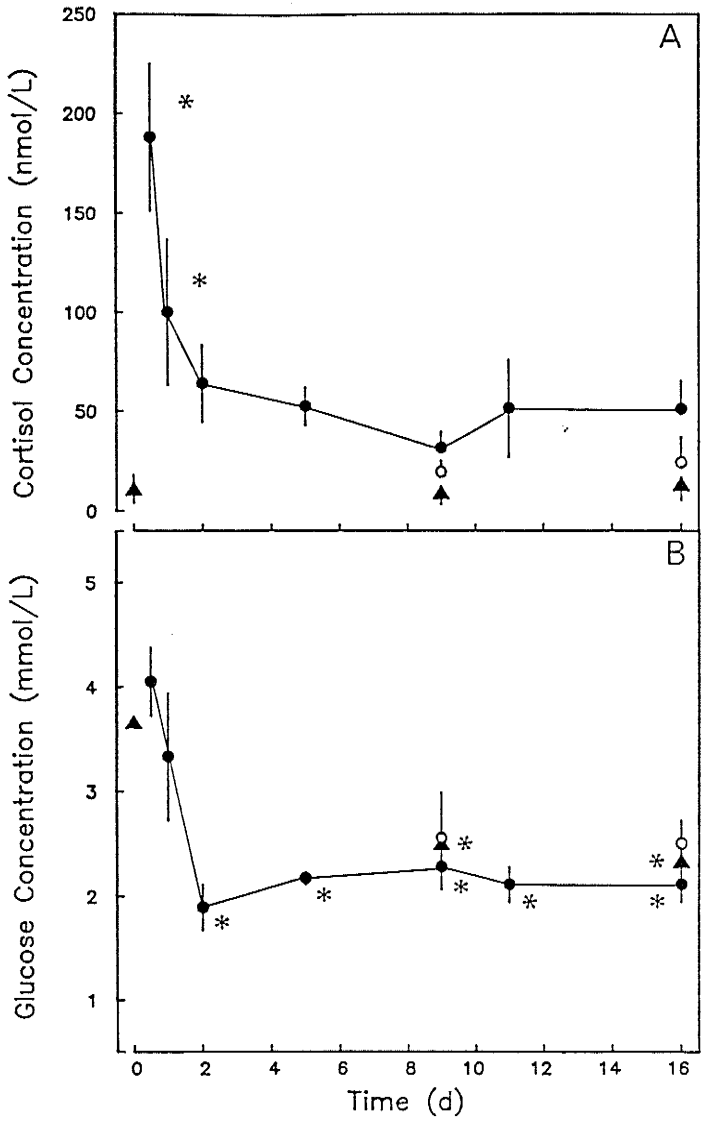
3.5 RESULTS AND DISCUSSION

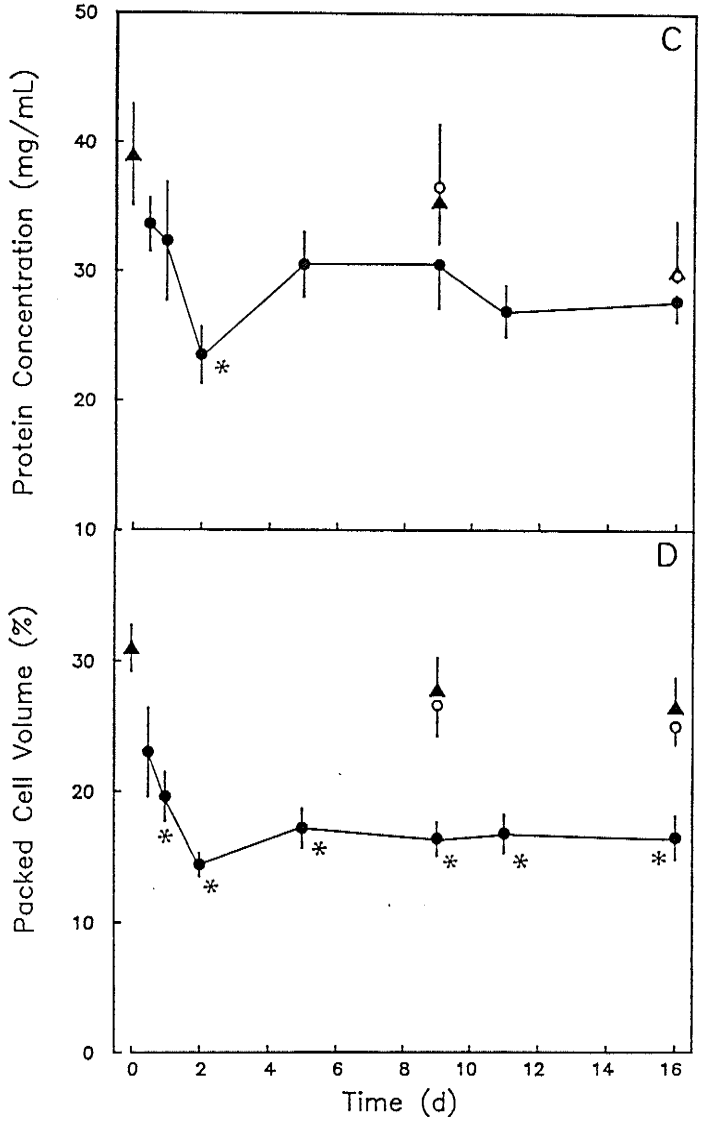
3.5.1 Experiment 1: Effects of Cannulation

Plasma cortisol levels were generally less than 30 nmol/L in trout sampled prior to cannulation (Fig. 3.1A). Following anesthetization, surgery and confinement, plasma cortisol was elevated at 6 and 24 h, but by 48 h fell to approximately 50 nmol/L and remained at this level for at least 16 d. These findings agree generally with those in free-swimming cannulated trout (Bry and Zohr 1980) and confined non-cannulated trout (Swift 1981b).

Neither plasma glucose (Fig. 3.1B) nor protein (Fig. 3.1C) were altered in cannulated fish relative to unoperated controls, although both parameters fell before 2 d, probably due to starvation. The lack of alteration in plasma glucose is similar to previous findings in confined fish (Ince and Thorpe 1976; Soivio and Oikari 1976; Swift 1981b), where the expected increase associated with anesthetization and handling did not occur (Houston et al. 1971; Soivio et al. 1977). Plasma protein levels were within normal ranges reported for rainbow trout (Hille 1982; Miller et al. 1983).

FIGURE 3.1. Temporal changes of (A) plasma cortisol, (B) plasma glucose, (C) plasma protein, and (D) packed cell volume after aortic cannulation (●). Free-swimming, non-cannulated control fish from the holding tanks (▲) and cannulated fish bled only twice (o) are indicated. Values represent mean ± SE from 5 fish. Significant differences from time 0 are indicated (*).


A
B
Cortisol Concentration (nmol/L)
Glucose Concentration (mmol/L)
Time (d)


C
D
Protein Concentration (mg/mL)
Packed Cell Volume (%)
Time (d)

Packed cell volume did not differ between control trout and trout bled at 9 and 16 d (Fig. 3.1D). However, in fish bled repeatedly PCV stabilized at 50% of control values. The effects of repeated blood sampling in depressing PCV are well documented (Soivio et al. 1975; Casillas and Smith 1977; Cairns and Christian 1978; Lane 1979). Repeated blood sampling in conjunction with surgery likely caused the observed depression in PCV. My subsequent experiments in which fish were allowed to recover prior to sampling showed PCV values similar to those published for confined fish (Soivio et al. 1975; Swift 1981b). The amount of blood withdrawn was 7 to 8% of the estimated total blood volume for fish of 250-300 g (Nikinmaa et al. 1981). No effects of blood sampling on PCV were noted in large Atlantic salmon (*Salmo salar*) which had less than 2% total blood volume removed (Nichols and Weisbart 1984a).

In summary, the blood parameters stabilized by 6 d following cannulation and confinement. The minimum recovery period used in later experiments was 9 d.

3.5.2 Experiment 2: Diel changes in Cannulated and Non-cannulated Trout

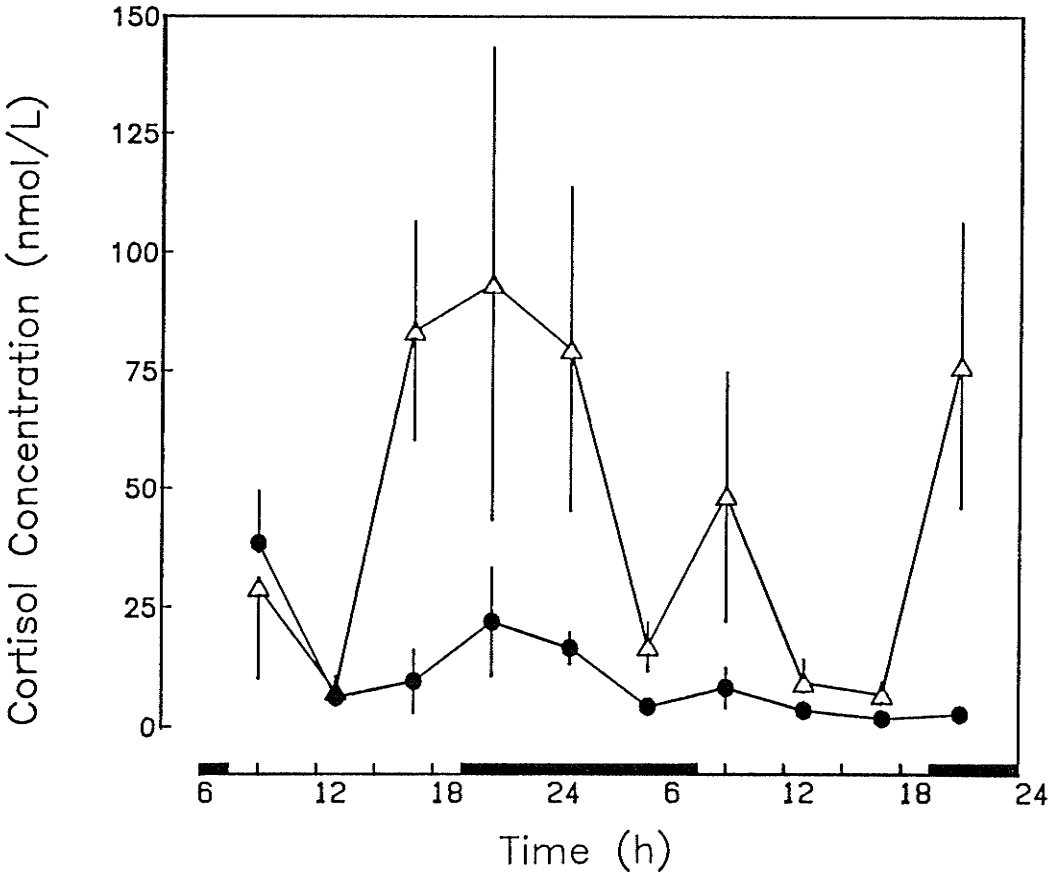
Diel changes in plasma hormone levels will complicate plasma kinetic studies because steady-state assumptions are violated. It is therefore necessary to establish if plasma cortisol levels undergo significant diel variation. Plasma cortisol for non-cannulated free-swimming trout was slightly elevated at 0900 and 2100 on day 1 (Fig. 3.2), but these changes were not evident the next day. In cannulated trout held in the same room and on the same water supply plasma cortisol increased on both days 1 and 2. Although these changes occurred mainly at sampling times

following a change in light intensity, they were in all likelihood due to prior blood removal. Owing to the logistic problem of maintaining sufficient cannulated fish, it was not possible to study cannulated trout sampled terminally at each time in the diel cycle. The blood volume removed from each fish at each sampling represented 1% of the total blood volume for a sum of 5% over the experiment. Similar elevations in plasma cortisol were noted by Bry (1982) who then adopted a 28-h rather than 4-h sampling interval in diurnal experiments with cannulated trout. Diel changes in cortisol have been associated with long photoperiod, feeding and low stocking densities (Bry 1982; Rance et al. 1982; Pickering and Pottinger 1983; Spieler and Noeske 1984). Fish in this study were held on an intermediate photoperiod (12 h), starved and the stocking density was low.

No consistent diel change occurred in plasma glucose and protein or PCV but all 3 parameters were significantly lower in the cannulated and serially-bled trout than in the free-swimming trout (data not shown). This trend was evident for glucose and protein in Expt 1 but was not significant. The larger sample size in Expt 2 presumably allowed better statistical discrimination.

In summary, the holding conditions (starvation and intermediate photoperiod) appeared to attenuate daily fluctuations in plasma cortisol and other measured parameters in free-swimming uncannulated fish. However, repetitive blood sampling of cannulated fish caused increases in plasma cortisol levels.

FIGURE 3.2. Plasma cortisol in cannulated trout (Δ) and in free-swimming and non-cannulated control fish (●) sampled at intervals during a 12-h photoperiod (light and dark periods indicated). Values represent mean ± SE of 5 fish.


Cortisol Concentration (nmol/L)
150
125
100
75
50
25
0
6
12
18
24
6
12
18
24
Time (h)

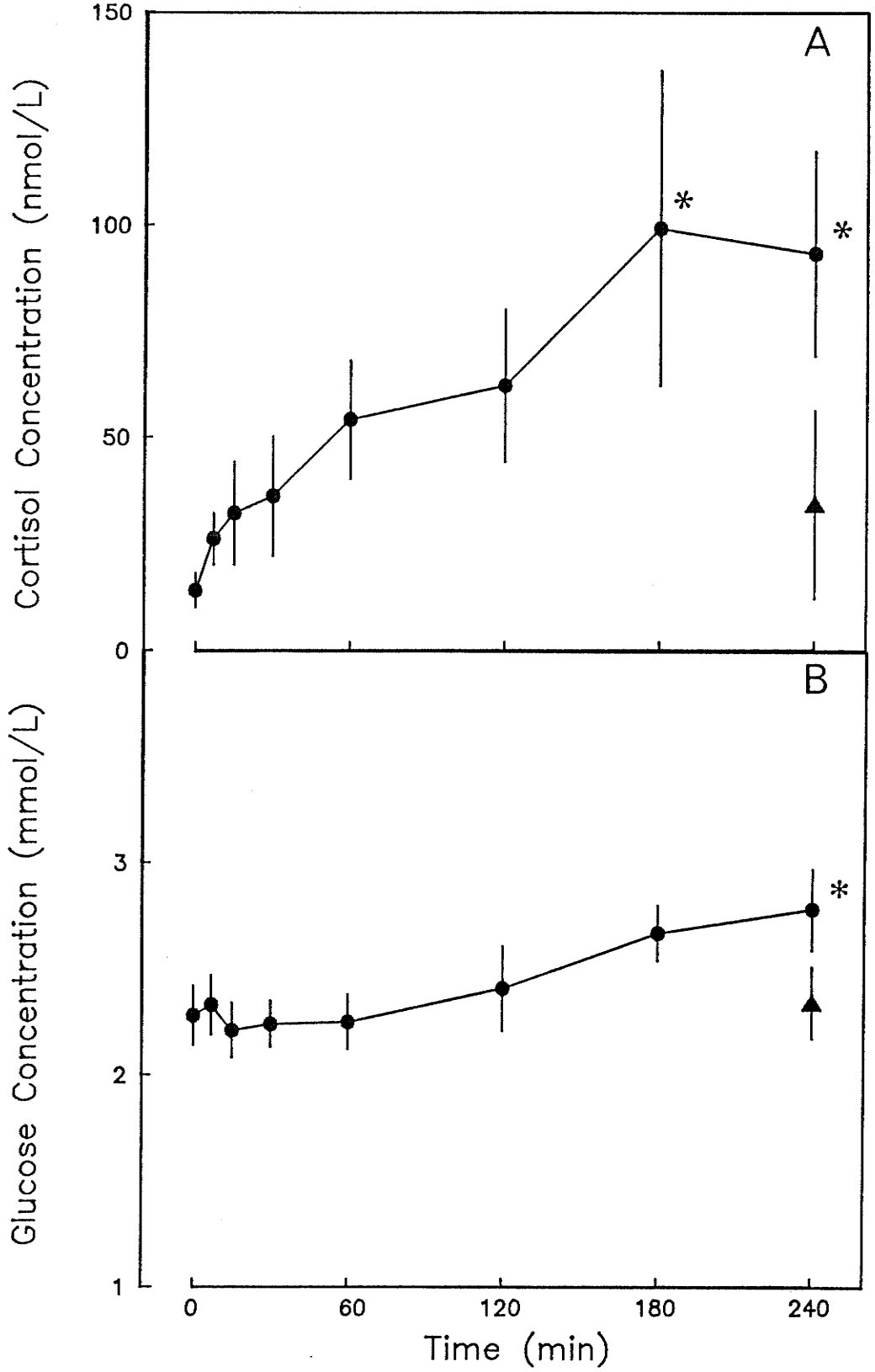
3.5.3 Experiment 3: Effects of Serial Blood Sampling

Blood removal was correlated with a progressive increase in plasma cortisol from 15 to 100 nmol/L at 180 min (Fig. 3.3A). Plasma cortisol levels of undisturbed cannulated trout at 240 min did not differ from 0-min values but were significantly different from values for serially-bled trout at 180 and 240 min. Therefore, as in other studies on rainbow trout (Bry 1982), at least part of the plasma cortisol elevation was due to blood removal. Nichols and Weisbart (1984b) repetitively bled large Atlantic salmon (1-4 kg) at 15-min intervals for 5 h. Because plasma cortisol did not consistently rise after their first sample, and the peaks were randomly timed, their evidence suggested a more episodic cortisol secretion pattern.

