COMPLETION REPORT

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Impact of Surface Water Acidification on Commercially and Recreationally Important Salmonid Fishes: Effects on Reproductive Success and Recruitment

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EXECUTIVE SUMMARY

- Reproductive failure has been cited as an important factor in the gradual process of fish population extinction in acidified surface waters. Effects of low environmental pH on salmonid reproduction were evaluated in this regard, using rainbow trout as a model.

- Adult trout were exposed to pH levels 4.5, 5.0, 5.5, and control (range 6.5 to 7.1) during the final 6 weeks of reproductive maturation. Plasma sex steroid levels and calcium concentrations revealed no gross physiological abnormality in adult trout. Plasma sodium levels tended to decrease in acid-exposed fish and fell sharply in fish exposed to pH 4.5 for 42 days.

- Reduced survival rates in unacidified water of the progeny of acid-exposed trout indicate that spermatogenesis and oogenesis are sensitive to pH levels 5.5 and below. Survival rates of the progeny of unexposed parents were reduced by exposure to pH levels 4.5, 5.0, and 5.5.

- These results demonstrate that gametogenic performance and subsequent survival and fitness of early life history stages of salmonids may be influenced by environmental acidity even at pH levels which do not elicit clinical indications of reproductive stress in adult fish.

- Exposure of juvenile rainbow trout to acidic conditions for 5 days altered plasma glucose, plasma sodium, and hematocrit levels, but not plasma cortisol, a primary stress response factor and frequently employed indicator of stress.

- Subjection of the juvenile trout to severe handling stress after acid exposure resulted in much greater elevations of plasma cortisol levels in fish exposed to pH 4.7 than in fish held at ambient pH (6.6).

- These results indicate that acid-stressed fish are more sensitive to additional stresses even though they may appear to be physiologically normal.

- Planning of management strategies such as stocking fish in acidified waters must take into account the possibility that acid-exposure of juvenile trout may exacerbate responses to additional stress.

ABSTRACT

Reproduction of salmonid fishes in acidic water was studied, using the rainbow trout (*Salmo gairdneri*) as a model. Adult trout were exposed to pH levels 4.5, 5.0, 5.5, and control (6.5 to 7.1) during the final 6 weeks of reproductive maturation. Reduced survival rates of the progeny of acid-exposed females through 7 days of development, hatching, and yolksac absorption demonstrate that oogenesis is sensitive to environmental acidification. Similar reductions in the survival of the progeny of acid-exposed males indicate the sensitivity of spermatogenesis to low ambient pH. The progeny of unexposed adults had lower survival rates through 7 days of development and hatching when they were reared at pH levels 4.5, 5.0, and 5.5 than did embryos reared at the control pH level. No embryos exposed to pH 4.5 survived to the eyed stage. Plasma estradiol-17β, androgen, and 17α-hydroxy-20β-dihydroprogesterone concentrations in acid-exposed adult trout revealed no gross physiological abnormalities due to acid stress. Plasma calcium levels in adult females showed no apparent effects due to low pH, but were decreased in adult males exposed to pH 4.5 for 42 days. Plasma sodium levels fell slightly in adult fish after 7 days of exposure to pH levels 4.5, 5.0, and 5.5 and continued to decline in fish exposed to pH 4.5. We conclude that salmonid gametogenesis and early ontogeny are likely to be affected by environmental acidification.

TABLE OF CONTENTS

INTRODUCTION 1

Acidic deposition and water quality alterations 2

Fish mortalities and toxicity bioassays 3

Physiological responses to ambient acidity 6

Population extinction and reproductive failure 8

METHODS 12

RESULTS 17

DISCUSSION 23

BIBLIOGRAPHY 30

APPENDIX 1 41

References 57

Table 1 63

Figures 64

LIST OF TABLES

Table		Page
1	Survival rates of the progeny of acid-exposed and unexposed adult rainbow trout	18
2	Calcium and sodium concentrations in plasma of acid-exposed adult rainbow trout	21
3	Estradiol-17β, androgen, and 17α-hydroxy-20β-dihydroprogesterone concentrations in plasma of acid-exposed adult rainbow trout	22
1	Appendix	63
	Mean plasma cortisol, plasma glucose, plasma sodium, and hematocrit in juvenile rainbow trout after continuous exposure to reduced environmental pH	

LIST OF FIGURES

Appendix:		Page
Figure 1	Responses of plasma cortisol in juvenile rainbow trout subjected to a 30-s handling stress after 5 days of continuous exposure to low environmental pH	64
Figure 2	Responses of plasma glucose in juvenile rainbow trout subjected to a 30-s handling stress after 5 days of continuous exposure to low environmental pH	66
Figure 3	Responses of plasma sodium in juvenile rainbow trout subjected to a 30-s handling stress after 5 days of continuous exposure to low environmental pH	68
Figure 4	Responses of hematocrit in juvenile rainbow trout subjected to a 30-s handling stress after 5 days of continuous exposure to low environmental pH	70

PREFACE

This completion report fulfills the concerns of an Interagency Agreement (No. AD-14F2A100) entitled "Impact of Surface Water Acidification on Commercially and Recreationally Important Salmonid Fishes: Effects on Reproductive Success and Recruitment" between the United States Environmental Protection Agency's Corvallis Environmental Research Laboratory and the United States Fish and Wildlife Service's Oregon Cooperative Fishery Research Unit. Work for this Interagency Agreement was carried out under a Fish and Wildlife Service Research Work Order (No. 14-16-0009-1512, WO No. 1).