Plasma glucose levels increased slightly but significantly in serially bled trout (Fig. 3.3B). Soivio et al. (1975) also noted elevation of plasma glucose in repetitively sampled rainbow trout. Mean PCV (20.9 ± 1.6%) and plasma protein 17.5 ± 0.8 mg/mL) did not change with time and did not differ from values for terminally-bled cannulated trout (data not shown).

It is concluded that when using trout of the size used (≈300 g), repetitive blood sampling acts as a stressor (as judged by plasma glucose and cortisol), making it unwise to estimate cortisol PCR by any technique involving serial blood removal.

FIGURE 3.3. Temporal changes of (A) plasma cortisol and (B) plasma glucose after pulse delivery of 20 µL of saline-ethanol vehicle into serially-sampled cannulated trout (●). Cannulated trout bled only once at 240 min are indicated (▲). Values represent mean ± SE of 8 fish. Significant differences from time 0 are indicated (*).


150
100
50
0
A
Cortisol Concentration (nmol/L)
*
*
3
2
1
B
Glucose Concentration (mmol/L)
*
0
60
120
180
240
Time (min)

3.5.4 Experiment 4: Effects of Vehicle Infusion

Infusion of saline:ethanol vehicle caused a major increase in plasma cortisol at 2 h (Fig. 3.4). At 24 h plasma cortisol was 110 nmol/L. When vehicle infusion commenced 24 h prior to removal of the first blood sample, plasma cortisol did not differ from that of trout which had not been infused, and plasma cortisol levels showed no significant increase with time. By 24 h plasma cortisol had regained initial levels of 50 nmol/L. Plasma glucose (1.6 ± 0.1 mmol/L), protein (21.3 ± 3.5 mg/mL) and PCV (21.4 ± 1.8%) were not influenced by infusion for 24 h (data not shown).

The direct cause of the large increase in cortisol was not examined. Introduction of vehicle at 70 µL/h should not alter blood volume because this value represents 3% of the glomerular filtration rate (adjusted to equal fish size; Holmes and McBean 1963). The effects of type of injection vehicle or pump vibrations await further study. Use of larger fish may alleviate some of the observed increase in plasma cortisol.

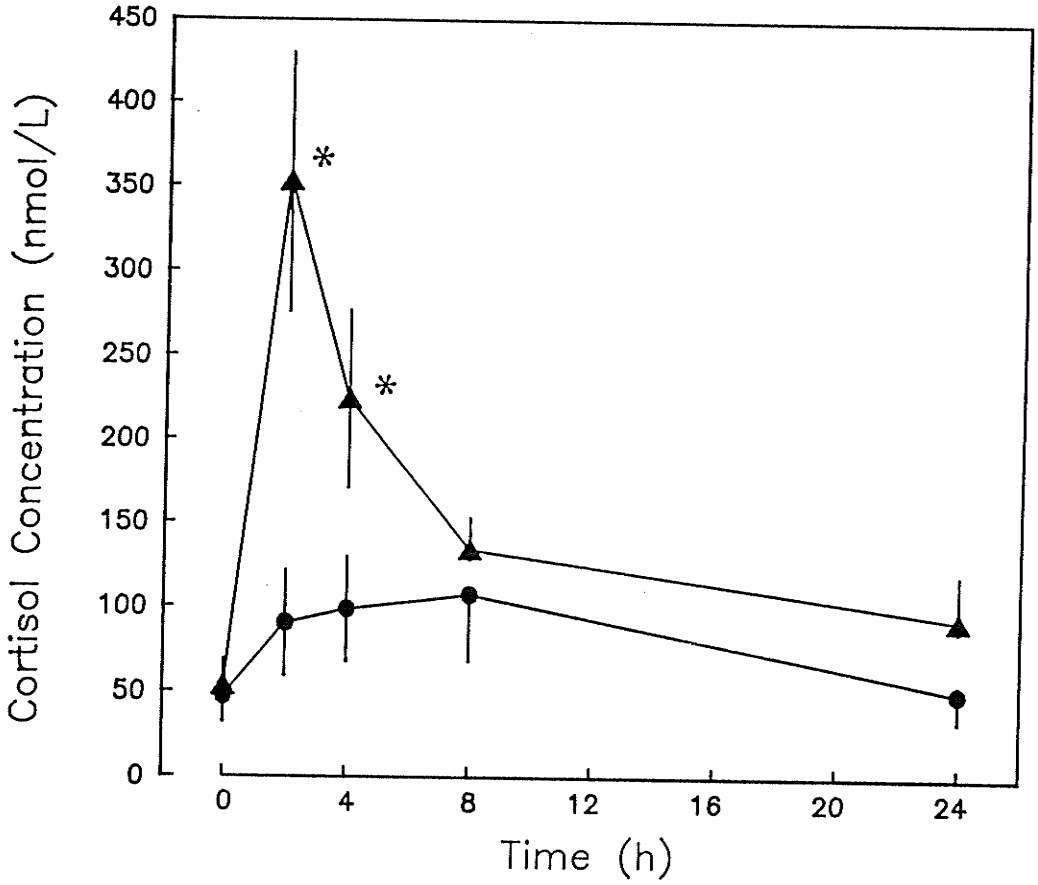
It is concluded that a potentially feasible protocol for estimation of cortisol PCR would be to:

i) infuse saline:ethanol vehicle for 24 h;

ii) infuse labelled cortisol through a cannula until constant plasma cortisol radioactivity is obtained; and

iii) remove a single terminal blood sample for measurement of radioactive and total cortisol.

FIGURE 3.4. Temporal changes of plasma cortisol in trout continuously infused (70 µL/h) with saline-ethanol vehicle. Fish sampled the same day as infusion began (▲) and fish sampled 24 h after initiation of infusion (●) are indicated. Each point represents mean ± SE from 4 terminally sampled fish. Significant difference from time 0 (*).


Cortisol Concentration (nmol/L)
450
400
350
300
250
200
150
100
50
0
*
*
0
4
8
12
16
20
24
Time (h)

3.5.5 Experiment 5: Effect of Acid on Plasma Cortisol Levels and Kinetics

Following infusion of labelled cortisol, radioactivity in the plasma TLC fraction attained a steady state by 16 h (Fig. 3.5). Trout used to determine the cortisol PCR were bled at 24 h (Table 3.1). Cortisol PCR was unaltered after acid exposure but the cortisol PDR was increased almost 4-fold by acid treatment largely due to an increase in the plasma cortisol concentration. Ashcom (1979) following plasma cortisol patterns in brook trout from water at pH 4.0, also suggested that the plasma clearance rate was not appreciably altered by acid exposure. Because trout were chronically confined and starved, it is possible that they displayed maximum PCR for cortisol and were refractory to further acid stress.

In addition, the results emphasize that clearance rates calculated using total or extractable radioactivity (Ilan and Yaron 1983; Redding et al. 1984; Patino et al. 1985) can underestimate the clearance of authentic cortisol by 2- to 4-fold. Present clearance values calculated using total radioactivity are roughly comparable to values (53-83 mL/h per kg) reported by Redding et al. (1984). The PCR values for cortisol (TLC fraction) correspond to values described for sockeye salmon, *Oncorhynchus nerka* (Donaldson and Fagerlund, 1968, 1970) and Atlantic salmon (Nichols and Weisbart 1985) but are considerably greater than values recorded for other species (Owen and Idler 1972; Leloup-Hatey 1974, 1976; Goodman and Butler 1976).

FIGURE 3.5. Profile of plasma radioactivity in TLC fraction following a priming dose and constant infusion of [^{3}H]cortisol into cannulated trout. Each point represents mean ± SE from 4 terminally sampled fish. Thus a total of 20 fish was infused.

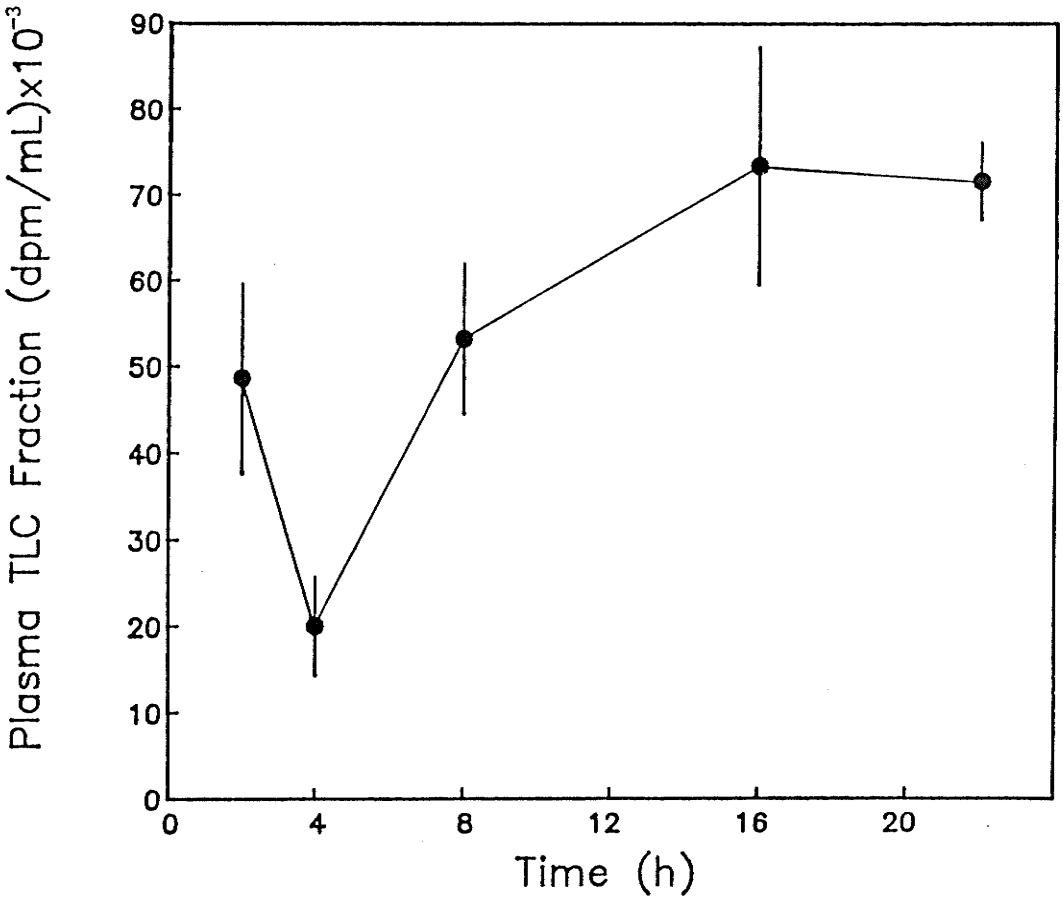

Plasma TLC Fraction (dpm/mL)x10^{-3}
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
Time (h)

TABLE 3.1 Effect of acid exposure for 7-10 d on cortisol PCR and selected blood parameters of cannulated rainbow trout. Values represent mean (SE) from 12 fish and are based on a body weight of 1 kg. Significant differences from pH 7.7 are indicated (*).

Parameter	pH 7.7	pH 5.0
Plasma Cortisol (nmol/L)	60 (14)	134 * (19)
Cortisol PCR (TLC fraction) (mL/h)	303 (42)	354 (62)
Cortisol PCR[1] (extractable) (mL/h)	139 (37)	159 (39)
Cortisol PCR[1] (total) (mL/h)	75 (24)	125 (35)
Cortisol PDR (nmol/h)	15.5 (3.3)	59.1 * (12.7)
PCV (%)	20.2 (1.2)	28.5 * (4.0)
Plasma Glucose (mmol/L)	2.7 (0.3)	4.0 * (0.5)
Plasma Protein (g/L)	25 (1)	31 * (3)

[1] PCR calculated using extractable or total radioactivity instead of TLC fraction to facilitate comparisons to other studies.

Elevations in plasma cortisol, glucose, protein and PCV (Table 3.1) corresponded to previous results in acid-stressed trout (PART 1; McDonald 1983; Giles et al. 1984). It is worth noting that plasma glucose, protein and packed cell volume generally were not clearly correlated with increased plasma cortisol in Expts 1 to 4. In support, large increases in plasma cortisol were induced in Atlantic salmon by ACTH injections (Nichols and Weisbart (1984b) but no changes were found in plasma glucose. These and the present findings contrast with those in other teleosts where cortisol has been implicated in the control of blood glucose (Chan and Woo 1978; Leach and Taylor 1980, 1981). Further study of the control of plasma glucose in acid-exposed fish and fish stressed in other ways appears necessary.

3.6 CONCLUSIONS

The effects of factors which may influence plasma cortisol levels in kinetic studies (cannulation, diurnal change, serial sampling and vehicle infusion) were evaluated. A feasible protocol for estimation of cortisol PCR and PDR by constant infusion in minimally stressed fish was applied to trout exposed to water at pH 5.0. Acidic conditions did not alter cortisol PCR but due to elevated plasma cortisol levels, cortisol PDR was increased. Low pH elevated plasma glucose corroborating previous findings (PARTS 1 and 2). Increased PCV and plasma protein under acidic conditions was supportive of impaired fluid balance.

PART 4
EFFECTS OF LOW AMBIENT pH AND ALUMINUM ON PLASMA KINETICS OF CORTISOL, T_3 AND T_4 [1]

4.1 SYNOPSIS

Plasma kinetics for cortisol, T_3 and T_4 and other blood parameters were examined in cannulated rainbow trout exposed for 7 d to a sublethal ambient pH of 4.7 (H_2SO_4), an ambient pH of 4.7 plus Al ($AlKSO_4$, 20 µmol/L) or an ambient pH of 7.7 (controls). Sublethal pH alone increased the cortisol level and the cortisol PDR but did not change the cortisol PCR; it did not modify plasma T_3 and T_4 levels or their kinetics; it increased plasma glucose and decreased plasma Cl^- and osmolality with no change in PCV. Low pH in combination with Al increased the cortisol plasma level, PCR and PDR; it increased the T_4 PDR, but decreased the T_3 PCR and T_3 plasma appearance rate (PAR); it also increased plasma glucose and depressed plasma Cl^- and osmolality but increased PCV. It is concluded that Al exacerbates the effects of low ambient pH to increase cortisol production and decrease T_3 production.

[1] Previously published (Brown et al. 1990a).

4.2 INTRODUCTION

The short term laboratory responses of freshwater teleost fish to low ambient pH include ion loss from body fluids (McDonald 1983). Over a longer period, field and laboratory studies suggest that low pH reduces growth (Tam and Payson 1986; Tam et al. 1988), reflecting possible shifting of resource use from somatic and gonadal growth towards maintenance of homeostasis (Tam et al. 1988). In nature, these effects of low pH are potentially complicated by the effects of metals, particularly aluminum, which may be liberated from catchment soils by depressed pH (LaZerte 1984). The endocrine aspects underlying these physiological changes, while incompletely understood, implicate both the interrenal and thyroid systems. Low pH alone, or in combination with Al elevates plasma cortisol levels (PARTS 1 and 2; Brown et al. 1986a; Goss and Wood 1988; Tam et al. 1987) and causes sustained activation of pituitary-corticotrophs while depressing thyrotroph activity (Tam et al. 1988). Plasma T_4 and T_3 may also be influenced by low pH (PARTS 1 and 2; Edwards et al. 1987), but the response profile is incompletely defined. To date, there has been one other study on the effects of Al on the fish thyroid system (Whitehead and Brown 1989). Moreover, evaluating the response of both the interrenal and thyroid systems to an environmental stressor provides a first step towards testing the hypothesis that interrenal activity antagonizes thyroid function (Redding et al. 1986).

To obtain a more complete perspective on the interrenal and thyroid responses to low ambient pH and aluminum, I investigated the effects of these agents alone or in combination, on plasma levels of cortisol, T_3 and

T_4 and on the plasma kinetics of these hormones. This was done to evaluate the rates of hormone addition to and removal from the plasma pool. Measurement of plasma glucose, osmolality, Cl^- and PCV were used to substantiate previously described effects of low pH and Al.

4.3 MATERIALS AND METHODS

4.3.1 Fish Maintenance

Rainbow trout (2-3 yrs; weight 403 ± 14 g; $\hat{x}$ ± SE, N = 115) were held under conditions described previously (see PARTS 1-3). Fish were fed at a ration of 1.5% wet body weight per day and were acclimated to laboratory holding conditions at least 3 weeks prior to their use in experiments. Experiments were conducted between February and August.

4.3.2 Pulse Injection of $[^{125}I]T_3$

Fish were cannulated (see PART 3) and allowed to recover in 60-L aquaria for 7 d prior to use in experiments. All fish had a 600-µL blood sample removed just prior to injection. Plasma was separated by centrifugation and 100 µL set aside for T_3 radioimmunoassay. For each fish 100 µL of its own plasma was used to resuspend $[^{125}I]T_3$ (780 mCi/µmol, Amersham) which had been dried in a microcentrifuge tube (500 µL). The plasma and tracer were aspirated into a section (45 cm) of cannula tubing and it was attached to the cannula implanted into the fish. The $[^{125}I]T_3$ in the plasma vehicle was injected into the fish and the contents of the tubing immediately flushed into the fish with the remaining plasma (200 µL) followed by 300 µL trout saline. The piece of

tubing which had contained the tracer was removed and a new piece containing saline attached. After corrections for iodide contamination and residual counts in the microcentrifuge tube and injection cannula, it was calculated that each fish received 0.73 ± 0.02 µCi [^{125}I]T_3. Preliminary experiments indicated that residual radioactivity in the cannula attached to the fish was low (<200 cpm) and did not contaminate subsequent blood samples. Blood samples (200 µL) were removed after discarding the cannula dead volume at 5, 13, 45, 120, 480, 960 and 1440 min. At 2880 min a large terminal blood sample (1 mL) was removed. The total blood volume removed prior to the last sample was <10% based on a blood volume of 3.0% body weight (see section 4.4.2).

Plasma volume (V_p) was determined in a separate group of 5 cannulated fish. After removing an initial blood sample (300 µL) fish were injected through the cannula with 0.056 ± 0.002 µCi [^{125}I]fibrinogen (1.2 mCi/mg, ICN). Tracer was immediately flushed into the fish with plasma (200 µL) and fish saline (400 µL). The injected dose was corrected for iodide contamination (10.9%) and loss due to adherence to injection tubing and syringe. Preliminary experiments indicated that residual counts in the cannula after injection were low (<50 cpm) and did not affect counts obtained in subsequent blood samples. After discarding the cannula dead volume, 150 µL of blood was sampled from the cannula using heparinized syringes at 2.5, 5.5, 10.5, 15.5, 20.5 and 25.5 min. Plasma samples (50 µL) were analyzed for [^{125}I]fibrinogen after separation from contaminant radioiodide on Pharmacia PD10 columns. A single exponential function $Y(t) = Ae^{-at}$ was fitted to the data for each fish, where Y represents the fraction of [^{125}I]fibrinogen in 1 mL of plasma and t represents the time

after [^{125}I]fibrinogen injection. The V_p was calculated from $V_p = 100/A$ and the blood volume = V_p + ((PCV/100) X V_p) (Nikinmaa et al. 1981).

A three-compartment model was used for the analysis of T_3 kinetics. A similar model was used previously for studying T_3 kinetics in rats (Distefano et al. 1982). In addition, the independent data for plasma volume were included in the fitting procedure at time 0. The curves describing loss of [^{125}I]T_3 (% dose/mL) were analyzed by the graphical curve peeling method (Shipley and Clark 1972) as previously applied to plasma T_3 and T_4 kinetic data in trout (Eales et al. 1982, 1986). This provided estimates of A_1, a_1, A_2, a_2, A_3 and a_3 for the nonlinear regression equation $Y(t) = A_1e^{-a1t} + A_2e^{-a2t} + A_3e^{-a3t}$, where Y represents the fraction of [^{125}I]T_3 in 1 mL of plasma. These values were then used to provide starting values for the computerized nonlinear, weighted least-squares regression described by Distefano et al. (1982). The computer derived terms were then used to calculate T_3 PCR, T_3 plasma appearance rate (PAR) and T_3 pool sizes exactly as outlined by Distefano et al. (1982) for a three-pool mammillary model. The production of T_3 from T_4 can and does occur in several body tissues and the T_3 formed does not necessarily have to traverse the plasma pool prior to degradation. Therefore, using plasma data to estimate plasma degradation of T_3, one can only obtain a minimum estimate, more appropriately termed the PAR (Distefano 1986) and T_3 PAR = PCR X PC, where PC represents the plasma hormone concentration determined by radioimmunoassay.

4.3.3 Infusion of Labelled Hormones

Plasma kinetics of cortisol, T_3 and T_4 were also assessed using the previously described constant infusion technique (PART 3). After 3 d ([^{125}I]T_3) or 5 d ([^{3}H]cortisol and [^{125}I]T_4) of exposure to an ambient pH of 4.7, pH 4.7 plus Al or pH 7.7 (controls), trout saline:plasma (9:1, v/v) was infused through the cannula using infusion pumps (see PART 3) at a rate of 50-60 µL/h. At 24 h, the pumps were momentarily stopped, and after elimination of the cannula dead volume, an initial blood sample (100 µL) was removed prior to labelled hormone infusion. No additional blood was removed until infusion was completed. After removal of the initial sample the fish were injected through the cannula with a 1-µCi priming dose of the respective tracer and the syringes containing vehicle exchanged for those also containing [1,2,6,7-^{3}H(N)]cortisol (85.5 Ci/mmol, New England Nuclear), [^{125}I]T_3 (780 mCi/µmol, Amersham) or [^{125}I]T_4 (900 mCi/µmol, Amersham). The amount of radioactivity infused was 0.2 µCi/h for T_3 and T_4.

The time required after labelled cortisol infusion to attain constant plasma cortisol radioactivity indicating steady state was 16 h (PART 3). Routinely, labelled cortisol was infused for 24 h. Based on experiments conducted in this study T_4 and T_3 required 24 h and 96 h of infusion respectively, to attain steady-state in plasma. In all cases, fish were exposed to low pH or low pH and aluminum for 7 d before sampling. At this time trout were rapidly anesthetized in pH-neutralized TMS (0.76 mmol/L) and blood was removed from the caudal vessels using 3- or 5-mL heparinized syringes. Plasma was obtained by centrifugation and stored at -70 °C until analyzed.

The PCR values were calculated from the known rates of labelled hormone infusion and the PDR for cortisol and T_4 were calculated as outlined in PART 3. The T_3 PAR was calculated as described previously (section 4.3.2)

4.3.4 Identification of Labelled Hormones in Plasma and Infusion or Injection Media

[^{3}H]Cortisol. Plasma (300 µL) and infusion medium (50 µL) were incubated with non-radioactive cortisol (28 nmol) and a known amount of [^{14}C]cortisol; (55 mCi/mmol, New England Nuclear) and extracted with 5 mL dichloromethane as outlined in PART 3. The dichloromethane extracts were dried, redissolved in dichloromethane:methanol (9:1, v/v), applied to TLC plates and the plates developed in dichloromethane:methanol:water (150:9:0.5, v/v) (see PART 3). The area containing cortisol was visualized under short-wave UV radiation, aspirated, eluted into liquid scintillation vials, dried and counted (PART 3). Recovery of [^{14}C]cortisol was used to correct for procedural losses of [^{3}H]cortisol for each individual sample.

To provide estimates independent of the TLC procedure, [^{3}H]cortisol was determined in plasma by high performance liquid chromatography (HPLC). The HPLC system consisted of 2 Model 302 solvent pumps, a Model 231 sample injector, a Model 704 system controller, a Model 620 data module, a Model 116 dual channel UV absorbency detector set at 254 nm, and a Model 201 fraction collector (Gilson Medical Electronics). Residues from the dichloromethane extracts were redissolved in mobile phase and injected onto a 3-micron Adsorbosphere HS C_{18} column, 4.6 mm i.d. by 104 mm length with an attached 10-mm Adsorbosphere cartridge guard column

(Alltech Associates Inc.). Corticosteroids were eluted with a 25-min gradient of 50-70% methanol and water (O'Hare and Nice 1981) delivered at a flow of 0.5 mL/min. Fractions were collected at 0.5-min intervals, dried and counted.

Non-radioactive cortisol, cortisone, corticosterone and 11-deoxycortisol standards were purchased from Sigma or Steraloids. Dichloromethane (Caledon Lab.) and methanol (Fisher) were HPLC grade. Distilled water was purified using a MilliQ water system (Millipore).

$[^{125}I]T_3$ and $[^{125}I]T_4$. Plasma $[^{125}I]T_3$ and $[^{125}I]T_4$ and infusion or injection media were separated by a technique (Sephadex/Ab) in which excess antibodies specific for T_3 or T_4 (Calchemical Lab., Calgary, Alta.) sequentially elute $[^{125}I]T_3$ or $[^{125}I]T_4$ from plasma samples directly applied to miniature G-25 Sephadex columns (Eales 1977).

4.3.5 Exposure to Acid and Al

Fish (6 per treatment) were cannulated via the dorsal aorta as described in PART 3. After cannulation fish were not fed and were allowed to recover for 7 d after surgery. Following recovery, trout were held for 7 d in individual restraining chambers (60-L aquaria) supplied with acidified water (pH 4.7, H_2SO_4), acidified water plus 20 µmol Al/L or control water (pH 7.7). Low pH water was obtained using the acidification-decarbonation system described in PART 1. Water temperature averaged 12.8 ± 0.8 °C ($\hat{x}$ ± SE) and free CO_2 concentration was 44 ± 6 µmol/L ($\hat{x}$ ± SE). Instrument calibrations were checked daily against a standardized pH meter. Stock solutions of Al ($AlKSO_4$) were prepared in deionized water and acidified with 1 mL of concentrated HNO_3

per liter. The stock solution was perfused into the incoming water line of each test chamber using multichannel peristaltic pumps. Each test chamber received at least 2 L of aerated water per gram of fish each day.

4.3.6 Measurement of Aluminum

Procedures for fractionation and measurement of Al were similar to those schemes described in PART 2. Levels of fluoride and total inorganic SO_4^{2-} levels were measured as in PART 2. Calculations of Al species in the monomeric fraction were performed according to Driscoll (1984). A complete set of Al measurements and speciation calculations was conducted on water collected from the outflow of each test chamber after 1 and 7 d of exposure. All Al measurements were completed on the day of sampling.

4.3.7 Blood Analyses

Radioimmunoassays for cortisol, T_3 and T_4 were performed as described previously (PART 1). Hormone measurements were conducted on both the initial and terminal plasma samples to ensure minimal change (<20%) occurred during the course of the experiment. Plasma cortisol could be measured in fish infused with [^{3}H]cortisol because the cortisol assay used cortisol-3-(*O*-carboxymethyl)-oximino-(2-[^{125}I]iodohistamine) as tracer. The [^{3}H]cortisol does not interfere with ^{125}I tagged cortisol which was counted in an LKB CompuGamma counter. For [^{125}I]T_3- and [^{125}I]T_4-infused fish, plasma radioactivity was elevated 20-fold prior to assay with [^{125}I]T_3 or [^{125}I]T_4 to limit error in plasma hormone determinations to <5%. Plasma glucose, osmolality and chloride were measured as described previously (PART 2). Packed cell volume (PCV) was determined in

triplicate on whole blood using Clay-Adams heparinized microhematocrit tubes.

4.3.8 Statistics

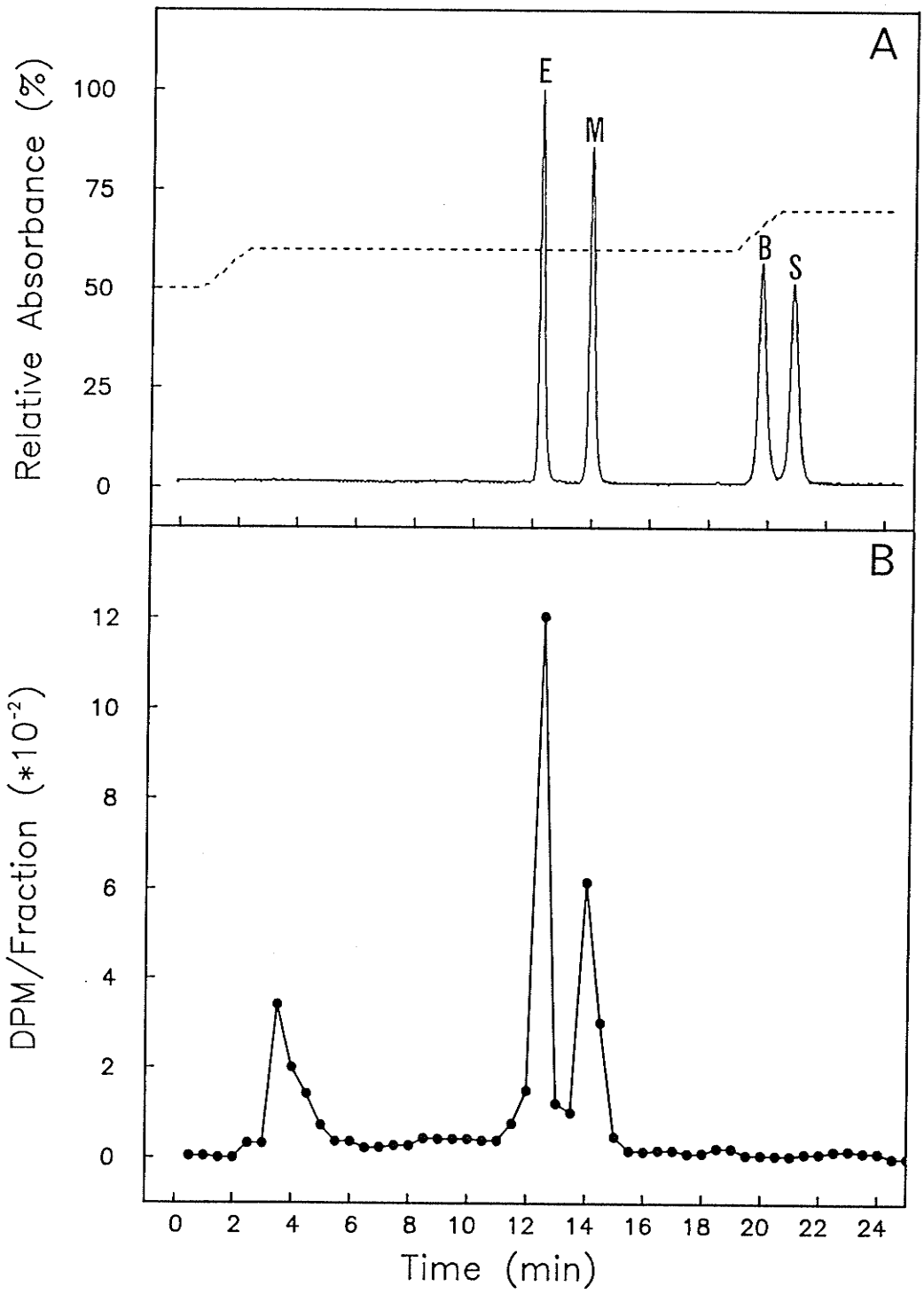
Bartlett's test for homogeneity of variance was applied to data and where required, data were transformed according to Taylor's power law to obtain more uniform variance (Southwood 1978). For clarity of presentation, untransformed arithmetic means and standard errors are given in the Results. One-way ANOVA was computed and Tukey's Studentized Range test was used to distinguish differences between group means. Probability levels of <0.05 were considered significant.

4.4 RESULTS

4.4.1 Recovery and Separation of Radioactive Materials in Plasma

Cortisol. Recovery of added [^{14}C]cortisol from plasma by extraction with dichloromethane was 84.5 ± 2.8% ($\hat{x}$ ± SE, N = 23). Cortisol and cortisone were completely separated using HPLC (Fig. 4.1A). The elution profile of unlabelled standards was unaltered by co-separation with plasma from [^{3}H]cortisol-infused fish and a major radioactive peak coincided with the cortisol peak (M) (Fig. 4.1B). The first peak corresponded to the void volume. The second peak corresponded to cortisone (E). Although the measured radioactivity co-eluted with the added cortisol standard, radioactive components other than cortisol could contribute to this fraction. I therefore refer to the radioactive fraction

FIGURE 4.1. Separation of corticosteroids by reverse phase HPLC. A- Relative absorbance at 254 nm of 200 ng standards of cortisone (E), cortisol (M), corticosterone (B), and 11-desoxycortisol (S); dashed line indicates % methanol in gradient. B-Elution profile from a fish infused with [^{3}H]cortisol.


A
E
M
B
S
Relative Absorbance (%)
100
75
50
25
0
B
DPM/Fraction (*10-2)
12
10
8
6
4
2
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (min)

co-eluting with standard cortisol as the M fraction because recrystallization to constant specific activity was not done. When the routinely used TLC procedure (PART 3) and HPLC were compared, similar estimates of radioactive material co-eluting with standard cortisol were obtained (Table 4.1).

T_3 and T_4. The Sephadex/Ab extraction of plasma provided recoveries of added [^{125}I]T_3 and [^{125}I]T_4 that exceeded 93.5% with less than 1.5% cross-contamination of fractions. Following infusion of [^{125}I]T_3 and [^{125}I]T_4, plasma radioactivity associated with T_4 antisera attained a steady state by 18 h (Fig. 4.2A); while radioactivity associated with T_3 antisera required 72 h to achieve steady state (Fig. 4.2B).

4.4.2 Plasma Volume and T_3 Kinetics by Pulse Injection

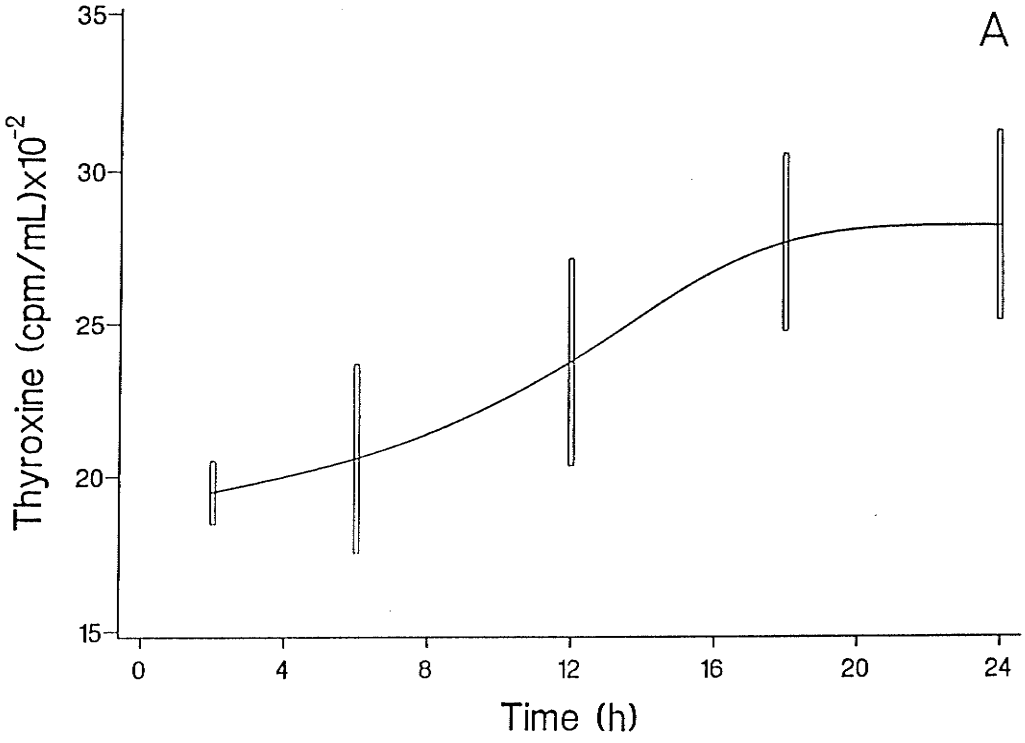
The mean values of coefficients and exponents of the best single exponential fit to the fibrinogen kinetics data for individual fish were, respectively A = 4.4 ± 0.3 % dose/mL per kg and a = -0.012 ± 0.002 (min^{-1}). Estimates of V_p in the fish injected with [^{125}I]fibrinogen (Fig. 4.3) averaged 23 ± 2 mL per kg. Mean PCV was 28.9 ± 1.9% and total blood volume was calculated at 29.6 ± 2.2 mL per kg.

For each fish examined, loss of plasma [^{125}I]T_3 with time conformed significantly better to a 3-compartment model than a 2-compartment model (Fig. 4.4). The mean T_3 PCR estimated from fitting 3-compartment models was 6.0 ± 0.4 mL/h per kg (Table 4.2). The plasma pool (Q_p) represented only 6.2% of the total T_3 pool (Q_d) in trout (Fig. 4.5). The first fast tissue pool (Q_2)contained 12.3% and the second slow tissue pool (Q_3) the remaining 81.5% of Q_d.

TABLE 4.1 Estimates of plasma radioactive materials corresponding to authentic cortisol. Plasma of [^{3}H]cortisol-infused fish was examined by TLC and HPLC. Percent difference was calculated relative to HPLC measurements. Values represent mean of 3 replicate analyses from randomly selected fish.

Fish	HPLC (dpm/mL)	TLC (dpm/mL)	% Difference
A	6196	6940	-12.0
B	3334	2881	13.6
C	4239	4399	2.4
D	3036	3537	-16.5
E	4618	4237	8.3

FIGURE 4.2. Increase in plasma ^{125}I-labelled materials following a priming dose and constant infusion. A-Radioactivity eluted by T_4 antibody from plasma of $[^{125}I]T_4$-infused fish. B-Radioactivity eluted by T_3 antibody from plasma of $[^{125}I]T_3$-infused fish. Each point represents mean ± SE from 4 terminally sampled fish. Total number of fish infused was 20 for each hormone.


A
Thyroxine (cpm/mL)x10^{-2}
35
30
25
20
15
0
4
8
12
16
20
24
Time (h)

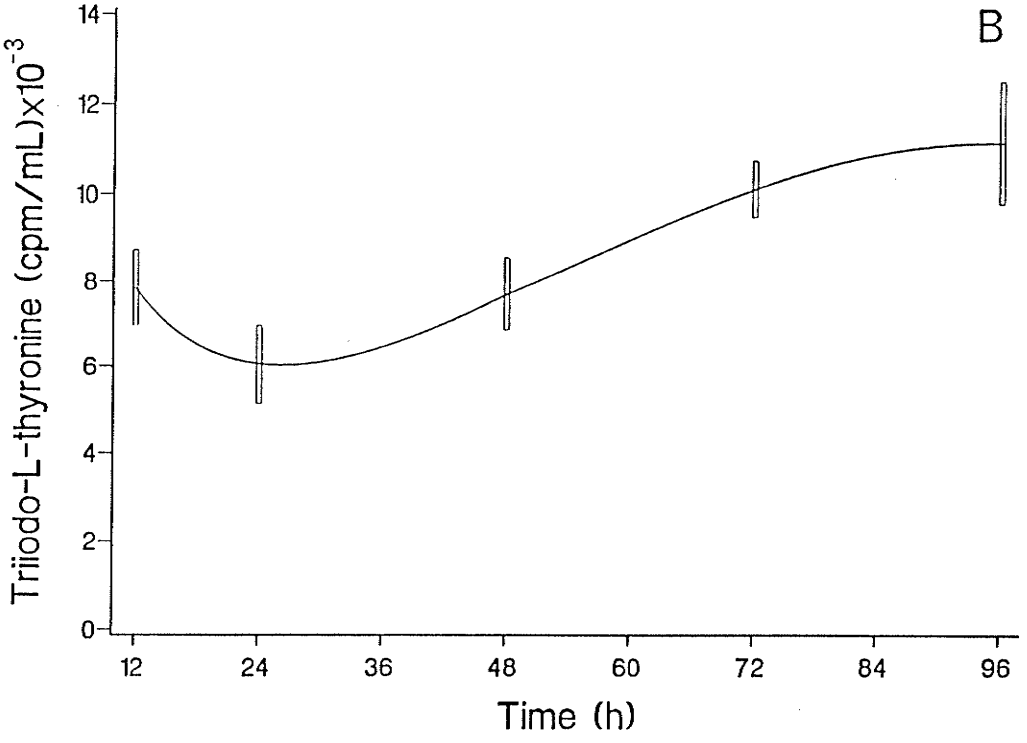

B
Triiodo-L-thyronine (cpm/mL)x10^{-3}
14
12
10
8
6
4
2
0
12
24
36
48
60
72
84
96
Time (h)

FIGURE 4.3. Plasma [^{125}I]fibrinogen from plasma after its injection in 5 rainbow trout (A-E). The data points were fitted with a single exponential function (■——■) to determine t_0 tracer concentration and hence the plasma volume (V_p).

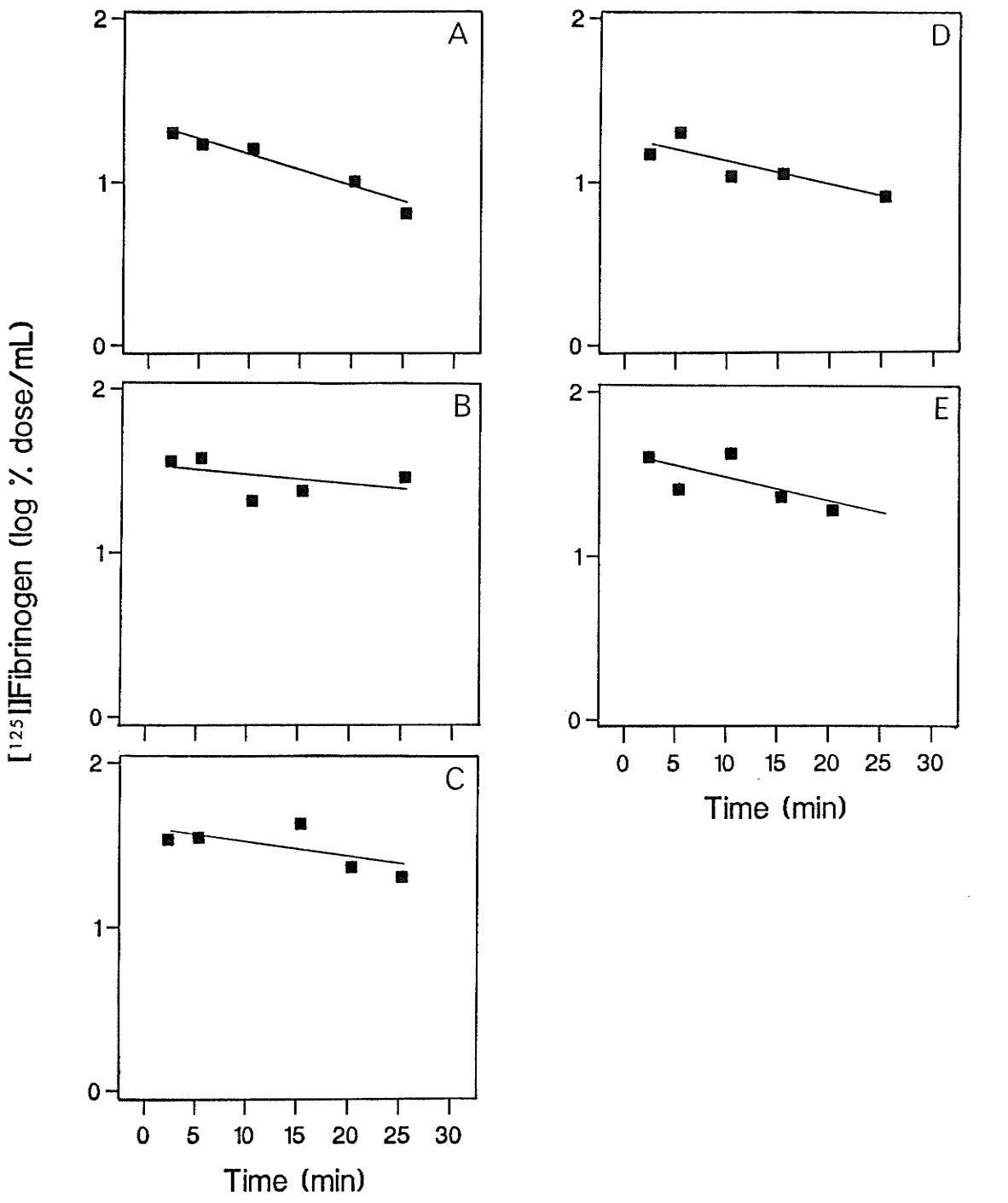

[125I]Fibrinogen (log % dose/mL)
A
B
C
D
E
2
1
0
0 5 10 15 20 25 30
Time (min)

FIGURE 4.4. Disappearance of plasma [^{125}I]T_3 after its injection in 5 rainbow trout (A-E). Each point represents the mean of duplicate analyses. The best fit 3-exponential functions (●——●) were used to calculate T_3 kinetic parameters (Table 4.2).

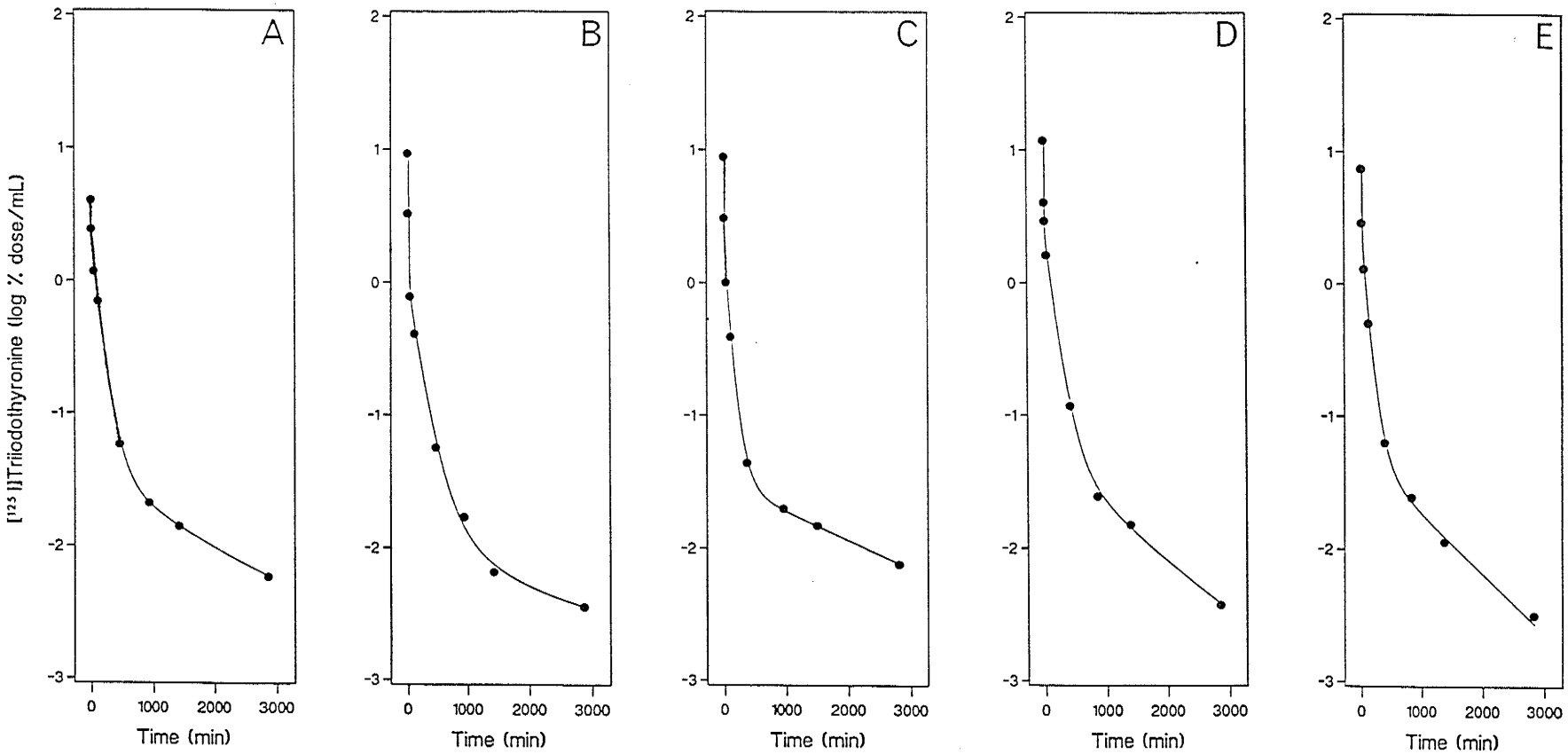
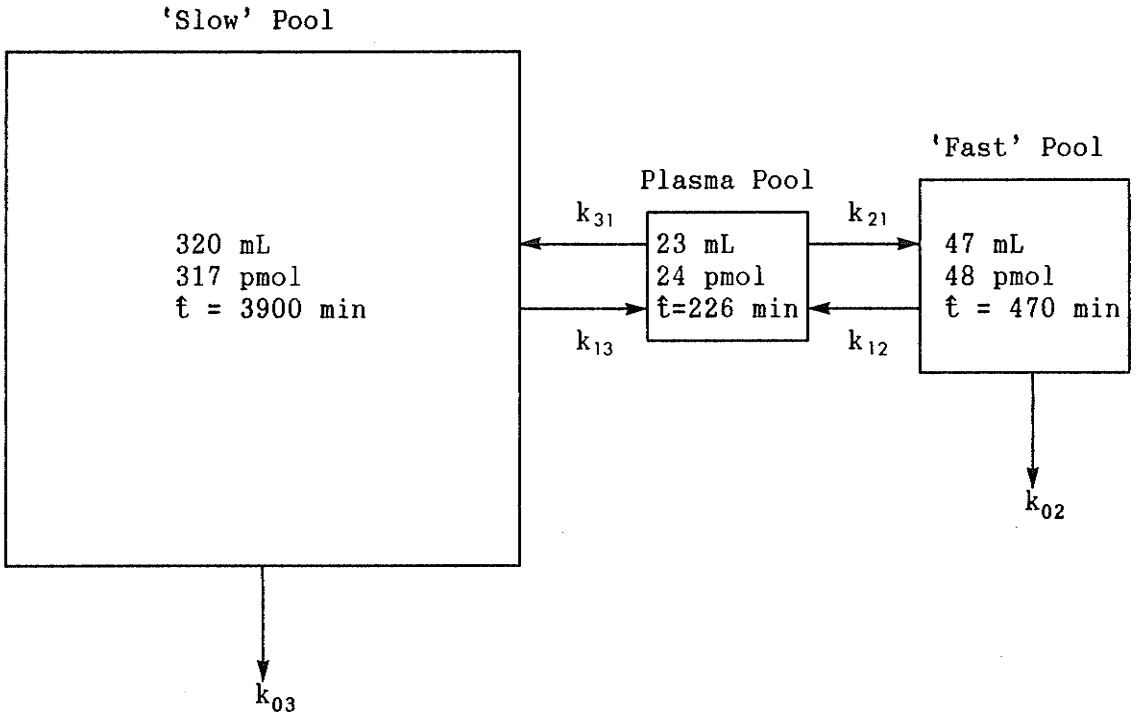

[123 I]Triiodothyronine (log % dose/mL)
A
B
C
D
E
2
1
0
-1
-2
-3
0
1000
2000
3000
Time (min)

TABLE 4.2 Estimates of kinetic parameters for rainbow trout injected with $[^{125}I]T_3$. Kinetic parameters are based on a body weight of 1 kg.

	Individual Fish					
PARAMETER	A	B	C	D	E	Population ($\hat{x}$ ± SE)
A_1 (% dose/mL)	3.11	3.32	3.17	2.54	3.38	3.10 ± 0.15
a_1 (min^{-1})	-0.706	-0.111	-0.171	-0.177	-0.357	-0.304 ± 0.108
A_2 (% dose/mL)	1.24	0.823	1.12	1.14	1.20	1.10 ± 0.074
a_2 (min^{-1})	-0.0055	-0.0033	-0.0078	-0.0070	-0.0048	-0.0057 ± 0.001
A_3 (% dose/mL)	0.231	0.146	0.222	0.276	0.267	0.228 ± 0.023
a_3 (min^{-1})	-0.00026	-0.00018	-0.00022	-0.00045	-0.00038	-0.0003 ± 0.00005
T_3 (pmol/mL)	1.2	0.6	0.9	1.1	1.5	1.1 ± 0.2
T_3 PCR (mL/h)	5.4	5.5	5.1	7.6	6.2	6.0 ± 0.4
T_3 PAR (pmol/h)	6.5	3.3	4.8	8.4	9.1	6.4 ± 1.1
V_p (mL)	21.8	23.3	22.2	25.3	20.6	22.6 ± 0.8
V_2 (mL)	44.6	65.4	41.3	38.6	44.5	46.9 ± 4.8
V_3 (mL)	298	472	345	246	239	320 ± 43
V_d (mL)	365	560	409	310	304	390 ± 47
Q_1 (pmol)	36.2	14.0	21.1	27.8	29.9	23.8 ± 2.8
Q_2 (pmol)	53.5	39.2	39.2	42.5	64.5	47.8 ± 4.9
Q_3 (pmol)	358	283	328	271	347	317 ± 17
Q_d (pmol)	438	336	368	341	456	388 ± 25

FIGURE 4.5. Three-compartment model for $[^{125}I]T_3$ kinetics in rainbow trout. The plasma pool represents the compartment into which $[^{125}I]T_3$ was injected and from which samples were withdrawn for analysis. The 'fast' pool represents the tissue compartment most rapidly exchanging with plasma (rates k_{21} and k_{12}). The 'slow' pool represents the remaining tissue compartment (rates k_{31} and k_{13}). It is assumed that T_3 loss only occurs in the tissue pools (rates k_{02} and k_{03}). No loss of T_3 is assumed to occur from plasma. The single pass mean transit time for T_3 molecules in each pool are indicated ($\hat{t}$).



$$-k_{11} = k_{21} + k_{31} > 0$$

$$-k_{22} = k_{12} + k_{02} > 0$$

$$-k_{33} = k_{13} + k_{03} > 0$$

$$c_1 = k_{12}k_{21} > 0$$

$$c_2 = k_{13}k_{31} > 0$$

4.4.3 Exposure to Acid and Aluminum

There was no mortality during the 7 d exposures to pH 7.7, pH 4.7 or pH 4.7 plus 20 µmol/L Al. In the acid treated tanks, the pH ranged from 4.6 to 4.9. Concentrations of total Al were close to nominal values averaging 21.6 ± 0.73 µmol/L ($\hat{x}$ ± SE, N = 28). Labile Al consisted largely of AlF species and was 19.2 ± 0.6 µmol/L ($\hat{x}$ ± SE, N = 28), representing 88.7% of total Al.

4.4.4 Effects of Low pH and Aluminium on Plasma Hormone Levels and Kinetics

Trout exposed to pH 4.7 for 7 d had increased plasma cortisol levels and cortisol PDR relative to controls (Table 4.3). Neither plasma T_3 and T_4 concentrations, nor their kinetics were altered by low pH. Low pH plus Al increased plasma cortisol concentration, cortisol PCR and cortisol PDR to a greater extent than did acid alone. Although T_4 PDR was increased and T_3 PCR and T_3 PAR were reduced by low pH plus Al, plasma T_3 and T_4 levels did not change.

4.4.5 Non-hormonal Blood Parameters

Low ambient pH (4.7) for 7 d increased plasma levels of glucose, decreased plasma levels Cl^- and osmolality but did not alter PCV (Table 4.4). Adding Al to low pH water further increased plasma glucose and decreased osmolality, and also elevated PCV.

TABLE 4.3 Effects of low pH and 20 µmol Al/L for 7 d on plasma levels, PCR, PDR or PAR of cortisol, T_3 and T_4 in cannulated trout. Values represent mean (SE) of 6 fish and are based on a body weight of 1 kg. Significant differences from value at pH 7.7 are indicated (*).

PLASMA PARAMETER	pH 7.7	pH 4.7	pH 4.7 + Al
Cortisol (nmol/L)	120 (50)	400 (180)	870 * (310)
Cortisol PCR (mL/h)	176 (28)	213 (59)	260 * (49)
Cortisol PDR (nmol/h)	22 (6)	70 * (30)	249 * (94)
T_3 (nmol/L)	1.5 (0.2)	1.6 (0.6)	1.5 (0.7)
T_3 PCR (mL/h)	6.1 (0.7)	6.2 (2.1)	3.8 * (1.8)
T_3 PAR (pmol/h)	9.1 (1.1)	8.9 (2.1)	5.8 * (1.8)
T_4 (nmol/L)	2.3 (0.4)	1.7 (1.3)	3.4 (0.7)
T_4 PCR (ml/h)	16.4 (0.9)	17.8 (1.8)	16.6 (2.2)
T_4 PDR (pmol/h)	36.1 (0.5)	28.8 (5.0)	51.4 * (7.2)

TABLE 4.4 The effects of low pH and 20 µmol Al/L on plasma glucose, chloride, osmolality and on packed cell volume (PCV) in cannulated trout. Values represent mean (SE) for the 24 fish used in each treatment group in the kinetic studies. Significant differences from the pH 7.7 group are indicated (*).

PARAMETER	pH 7.7	pH 4.7	pH 4.7 + Al
Glucose (mmol/L)	4.0 (0.2)	6.8 * (0.8)	11.7 * (2.4)
Chloride (mmol/L)	134 (1)	127 * (4)	123 * (5)
Osmolality (mosmol/kg)	303 (5)	288 * (8)	278 * (8)
PCV (%)	26.3 (1.7)	27.0 (2.0)	36.6 * (2.1)

4.5 DISCUSSION

Cortisol PCR and PDR in cannulated control trout were comparable to values determined previously (PART 3) and agreed generally with values given for other salmonids held in freshwater (Nichols and Weisbart 1985; Nichols et al. 1985). However, plasma cortisol levels in cannulated control fish were double those found previously (PART 3). The cause of this difference was not immediately apparent. Nevertheless, plasma cortisol concentrations were lower than other published values for cannulated rainbow trout (Goss and Wood 1988) and increased due to low pH and Al.

The higher plasma cortisol levels at low pH were similar to those described previously (PARTS 1-3; Goss and Wood 1988). As I reported earlier, low pH did not affect cortisol PCR (PART 3). Addition of Al to acidic water increases plasma cortisol markedly, corroborating other recent findings (Goss and Wood 1988; Wood et al. 1988a). Due largely to the increased plasma levels of the hormone, cortisol PDR was elevated by acid and more so by acid plus Al. This suggests that under these conditions, measurement of plasma cortisol may provide a reliable index of cortisol secretion rate from the interrenal.

The role of cortisol during acid and acid/Al exposure remains to be defined. Elevated cortisol has been linked to chloride cell proliferation in low Ca^{2+} water (Perry and Wood 1985) and to enhanced whole body Ca^{2+} uptake (Flik and Perry 1989). Both effects may aid in maintaining ion balance in low pH and high Al environments (Goss and Wood 1988; Wood et al. 1988a).

Plasma T_3 and T_4 levels in the cannulated trout were similar to those reported previously in non-cannulated trout (PART 1). Estimates of T_3 PCR determined by the constant infusion technique (6.1 mL/h per kg) closely agreed with those derived by multicompartmental analysis following injection of a single tracer bolus (6.0 mL/h per kg). Values derived from previous single injection studies in fish employed 2-compartment models (Eales 1979b; Eales et al. 1986) and while results are not strictly comparable, the estimates of T_3 PCR tended to be similar (3.7-12 mL/h per kg). In general, estimates of T_4 PCR by constant infusion were consistent with those (5.4-34.5 mL/h per kg) determined for salmonids by multicompartmental analysis (Eales et al. 1982; Specker et al. 1984). The plasma volume determined from kinetic analysis of [^{125}I]fibrinogen loss was similar to the values (20-33 mL/kg) previously reported for rainbow trout using a variety of different materials to trace plasma volume (Conte et al. 1963; Duff et al. 1987; Nichols 1987; Gingerich et al. 1989).

Although I generally observed depressed plasma T_3 in response to low pH alone (PART 1) the effect was not evident in this study. The trout used before were smaller and 8 to 10 d of low pH exposure were required to induce plasma T_3 effects. Perhaps the 7 d low pH exposure was of insufficient duration to lower plasma T_3 levels in these larger cannulated trout. Unaltered hepatic T_4 5'-MD (5'-monodeiodinase) in trout exposed for 7 d to low pH without Al supported the plasma and kinetic measurements in suggesting no significant alteration in T_3 production due to these conditions (Brown et al. 1990a). Lack of a clear response of plasma T_4 to low ambient pH (with no Al present) was consistent with the earlier findings in non-cannulated fish where plasma T_4 was only

occasionally increased following similar treatments (PARTS 1 and 2). In contrast, increased T_4 levels were observed following low pH exposure in brown trout (Edwards et al. 1987; Brown, J.A. et al. 1989).

The indices of peripheral thyroid function, assessed in fish exposed to low pH and Al, support the hypothesis that extrathyroidal conversion of T_4 to T_3 was impaired due to Al addition. Reduced T_3 PCR and T_3 PAR occurring simultaneously with unchanged or increased T_4 PCR and T_4 PDR imply lowered T_3 production from T_4. Hepatic T_4 5'-MD represents a major step for plasma T_3 generation (de Luze 1982) and Al reduced both the affinity of this enzyme for the T_4 substrate and the amount of functional enzyme (Brown et al. 1990b). Recent measurements of tissue T_3 levels in trout exposed to acid and aluminum confirm this view because whole body T_3 levels were reduced by 80% under these conditions (Fok et al. 1990).

Altough low ambient pH and Al impaired peripheral thyroid function, the mechanism of the change was unclear. In addition to the potential direct effects of Al on aspects of thyroid activity, it is possible that the responses may be indirectly mediated. Cortisol represents a candidate for involvement because it is thought to exert antagonistic effects on thyroid indices (Eales 1979a; Redding et al. 1986). Low ambient pH increased interrenal secretory activity but did not alter peripheral thyroid function. However, the greater plasma cortisol response induced by low pH and Al may have been sufficient to cause the observed thyroid responses. Further experimental work is necessary to resolve the possibility (see PART 5).

The consequences of lower T_3 availability to overall fish performance may be speculative but worthy of comment. A consistent

response of fish to treatment with exogenous thyroid hormones is enhanced somatic growth (Higgs et al. 1982; Hilton et al. 1987). Coincidentally, recent studies document suppressed growth in trout treated with acid plus Al, but growth was not suppressed in trout exposed to low pH water alone (Sadler and Lynam 1987; Mount et al. 1988; Reader et al. 1988). Furthermore, chronic treatment with low Al levels, while protecting fish from ionoregulatory disturbances due to low pH, was detrimental to fish growth (Mount et al. 1988). Thus it appears that reduction of T_4 conversion to T_3, now considered the active hormone form (Eales 1985), could represent a factor limiting fish growth. Impairment of T_4 5'-MD activity by Al may represent the key component in this process.

The experiments demonstrating suppression by low pH of T_3 PAR and also T_4 5'-MD (Brown et al. 1990a) were conducted at moderately high ambient Ca^{2+} levels and with relatively high levels of Al. Further study using softer water and lower Al concentrations are required to determine the general applicability of the altered peripheral thyroid indices in Al exposure. Nevertheless, the trout were experiencing significant ionoregulatory disturbance attributable to low ambient pH and Al. The altered osmotic and carbohydrate parameters found in cannulated trout exposed to low ambient pH represent changes found previously (PARTS 1 and 2; McDonald 1983; Brown et al. 1986a). Aluminum exacerbated the effects of low pH on blood glucose, Cl^-, osmolality and PCV as described previously (Goss and Wood 1988; Wood et al. 1988b,c). However, the moderately high Ca^{2+} levels in Winnipeg city water likely ameliorated the effects of low pH and Al on ionoregulatory disturbances (Playle et al. 1989). The levels of labile Al used here were high but comparable to

environmental values reported for waters in the Laurentian area in Quebec (Hendershot et al. 1984) and the Birkenes catchment in Norway (Seip et al. 1984). Furthermore, AlF species, dominant in aqueous systems (Driscoll 1984; LaZerte 1984; Seip et al. 1984), are less toxic than free Al ions (Baker and Schofield 1982).

4.6 CONCLUSIONS

Low pH increases cortisol production, but by itself has minimal effects on thyroid hormone metabolism in rainbow trout. However, addition of Al intensifies cortisol production and also influences the thyroid system primarily by decreasing extrathyroidal T_3 generation, probably leading to diminished somatic growth. The possibility exists that some of the effects of acid and Al at 20 µmol/L on the thyroid system are mediated by elevated levels of cortisol.

PART 5
EFFECTS OF CORTISOL ON ASPECTS OF T_3 METABOLISM [1]

5.1 SYNOPSIS

Aspects of T_3 metabolism were studied in fed rainbow trout held at 11.5-14 °C and intraperitoneally implanted with hydrogenated corn oil (controls) or oil containing cortisol. Cortisol implants caused dose-related physiologic elevations in plasma cortisol lasting 2 to 3 weeks, loss in body weight and depression in plasma T_3 and free T_3 index with no consistent change in plasma T_4 or free T_4 index. Plasma T_3 clearance rate and T_3 PAR were both increased by cortisol, but with a significant decrease in muscle T_3 concentration as measured by HPLC. It is concluded that chronic physiologic cortisol treatment enhances plasma T_3 clearance, resulting in a decline in T_3 concentration in both plasma and tissue (muscle) compartments.

[1] Previously published (Brown et al. 1990b).

5.2 INTRODUCTION

In teleost fish, cortisol produced by the interrenal cells is thought to antagonize certain aspects of thyroid function. Evidence has accumulated linking exogenous cortisol treatment to reductions in size of pituitary thyrotrophs (Leatherland and Lam 1971), thyroid epithelial cell height (Van Overbeeke and McBride 1971), plasma T_4 response to TSH (Leatherland 1987), and circulating levels of thyroid hormones (Redding et al, 1984, 1986; Weisbart et al. 1987). However, contrasting effects have also been reported for fish exposed to cortisol. These include increased radioiodine uptake by the thyroid (Singh 1969), elevated or unchanged plasma thyroid hormones (Milne and Leatherland 1980; Leatherland 1987), and stimulated T_3 production by hepatic T_4 5'-MD activity (Vijayan et al. 1988).

Based on the above observations one cannot yet define the effects of cortisol on the teleost thyroid system. Current inconsistencies in the literature may be due in part to differences in species, physiologic state or mode of cortisol administration. However, cortisol doses have also differed widely, ranging from the upper physiologic range (Redding et al. 1986) to overtly pharmacologic (Milne and Leatherland 1980; Weisbart et al. 1987) which may have influenced the responses qualitatively as well as quantitatively. Finally, the methods and parameters used to assess thyroidal status have varied between studies and could also explain some of the literature discrepancies.

Current dogma suggests that T_3 is the active hormone at the receptor level (Bres and Eales 1986). Consequently I have focused on T_3

metabolism in rainbow trout and attempted to determine the effects of physiologic cortisol treatment not only on plasma levels and clearance rate but also on tissue (muscle) T_3 level. I have demonstrated that the cortisol-induced reduction in plasma T_3 is probably due to enhanced plasma T_3 clearance which could also explain the demonstrated muscle T_3 deprivation.

5.3 MATERIALS AND METHODS

5.3.1 Fish Maintenance

Rainbow trout (2-3 yrs; weight 376 ± 13 g; $\hat{x}$ ± SE, N = 196) were held as described in PARTS 1 and 2. Temperature varied from 11.5 °C to 14 °C but varied less than 1 °C during the course of an experiment. Fish were fed trout food at a daily ration of 1.5% of wet body weight and were acclimated to these conditions at least 4 weeks prior to experimental use. Experiments were conducted between March and September.

5.3.2 Implantation of Cortisol

Fish were lightly anesthetized in water containing pH-neutralized TMS (0.38 mmol/L) and NaCl (150 mmol/L) approximately iso-osmotic with fish plasma. Immediately after the fish lost equilibrium, they were blotted to remove excess moisture, weighed and injected with cortisol implants (Pickering and Duston 1983). Implants were prepared by suspending known concentrations of cortisol (0, 10 or 25 mg/kg) in hydrogenated corn oil (40 °C) and injecting the warmed liquid into the peritoneal cavity of each fish (1 mL/kg). Thus the volume of the implant was similar between

dose levels but was proportional to the size of each fish. Because the fish were held at 12 °C, the implants solidified and vestiges were present in the body cavity at the end of each experiment. The procedure was facilitated by placing the 1-mL glass syringes and needles (20 gauge) on a slide warming tray at 40 °C. Fish recovered from the procedure in anesthetic-free water within 3 min.

5.3.3 Blood Sampling and Analyses

Trout were routinely anesthetized in pH-neutralized TMS (0.76 mmol/L) and blood was removed from the caudal vessels using heparinized syringes and needles. Plasma was obtained by centrifugation and stored at -70 °C until analyzed. Concentrations of cortisol, T_3 and T_4 in plasma were measured by specific radioimmunoassay procedures (PART 1). Indices of free T_3 and free T_4 were estimated using miniature Sephadex G-25 columns and plasma diluted with phosphate buffer (pH 7.5) as outlined by Eales and Shostak (1985).

5.3.4 Plasma T3 Kinetics

Plasma T3 kinetics were assessed by constant infusion in fish cannulated via the dorsal aorta (see PARTS 3 and 4). Following recovery from the effects of cannulation (10 d), trout saline/plasma (9:1, v/v) was delivered through the cannula using infusion pumps (Harvard Apparatus) set at a rate of 50-60 µL/h. After 1 d the pumps were momentarily stopped; a preliminary blood sample (200 µL) removed via the cannula; the fish injected with a 1-µCi priming dose of [^{125}I]T_3 (780 mCi/µmol,

Amersham), and syringes with vehicle exchanged for those also containing $[^{125}I]T_3$.

The amount of radioactivity infused was 0.1 µCi/h. Infusions were routinely conducted for 4 d thereby insuring constant plasma radioactivity (see PART 4).

Plasma $[^{125}I]T_3$ was separated by the Sephadex/Ab technique in which excess antiserum specific for T_3 eluted $[^{125}I]T_3$ from plasma samples which had been directly applied to miniature G-25 Sephadex columns (Eales 1977).

The T_3 PCR (mL/h) and the T_3 PAR (pmol/h) normalized to a 1 kg fish were calculated as described in PART 4.

5.3.5 T3 Extraction from Muscle

Approximately 1 g skeletal muscle was excised from behind the head, dorsal to the lateral line, and anterior to the dorsal fin. The tissue was rinsed with NaCl solution (0.87%), minced with scissors, and homogenized (Polytron, 60 s, 22000 rpm) with 2 mL of potassium phosphate solution (20 µmol/L) containing propylthiouracil (PTU, 1 µmol/L). Iodothyronines were extracted from the homogenate by adding 5 mL of methanolic ammonia (99:1, v/v; with PTU) to the homogenizing tube and vortex mixing. After 15 min, the mixture was centrifuged at 1500 X *g* for 10 min at 4 °C and the supernatant transferred to evaporation tubes. The pellet was washed twice with 2 mL methanolic ammonia by resuspension followed by centrifugation. The pooled supernatant was evaporated at 40 °C to dryness.

Estimates of [^{125}I]T3 from the residue of muscle extracts and from randomly chosen plasma samples were obtained by HPLC. Residues from the dried muscle extracts or plasma (500 µL) were mixed with 0.25 N NaOH (500 µL), transferred to PrepSep C_{18} columns (900 mg, Fisher), and iodothyronines eluted with methanol (Rutgers et al. 1987). The dried elutes from the PrepSep extraction columns were reconstituted in acetonitrile and water (40:60, v/v) and 50 µL injected onto a 5-micron Econosphere C_{18} column (Alltech Associates Inc.). Iodothyronines were eluted using a 35-min linear gradient of 40-45% acetonitrile/0.1% trifluoroacetic acid (TFA) and water/TFA (0.1%) at a flow of 1 mL/min (Hays and Hsu 1987). Fractions were collected at 1-min intervals and ^{125}I activity determined. Methanol and acetonitrile (Caledon lab.) and TFA (Pierce Chemical Co.) were HPLC grade. Distilled water was purified using a MilliQ water system (Millipore). Authentic iodocompounds used as standards were: T_3 and T_4 (Sigma); 3,5',3-triiodo-L-thyronine (RT_3) (Calbiochem); triiodothyroacetic acid (T_3A), tetraiodothyroacetic acid (T_4A) (Cyclochemical Co.); 3,5-diiodo-DL-thyronine (T_2), diiodotyrosine (DIT) (Nutritional Biochemical Co.). The T_3 level in muscle tissue was calculated (Van Doorn et al. 1985) from the steady state value of labelled hormone in muscle (T, dpm/g), the percentage recovery of T_3 from tissue (R), the steady state [^{125}I]T_3 in plasma (C), and the plasma total T_3 concentration (PC); tissue T_3 (nmol/kg) = (T/C) X (100/R) X 1000 X PC.

5.3.6 Statistics

Bartlett's test (Expts 1 and 2) or the Fmax-test (Expts 3 and 4) for homogeneity of variance were applied to data (Snedecor and Cochran

1971). Where required, data were transformed according to Taylor's power law to obtain more uniform variance (Southwood 1978). For clarity of presentation, arithmetic means and standard errors are given in results. Two-way ANOVA (Expts 1 and 2) was computed. Tukey's studentized range test was used to evaluate differences between group means. Differences between initial and final weights were compared using paired *t*-tests. In Expts 3 and 4, differences between group means were compared by the *t*-test. Probabilities of <0.05 were considered significant.

5.4 RESULTS

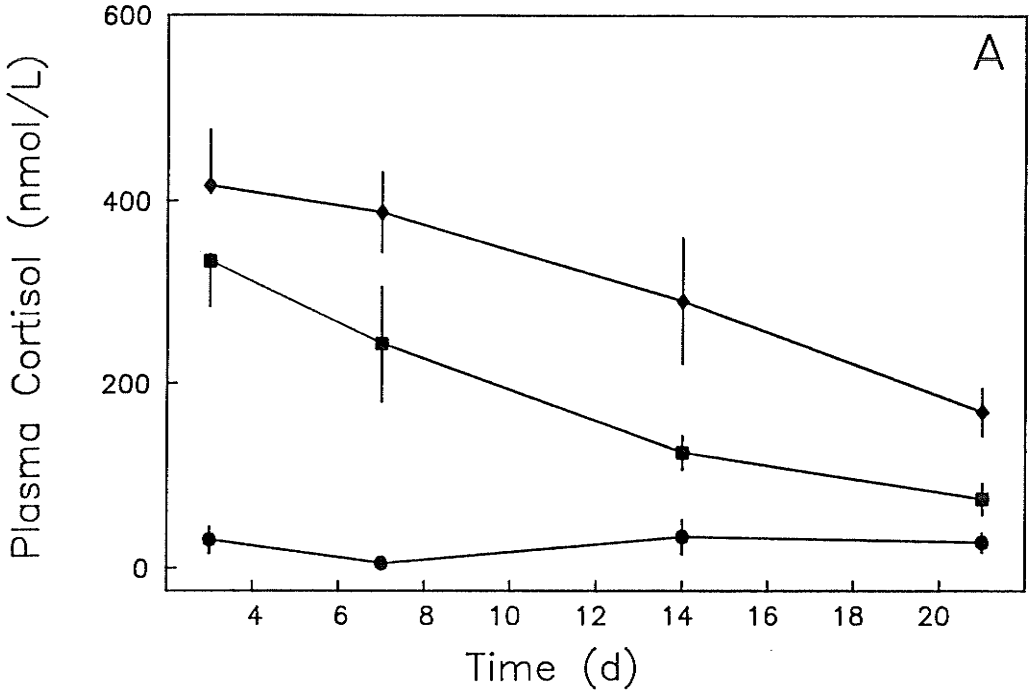
The effects of cortisol implants on the thyroid system were examined in 4 experiments.

5.4.1 Experiment 1: Effect of Cortisol Implants (3 to 21 d) on Plasma T_3, Plasma T_4 and Body Weight.

Fish were administered implants with three concentrations of cortisol (0, 10 or 25 mg/kg) and blood removed after 3, 7, 14 and 21 d. Fish were fed 1.5% wet body weight the day after receiving the implants and every second day thereafter. Groups were not fed the same day they were scheduled for sampling.

Corn oil implants containing 10 and 25 mg cortisol/kg elevated plasma cortisol levels for 14 and 21 d respectively (Fig. 5.1A). The plasma cortisol level in fish receiving simple corn oil implants was low (<30 µmol/L). From 3 d onwards cortisol caused a loss in body weight relative to controls (Table 5.1).

FIGURE 5.1. Plasma cortisol levels in rainbow trout receiving corn oil implants containing cortisol: A-fish exposed (Expt 1) at 0 (●), 10 (■), or 25 (♦) mg/kg; B-fish exposed (Expt 2) at 0 (●), or 10 (■) mg/kg and unhandled fish not given implants (▲). Values represent mean ± SE of 5-6 fish.


A
Plasma Cortisol (nmol/L)
600
400
200
0
4
6
8
10
12
14
16
18
20
Time (d)

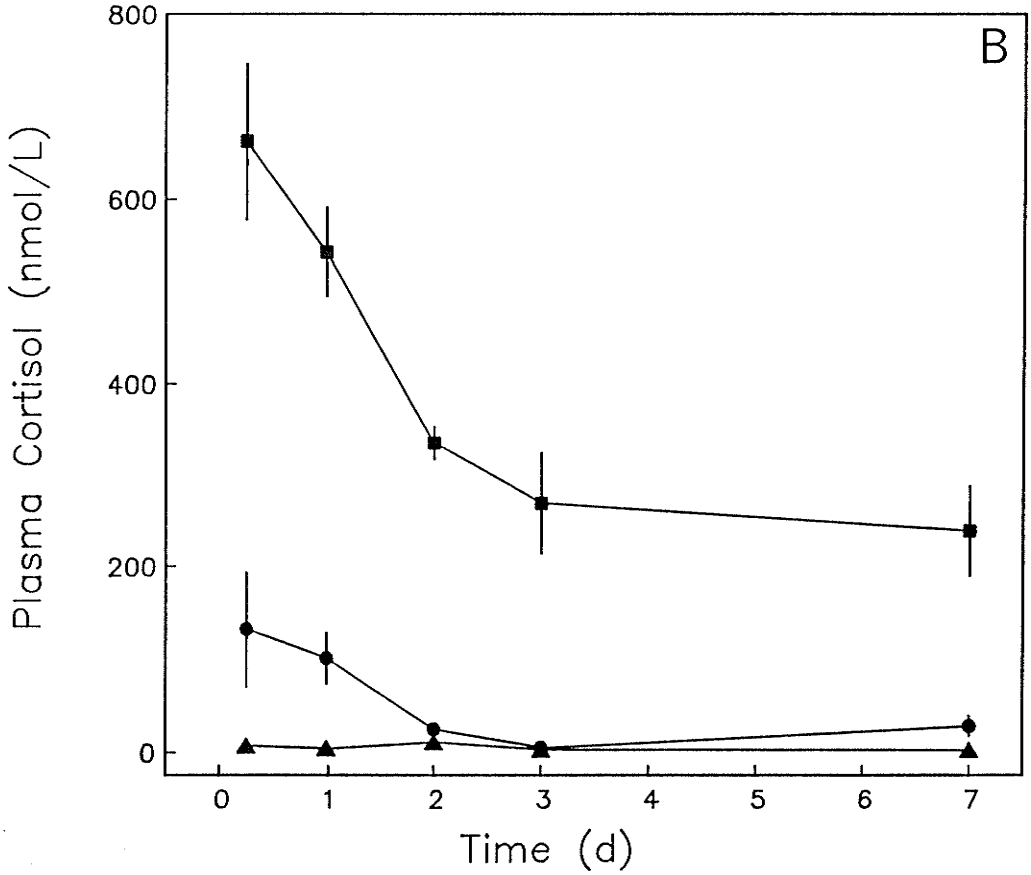

B
Plasma Cortisol (nmol/L)
800
600
400
200
0
0
1
2
3
4
5
6
7
Time (d)

TABLE 5.1 Effects of cortisol implants (0, 10 and 25 mg/kg) on plasma concentrations of T_3 and T_4 and percentage body weight loss in rainbow trout (Expt 1). Values represent mean (SE) of 5-6 fish. An asterisk indicates differences relative to controls (0 mg/kg implant). A square indicates differences relative to initial body weight.

PARAMETER	IMPLANT DOSE (mg/kg)	TIME (d) 3	7	14	21
Plasma T_3 (nmol/L)	0	2.7 (0.9)	2.5 (0.4)	3.6 (1.0)	2.9 (0.3)
	10	0.8* (0.2)	2.5 (0.3)	1.7* (0.1)	2.1 (0.6)
	25	0.9* (0.4)	2.0 (0.4)	0.9* (0.3)	2.5 (0.4)
Plasma T_4 (nmol/L)	0	1.9 (0.6)	2.9 (0.5)	2.9 (0.8)	2.0 (0.2)
	10	3.2 (0.9)	2.4 (0.5)	2.3 (0.8)	3.4 (0.2)
	25	2.9 (0.3)	2.5 (0.3)	1.8 (0.4)	2.2 (0.3)
Weight Loss (%)	0	1.8 (0.9)	1.1 (0.9)	1.0 (0.5)	0.4 (0.6)
	10	4.6 ■ (1.4)	4.5*■ (0.8)	4.2*■ (0.6)	2.2 (2.0)
	25	5.7*■ (0.7)	4.2*■ (0.7)	5.8*■ (2.4)	4.9*■ (1.3)

The response was more sustained at the higher cortisol dose. Plasma T_3 was significantly reduced by cortisol treatment at 3 and 14 d, but the effect was complicated by an apparent temporary recovery at 7 d. By 21 d, T_3 levels in the treatment groups did not differ from the control. Plasma T_4 appeared refractory to cortisol exposure.

There was no mortality in fish receiving intraperitoneal implants of corn oil or oil plus cortisol. However after 10 d, outward symptoms of disease appeared in two fish given the highest cortisol dose (25 mg/kg). These fish were immediately removed from their tanks and were not analyzed.

5.4.2 Experiment 2: Effect of Cortisol Implants (6 h to 7 d) on Plasma T_3, Plasma T_4 and Body Weight.

Fish implanted with cortisol (0 and 10 mg/kg) were bled after 6 h and 1, 2, 3 and 7 d and otherwise treated as in Expt 1.

Plasma cortisol levels were highest 6 h post-implantation (Fig. 5.1B). Relative to unhandled cohorts, fish given implants without cortisol also had mildly elevated plasma cortisol, which returned to unhandled levels by 2 d. After 3 d, cortisol implants caused a loss in body weight relative to oil-implanted controls (Table 5.2). At 1 d, plasma T_3 was lowered in fish given oil implants with cortisol. Following this initial depression, T_3 levels recovered towards those found in unhandled controls. In the fish treated with cortisol, plasma T_3 was lower between 3 and 7 d. Plasma T_4 showed a decrease at 1 and 2 d which recovered to control values at 3 d (Table 5.2).

TABLE 5.2 Effects of cortisol implants (0 and 10 mg/kg) on plasma concentrations of T_3 and T_4 and percentage body weight loss in rainbow trout (Expt 2). Values represent mean (SE) of 5-6 fish. NI represent unhandled control fish not receiving an implant. Asterisk and triangle indicate differences relative to 0 mg/kg implant and NI fish, respectively. The square indicates differences relative to initial body weight.

PARAMETER	IMPLANT DOSE (mg/kg)	TIME				
		6 h	1 d	2 d	3 d	7 d
Plasma T_3 (nmol/L)	NI	3.8 (0.3)	3.9 (0.6)	3.7 (0.8)	3.9 (0.6)	4.1 (0.6)
	0	2.8 (0.2)	2.4 (0.8)	2.4 (0.4)	4.5 (0.9)	5.0 (0.5)
	10	3.2 (0.3)	1.7 ▲ (0.6)	3.0 (0.4)	3.1 (0.6)	2.7*▲ (0.7)
Plasma T_4 (nmol/L)	NI	2.9 (0.3)	3.4 (0.4)	3.9 (0.5)	3.4 (0.5)	3.4 (0.5)
	0	2.3 (0.7)	3.8 (1.1)	3.4 (0.5)	3.4 (0.5)	3.4 (0.7)
	10	4.3 (1.9)	0.9*▲ (0.2)	2.2 ▲ (0.5)	4.3 (0.5)	3.6 (0.7)
Weight Loss (%)	0	0.3 (0.8)	2.3 ■ (0.5)	1.1 (1.4)	1.7 (0.6)	1.6 (0.7)
	10	1.2 (0.2)	1.3 (0.6)	2.6 (0.4)	5.2*■ (0.7)	5.9*■ (0.5)

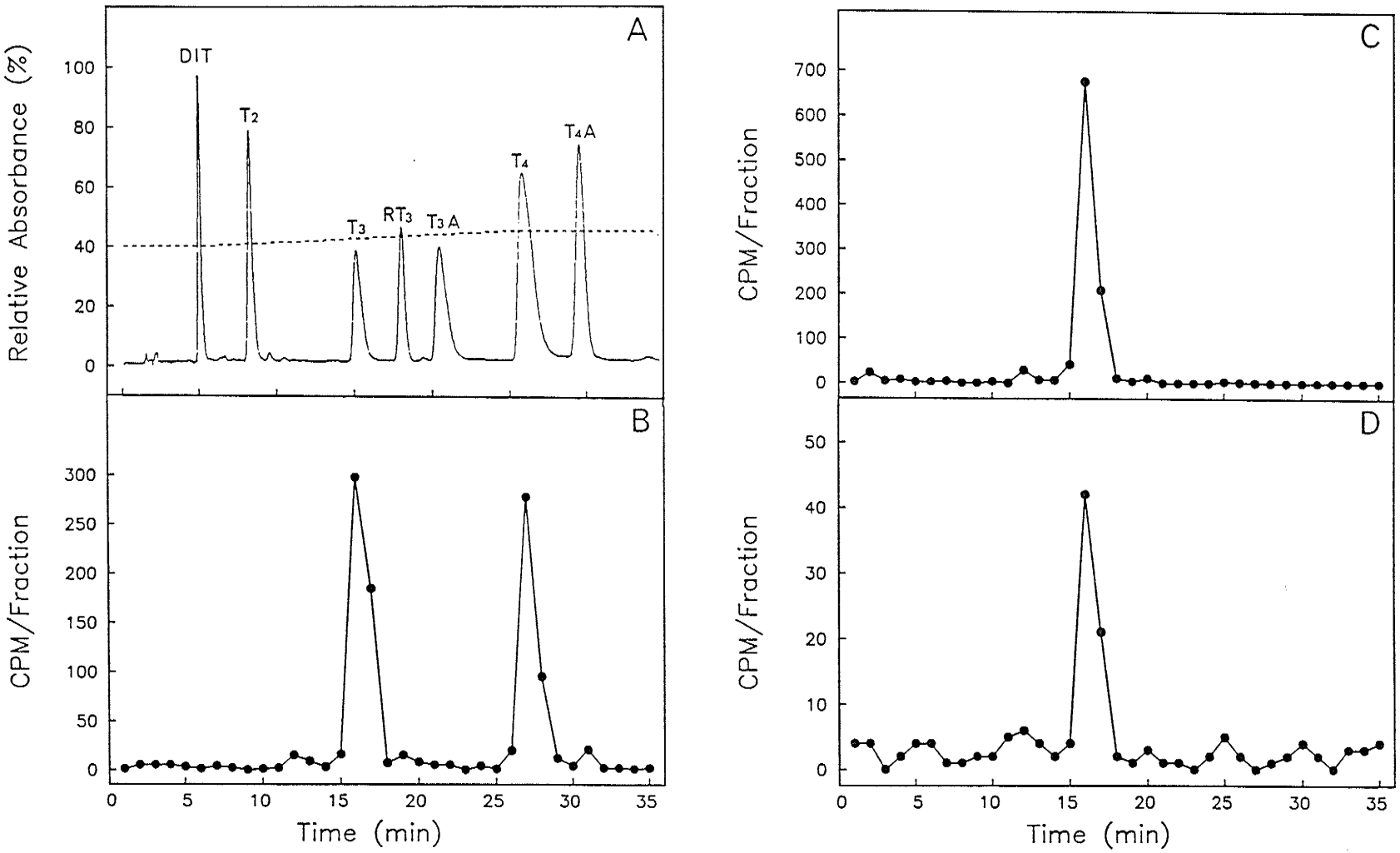
Free T_3 and T_4 indices were examined in plasma samples taken at 3 and 7 d. After 3 d the free T_3 index did not differ between cortisol-exposed (0.12 ± 0.03 nmol/L, N = 6) and control (0.13 ± 0.03 nmol/L, N = 6) fish. The free T_3 index in fish exposed 7 d to cortisol (0.077 ± 0.02 nmol/L, N = 6) was lower than in controls (0.13 ± 0.01 nmol/L, N = 6). No difference in free T_4 index was evident due to time of sampling; nor between cortisol-treated fish (0.33 ± 0.04 nmol/L; 3 and 7 d; N = 12) and control fish (0.25 ± 0.05 nmol/L; 3 and 7 d; N = 12).

5.4.3 Experiment 3: Effect of Cortisol on Plasma [^{125}I]T_3 Kinetics and Muscle T_3 Level.

Cannulated fish were given cortisol implants (0 and 10 mg/kg) and transferred to individual aquaria 3 d prior to [^{125}I]T_3 infusion. Because fish were infused for 4 d, the total exposure time to the implants equalled 7 d. Fish were not fed during infusion.

Recovery of [^{125}I]T_3 from plasma by the Sephadex/Ab technique exceeded 93.5%. The efficiency of methanolic ammonia extraction of[^{125}I]T_3 added to muscle homogenate was 64.6 ± 2.5% (N = 5). Recovery of [^{125}I]T_3 from plasma and resuspended methanolic ammonia extracts of muscle tissue by the solid phase C_{18} columns equalled 66.8 ± 2.5% (N = 10) and 62.0 ± 1.3% (N = 5), respectively. Chromatographic profiles for iodocompounds showed clear separation with the HPLC procedure (Fig. 5.2A,B). Elution profiles for plasma and muscle extracts of [^{125}I]T_3-infused fish each displayed a single peak corresponding to the T_3 standard (Fig. 5.2C,D).

FIGURE 5.2. Separation of iodocompounds by reverse phase HPLC: A-relative absorbance at 254 nm of standard (500 ng) diiodotyrosine (DIT), 3, 5-diiodo-DL-thyronine (T_2), 3,5,3'-triiodo-L-thyronine (T_3), 3,5',3-triiodo-L-thyronine (RT_3), triiodothyroacetic acid (T_3A), L-thyroxine (T_4), and tetraiodothyroacetic acid (T_4A) (dashed line indicates % acetonitrile in gradient); B-elution profile of PrepSep-C_{18} extracted [^{125}I]T_3 and [^{125}I]T_4 standards; C-elution profile of ^{125}I-labelled materials extractable from plasma of a cortisol (10 mg/kg)-exposed fish infused with [^{125}I]T_3; D-elution profile of ^{125}I-labelled materials extractable from muscle of a cortisol (10 mg/kg)-exposed fish infused with [^{125}I]T_3.


A
B
C
D
DIT
T2
T3
RT3
T3A
T4
T4A
Relative Absorbance (%)
CPM/Fraction
Time (min)

Estimates of plasma [^{125}I]T_3 by HPLC were 13.1 ± 3.0% (N = 5) lower than those found by the Sephadex/Ab method.

Cortisol implants, while increasing plasma cortisol, also elevated T_3 PCR and, to a lesser extent T_3 PAR, reduced plasma and muscle T_3 levels, promoted loss of body weight, but did not affect plasma T_4 (Table 5.3).

5.4.4 Experiment 4: Effect of Cortisol Implants on Plasma Free T_3 Index and Plasma Free T_4 Index.

Trout administered cortisol implants (0 and 10 mg/kg) were held for 7 d under conditions similar to those used in Expt 3. After anesthetization, blood was sampled from the caudal vessels. Cortisol exposure increased plasma cortisol, depressed plasma T_3 and free T_3 index, and lowered body weight (Table 5.4).

TABLE 5.3 Effects of exposure (7 d) to cortisol implants (0 and 10 mg/kg) on plasma levels of T_3, T_4 and cortisol and on T_3 PCR, T_3 PAR, muscle T_3 level and loss in body weight in rainbow trout (Expt 3). Values represent mean (SE) of 6 fish and are based on a body weight of 1 kg. Asterisks indicate significant differences relative to sham implants.

PARAMETER	SHAM IMPLANT	CORTISOL IMPLANT
Plasma T_3 (nmol/L)	2.7 (0.5)	1.6 * (0.3)
T_3 PCR (mL/h)	7.6 (1.3)	18.7 * (3.2)
T_3 PAR (pmol/h)	19.1 (3.7)	29.9 * (5.0)
Muscle T_3 (nmol)	0.58 (0.05)	0.44 * (0.07)
Plasma T_4 (nmol/L)	4.7 (1.0)	5.6 (1.5)
Plasma Cortisol (nmol/L)	44 (14)	196 * (42)
Weight Loss (%)	2.1 (0.5)	6.9 * (0.4)

TABLE 5.4 Plasma T_3, T_4 and cortisol levels, free T_3 and T_4 indices and loss of body weight in rainbow trout (Expt 4) exposed to cortisol implants (0 and 10 mg/kg) for 7 d. Values represent mean (SE) of 6 fish. Asterisks indicate significant differences relative to sham implants.

PARAMETER	SHAM IMPLANT	CORTISOL IMPLANT
Plasma T_3 (nmol/L)	6.1 (0.6)	2.8 * (0.7)
Free T_3 Index (nmol/L)	0.18 (0.02)	0.08 * (0.03)
Plasma T_4 (nmol/L)	5.6 (0.8)	7.8 (1.8)
Free T_4 Index (nmol/L)	0.41 (0.06)	0.58 (0.13)
Plasma Cortisol (nmol/L)	29 (11)	260 * (61)
Weight Loss (%)	1.4 (0.6)	5.9 * (0.9)

5.5 DISCUSSION

Intraperitoneal implants of hydrogenated corn oil containing cortisol elevated plasma cortisol in a dose-dependent fashion. Cortisol levels in the treated fish were similar to those reported in rainbow trout following severe acute handling stress (Barton et al. 1980; Woodward and Strange 1987) or exposure to toxic conditions (PARTS 1-3; Brown et al. 1986a; Goss and Wood 1988). The initial rapid decline in plasma cortisol implied higher levels at earlier times. While I cannot exclude the possibility that plasma cortisol exceeded levels considered physiological (<1200 nmol/L), it would occur for a very short time. The mildly elevated plasma cortisol in trout receiving simple corn oil implants most likely resulted from an increase in endogenous secretion due to the implantation procedure. This response was typical of the stress response observed following anesthetization and handling (PART 3; Barton and Peter 1982). The low plasma cortisol levels following this initial response were consistent with reported resting cortisol concentrations in rainbow trout (Barton et al. 1987; Pickering and Pottinger 1987).

The reduced weight observed in trout administered cortisol implants has been demonstrated repeatedly in salmonids (summarized by Pickering and Duston 1983). The present cortisol effect on body weight was not dose dependent, but the higher dose extended cortisol's duration of effect.

Recent reports have linked exogenous cortisol treatment with suppressed immunocompetence and increased susceptibility to a range of pathogens (Pickering and Duston 1983; Pickering and Pottinger 1985;

Tripp et al. 1987). In the my experiments only 2 fish (Expt 1) displayed disease symptoms. Following their elimination, no other fish exhibited overt signs of disease. Thus, I feel that potential exposure to pathogens does not represent a confounding factor in subsequent findings.

As shown in this study, cortisol depressed plasma T_3 in coho salmon (*Oncorhynchus kisutch*) (Redding et al. 1984), eel (Redding et al. 1986), and brook trout (Weisbart et al. 1987). In contrast, previous studies measuring plasma T_3 in rainbow trout administered cortisol implants (Leatherland 1987) or intraperitoneal cortisol injections (Milne and Leatherland 1980) showed only tendencies towards lower plasma T_3. The reason for the difference between this study and that by Leatherland (1987) is unclear. The study by Milne and Leatherland (1980) used very high cortisol doses and repetitive handling of fish.

Because I did not sample fish between the initial injection of corn oil and 6 h, the transient 100 to 200% elevation in plasma T_4 demonstrated previously following acute stress (Brown et al. 1978) was probably missed. Reduction in T_4 at 1 d was temporary and also occurred in cortisol-treated eels (Redding et al. 1986) and brook trout (Weisbart et al. 1987). In other investigations reporting unaltered T_4 following cortisol treatment (Redding et al. 1984; Leatherland 1987) plasma was sampled at a time when plasma T_4 had returned to control values in the present study. The high cortisol levels used by Milne and Leatherland (1980) increased plasma T_4.

Free T_3 and free T_4 levels have not been assessed previously during cortisol treatment in fish. Lack of a distinct effect of cortisol on the free T_4 index suggests that binding of T_4 to plasma protein was not altered. The lower free T_3 index in cortisol-treated fish was due to the

lower T_3 levels in plasma and not likely related to changes in plasma protein binding of T_3. In mammals, exogenous treatment with the synthetic glucocorticoid dexamethasone, reduced plasma protein binding of both T_4 and T_3 (Cavalieri et al. 1984).

The present measurements of T_3 PCR and PAR in control trout receiving corn oil implants closely agreed with values obtained in control fish from my previous study also employing the constant infusion technique (see PART 4). Moreover, T_3 PCR and PAR determined using constant infusion were similar to estimates obtained by multicompartmental analysis following injection of a single [^{125}I]T_3 bolus (PART 4; Eales 1979b; Eales et al. 1986). The elevated T_3 PCR in our cortisol-exposed trout was consistent with observations in eels where labelled T_3 loss from plasma was increased after cortisol injection (Redding et al. 1986). Due to the low plasma T_3 levels, the PAR was not increased to the same extent as T_3 PCR.

Estimates of muscle T_3 concentrations were somewhat lower than those determined previously using isotopic equilibrium or radioimmunoassay (Fok et al. 1990). Part of the difference may be methodological because labelled T_3 was separated by HPLC whereas Fok et al. (1990) used G-25 Sephadex chromatography. Muscle T_3 levels were lower than in most body tissues. However, muscle is the main body tissue and it represents the largest T_3 pool in fish (Fok et al. 1990). Therefore, the reduced muscle T_3 measured in the present cortisol-treated fish implied that whole body T_3 levels had been lowered.

The constant plasma total T_4 level and free T_4 index suggests that the substrate availability for hepatic T_4 5'-MD was not changed. This combined with the unaltered deiodinase activity in cortisol-exposed trout

reported by Brown et al. (1990b) implies that cortisol did not affect T_3 production. In support, the *in vivo* conversion of T_4 to T_3 was not altered by cortisol in eels (Redding et al. 1986). Additionally T_4 5'-MD was highest in liver compared to other tissues examined (D.L. MacLatchy, unpublished) and probably represents a major source for plasma T_3 (de Luze 1982).

Although a recent study by Vijayan et al. (1988) showed increased T_4 5'-MD activity in cortisol-implanted brook trout, the results of Brown et al. (1990b) do not conflict. In the study by Vijayan et al. (1988) the increased T_4 5'-MD occurred 56 d after administration of cortisol (50 mg/kg). Also, at that time, the cortisol implants did not affect plasma cortisol, T_3 or T_4 concentrations. The effects of cortisol in the present study would have likely occurred at an earlier time. Consequently the effect that Vijayan et al. (1988) observed might represent a secondary increase in T_3 production compensating for an initial loss in body T_3.

5.6 CONCLUSIONS

Overall, the finding that cortisol increased T_3 PCR without altering T_3 production by hepatic T_4 5'-MD and thereby reducing muscle T_3 is consistent with the idea that cortisol exerts antagonistic effects on thyroid function. The lower T_3 could mean that the amount of T_3 available for receptor binding was also reduced. Therefore T_3-dependent processes such as somatic growth may be impaired by high cortisol. The mechanism whereby cortisol actively induces elimination of T_3 requires further study involving excretory pathways.

PART 6

SUMMARY AND CONCLUSIONS

Low ambient pH increased interrenal activity and, in experiments of sufficient duration, reduced plasma T_3 levels, in addition to compromising aspects of ionoregulation (PARTS 1 and 2). High concentrations of Al in low pH water enhanced the effects of low pH alone in elevating plasma cortisol, depressing plasma T_3, and intensifying plasma ionic and glucose responses. In contrast, low concentrations of Al in low pH water generally promoted a return of all plasma values to control levels. At pH 4.7 the endocrine changes temporally followed reductions in plasma ionoregulatory properties and glucose. Thus the endocrine responses probably represented part of the compensatory processes dealing with the stress of ion loss. Furthermore, the endocrine responses occurred in both hard and soft water.

The effects of procedures used to assess hormone kinetics (cannulation, diurnal change, serial sampling and vehicle infusion) on plasma cortisol, glucose and protein and PCV were evaluated (PART 3). A potentially feasible protocol for estimation of cortisol PCR in minimally stressed fish would be to:

i) infuse saline vehicle for 24 h;

ii) infuse labelled cortisol until a steady state is obtained; and

iii) remove a single terminal blood sample for analysis.

Using these procedures, the kinetic studies (PARTS 3 and 4) indicated that low pH exposure (7-10 d) increased cortisol production (Fig.

6.1A, B) but by itself had minimal effects on thyroid hormone metabolism. However, addition of Al to low pH water intensified cortisol production (Fig. 6.1C) and also influenced the thyroid system primarily by decreasing extrathyroidal T_3 generation (Fig. 6.2A, B).

Physiologic doses of cortisol (PART 5) increased T_3 PCR without altering indices of T_3 production in the liver (Brown et al. 1990b) and thereby reduced muscle T_3 (Fig. 6.2C). These findings are consistent with the idea that cortisol antagonizes thyroid function. However, the mechanism whereby cortisol actively induces elimination of T_3 requires further study to examine the excretory pathways involved.

In addition to reducing T_3 PCR (PART 4), exposure to low ambient pH and AlF (7 d) lowered the affinity of hepatic 5'-MD for its substrate, T_4 (Brown et al. 1990a). By contrast, physiologic doses of cortisol similar to those observed during acid and AlF exposure stimulated T_3 PCR (PART 5) but had no effect on T_4 5'-MD activity (Brown et al. 1990b). Therefore, it is concluded that cortisol does not likely represent the substance directly mediating the response of T_4 5'-MD to low pH and AlF. However, the temporal relationships between exposure to either cortisol or acid/AlF and either T_3 PCR or T_4 5'-MD were not completely described in this study. Increased T_3 PCR during the early stages (<7 d) of acid and AlF stress when cortisol levels first became elevated could contribute to the overall reduction in tissue T3 levels. Together with the reduced ability to produce T_3 from T_4 substrate this could cause the profound reduction of tissue T_3 reported by Fok et al. (1990) in fish exposed to low pH water containing AlF.

FIGURE 6.1. Summary of low pH and AlF effects on the hypothalamic-pituitary-interrenal axis in fish. A-Stimuli from either the effects of external changes (e.g. environmental handling) or internal changes (e.g. osmotic imbalance) stimulate the release of corticotropin releasing hormone (CRH) from the hypothalamus. In turn, CRH causes secretion of adrenocorticotropin (ACTH) from the pituitary into the circulation. The ACTH increases the synthesis and release of cortisol from the interrenal cells (Donaldson 1981) which promotes energy and substrate mobilization and changes electrolyte balance. In control (pH 7.7) fish, the HPI axis functions at a low level. B-Exposure of rainbow trout to low pH (4.7) for 7 d causes a 3-fold increase in cortisol production. C-Exposure of fish to low ambient pH (4.7) and AlF (20 µmol/L) increases cortisol production 10-fold.

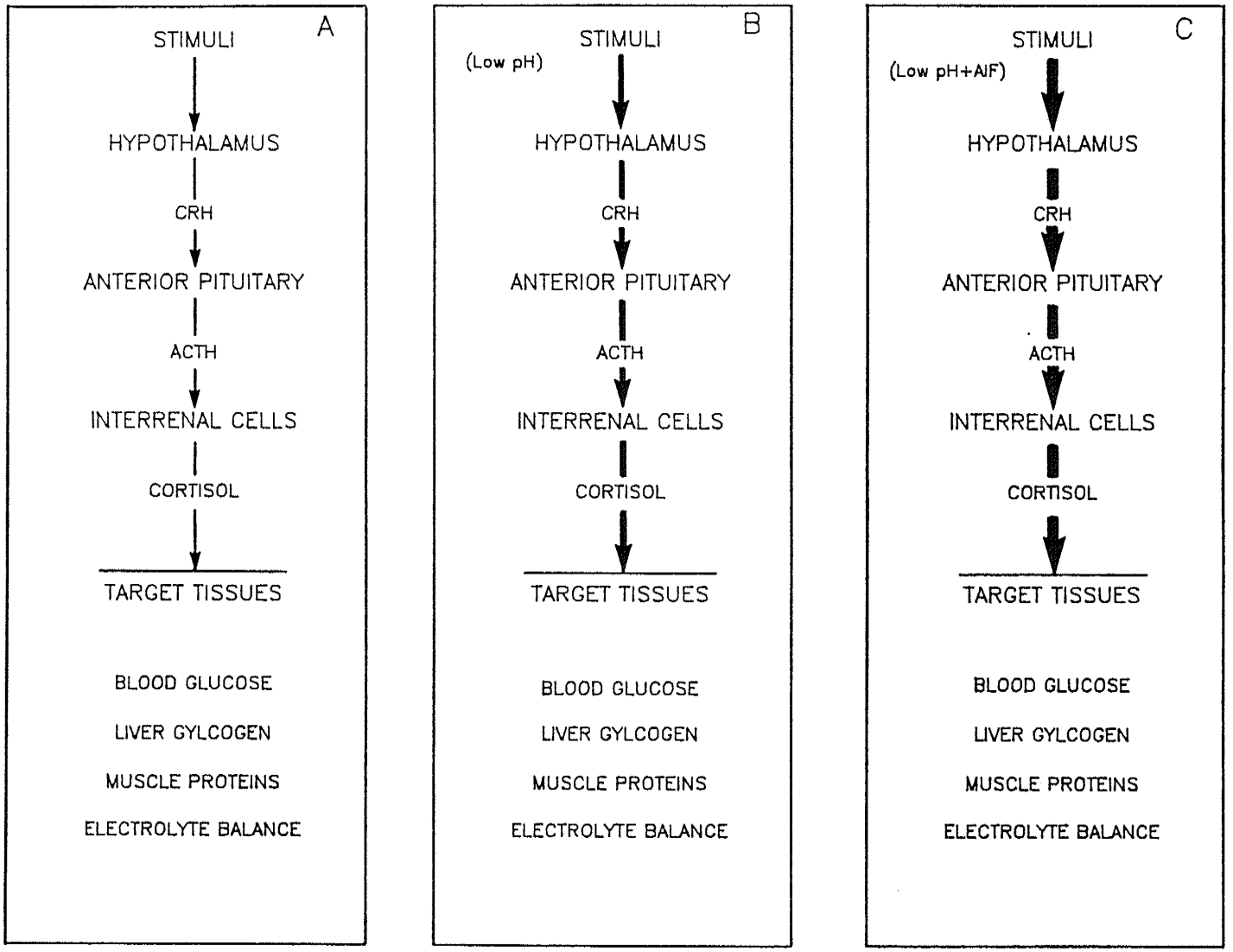
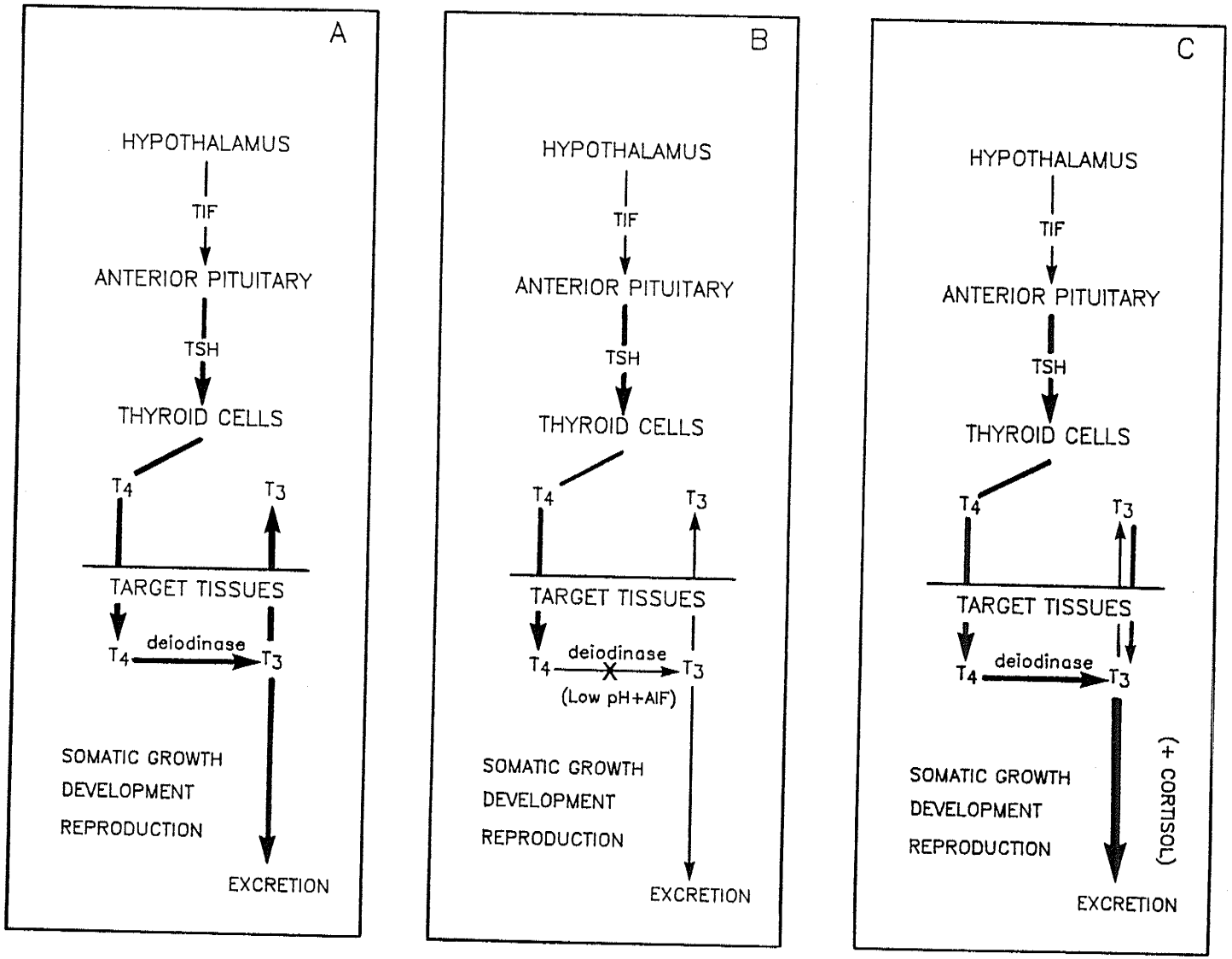

A
STIMULI
HYPOTHALAMUS
CRH
ANTERIOR PITUITARY
ACTH
INTERRENAL CELLS
CORTISOL
TARGET TISSUES
BLOOD GLUCOSE
LIVER GYLCOGEN
MUSCLE PROTEINS
ELECTROLYTE BALANCE
B
STIMULI
(Low pH)
HYPOTHALAMUS
CRH
ANTERIOR PITUITARY
ACTH
INTERRENAL CELLS
CORTISOL
TARGET TISSUES
BLOOD GLUCOSE
LIVER GYLCOGEN
MUSCLE PROTEINS
ELECTROLYTE BALANCE
C
STIMULI
(Low pH+AlF)
HYPOTHALAMUS
CRH
ANTERIOR PITUITARY
ACTH
INTERRENAL CELLS
CORTISOL
TARGET TISSUES
BLOOD GLUCOSE
LIVER GYLCOGEN
MUSCLE PROTEINS
ELECTROLYTE BALANCE

FIGURE 6.2. Summary of low pH and AlF effects on the hypothalamic-pituitary-thyroidal axis and peripheral conversion of T4 prohormone to T3 in fish. A-In control (pH 7.7) fish, thyrotropin (TSH) is spontaneously produced in pituitary thyrotropes and reguated by a hypothalamic inhibitory factor (TIF). TSH is carried via the circulatory system where it stimulates thyroidal production and release of T_4. T_4 is converted by a specific deiodinase in target or other tissues to T_3 which initiates the effects of thyroid hormones on growth, development and reproduction (Eales 1979a, 1985). B-Low ambient pH (4.7) and AlF (20 µmol/L) inhibits the production of T_3 from T_4. C-Cortisol increases plasma T_3 clearance rate thus reducing plasma and muscle T_3 levels. These findings suggests that mechanisms involved with T_3 metabolism or excretion are activated.


A
HYPOTHALAMUS
TIF
ANTERIOR PITUITARY
TSH
THYROID CELLS
T4
T3
TARGET TISSUES
T4
deiodinase
T3
SOMATIC GROWTH
DEVELOPMENT
REPRODUCTION
EXCRETION
B
HYPOTHALAMUS
TIF
ANTERIOR PITUITARY
TSH
THYROID CELLS
T4
T3
TARGET TISSUES
T4
deiodinase
T3
(Low pH+AIF)
SOMATIC GROWTH
DEVELOPMENT
REPRODUCTION
EXCRETION
C
HYPOTHALAMUS
TIF
ANTERIOR PITUITARY
TSH
THYROID CELLS
T4
T3
TARGET TISSUES
T4
deiodinase
T3
(+ CORTISOL)
SOMATIC GROWTH
DEVELOPMENT
REPRODUCTION
EXCRETION

The catabolic and gluconeogenic effects (Hadley 1984) of high cortisol production might be beneficial in facilitating production of energy (glucose) and substrates (solutes for osmoregulation) from muscle and fat. Cortisol also may stimulate chloride cell production (Perry and Wood 1985) to increase ion uptake (Flik and Perry 1989). These effects could promote adaption to the effects of low pH and Al. However, access to energy reserves and substrates not usually available to fish has the added consequence of diverting resources from reproduction and growth.

Chronic elevation of endogenous cortisol levels suppressed pituitary gonadotropin and circulating levels of sex steroids (Pickering 1989). Thus, sustained activation of the HPI axis due to low pH may represent a factor contributing to lower levels of reproductive hormones and poor reproductive performance observed in both feral (Freeman et al. 1983) and caged (Freeman and Sangalang 1985) Atlantic salmon from acidic rivers.

The reduced plasma T_3 concentrations and impaired T_3 kinetics in Al-exposed fish are of particular concern because T_3 plays an important role in somatic growth (Higgs et al. 1982; Leatherland 1982; Hilton et al. 1987) and in the early stages of female reproduction (Cyr et al. 1988). Recent studies document suppressed growth in trout treated with sublethal levels of acid plus Al, while growth was not suppressed in trout held in low pH water alone (Sadler and Lynam 1987; Mount et al. 1988; Reader et al. 1988). Gunn and Noakes (1987) reported latent effects of exposure (5 d) to low pH and Al lasting 30 d on growth and ontogenetic development in lake trout (*Salvelinus namaycush*) alveins. Furthermore, chronic treatment with low Al levels, while protecting fish from ionoregulatory disturbances due to low pH, was still detrimental to fish

growth (Mount et al. 1988). The environmental relevance of this observation merits further study because levels of Al judged to have little effect in bioassays may have long term consequences in terms of growth and reproductive performance. Reduction in T_4 conversion to T_3, now considered the active hormone form (Eales 1985), could be an important factor limiting fish growth in the presence of low ambient pH and Al.

It is worth noting that although plasma glucose represented a sensitive indicator of stress due to low pH and Al, it was not always clearly correlated with fluctuations in plasma cortisol (PARTS 1-3). Temporal inconsistencies between interrenal changes and plasma glucose exist in this and other studies (e.g. Nichols and Weisbart 1985; Kuhn et al. 1986). Thus the results show that other factors may be controlling plasma glucose levels during acid or acid and Al stress. However, cortisol may be necessary for the complete development of the glucose response to acid or acid and Al-induced stress.

Recently, Wendelaar Bonga and Balm (1989) suggested that the prolonged increase in plasma cortisol and interrenal activity in the rainbow trout (PARTS 1-3) resulted from the use of dying fish. There was mortality which I accurately documented but no fish upon which measurements were undertaken appeared moribund. The long-term responses (>3 d) I reported have since been documented in other studies in our laboratory on lake whitefish (*Coregonus clupeaformis*) (Scherer et al. 1986) and Arctic char (*Salvelinus alpinus*) (Jones et al. 1987), as well as by other laboratories for brook trout (Tam et al. 1987, 1988; Wood et al. 1988a) and brown trout (Brown, J.A. et al., 1989). The period during which interrenal activity was increased in these studies ranged from 7 to

240 d. Moreover, in a study I have not presented here, rainbow trout rapidly recovered from the effects of 21 d low ambient pH (4.7) exposure (Brown et al. 1986a) thus implying that the sampled fish were not dying. Recent evidence is consistent with long-term interrenal activation and the higher cortisol PDR found in rainbow trout exposed to low pH (PARTS 3 and 4). Sangalang et al. (1990) reported greater *in vitro* cortisol biosynthesis by interrenal tissue from caged Atlantic salmon (*Salmo salar*) held in the acidic (pH 4.7-5.2) Westfield River for 126 or 149 d relative to that in salmon held in the nearby but less acidic (pH 5.3-5.6) Medway River.

The field studies also support my laboratory observations concerning thyroid hormones. Holding Atlantic salmon in the acidic Westfield River for 126 or 149 d reduced plasma T_3 compared to levels found in salmon from the less acidic Medway River (Brown et al. 1990b). In brown trout held in an acidic Welsh stream during a 3-d episode of low pH and Al, plasma T_4 levels were increased (Whitehead and Brown 1989). This is a response I frequently observed in rainbow trout. Whitehead and Brown (1989) did not find reduced plasma T_3 but the exposure time may have been too short.

Evaluation of aspects of the HPT or HPI axes may eventually provide excellent means of assessing the responses of fish to toxic environmental stressors. Current information is largely limited to the HPI and it is not well related to effects on growth, reproduction or survival. Further research dealing with the effects of contaminants and endocrine functions is necessary to better understand modes of toxic action and relationships to ecologically relevant processes.

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