In addition to the specified tasks outlined in the Interagency Agreement, an ancillary study was conducted to optimize usage of the experimental fish-rearing facilities established for this agreement. This project on the "Effects of Prior Environmental Acidification on Physiological Stress Response of Juvenile Rainbow Trout to Acute Handling Stress" is described in the Appendix I of this report.

We wish to acknowledge the help provided to us for our studies by the staff of the Environmental Protection Agency's Western Fish Toxicology Station. In particular, we appreciate the help of Don Stevens in designing and setting-up the acidification dilution and delivery system and Gary Chapman for facilitating our work at the station. We also appreciate the help of Robert T. Lackey of the Corvallis Environmental Research Laboratory regarding the study design and the help of numerous students and staff of the Oregon Cooperative Fishery Research Unit, not identified in this report, with the rearing and breeding aspects of the study, as well as with laboratory analysis of samples.

Although the research described in this report has been funded wholly or in part by the Environmental Protection Agency through an Interagency Agreement, it has not been subjected to Agency's required peer and policy review and therefore does not necessarily reflect the views of the Agency.

INFLUENCES OF ENVIRONMENTAL ACIDIFICATION ON SALMONID REPRODUCTION

INTRODUCTION

Surface water acidification, presumably due in part to acidic precipitation, has been documented in northern Europe (Almer et al. 1974; Leivestad et al. 1976; Wright et al. 1980) and eastern North America, including the northeastern region of the United States (Haines 1981). The occurrence of acidic precipitation has been reported in other parts of the United States, such as the Los Angeles, San Francisco, Seattle, and Denver areas (Lewis and Grant 1980; Powers and Rambo 1981), and these areas contain lakes and streams that are susceptible to acidification (Omernik and Powers 1983). Thus, current and potential impacts of acidification on aquatic biota are receiving widespread attention.

Fish populations have been eliminated as a result of acidification in some systems and are endangered in others (Jensen Snekvik 1972; Schofield 1976; Harvey 1980). Because of the commercial and recreational importance of salmonid fisheries in Europe and North America, much of the concern has focused on the impacts of acidification on salmonid populations. The literature review in this section establishes the context for a study of the reproduction of rainbow trout (*Salmo gairdneri*) in a low pH environment, which will be reported in the following sections.

Acidic deposition and water quality alterations

The combustion of fossil fuels results in the emission of sulfur dioxide and nitrogen oxides into the atmosphere, where they undergo a complex process of transportation and transformation into sulfuric and nitric acids (Dovland and Semb 1980). Along with other chemicals from various natural and man-made sources, these acids may reach watersheds and aquatic systems through wet precipitation, dry deposition, impaction of aerosols, and adsorption of gases (Galloway and Cowling 1978).

The alterations of surface water chemistry by acidic deposition depend upon the nature of the receiving system and the quantity and mode of acidic input. The chemical composition of precipitation is modified as it moves through the watershed and interacts with the geological substrate, soil, and vegetation (Likens et al. 1979). The acid-neutralizing capacity of the bedrock and soils, hydrologic flow characteristics, soil and mineral types, and age of the soil influence the sensitivity of particular systems to acidification (Hendrey et al. 1980).

Temporal variations in acidification processes are also important. Heavy rains and rapid snowmelt may result in sharp episodic pH depressions and metal inputs (Gjessing et al. 1976; Schofield and Trojnar 1980). Chronic low pH conditions develop in waters in which the bicarbonate buffering system is gradually depleted (Henriksen 1980).

The characteristics of watersheds that determine water quality responses to acidic inputs are sufficiently divergent to warrant caution in describing "typical" conditions in impacted systems. Gjessing et al. (1976) thoroughly reviewed this subject. They noted increased acidity, decreased alkalinity, replacement of bicarbonate by sulfate as the predominant anion, and increased concentrations of calcium, magnesium, and aluminum as common effects of acidic precipitation on the water chemistry of susceptible lakes and streams.

Fish mortalities and toxicity bioassays

Observations of fish mortalities have been correlated with sudden episodic acidification events. Jensen and Snekvik (1972) described mortalities of Atlantic salmon (*Salmo salar*) in southern Norway associated with snowmelt. The pH of samples taken from a river during the fishkill ranged from 3.9 to 4.2. Similarly, an acidic pulse due to snowmelt coincided with a massive kill of brown trout (*Salmo trutta*) in the Tovdal River of Norway (Leivestad and Muniz 1976). Schofield (1977) reported mortalities of adult, yearling, and larval brook trout (*Salvelinus fontinalis*) held in tanks supplied with water from Little Moose Lake, New York, during a spring snowmelt. The relatively minor change in lake water pH and significantly elevated aluminum concentrations implicated aluminum as the dominant toxic factor rather than hydrogen ion. Grahn (1980) theorized that a kill of ciscoe (*Coregonus albula*) in an acidified Swedish lake resulted from the exposure of fish to precipitating aluminum hydroxide.

Bioassay studies have provided information about several factors that influence acid toxicity. Leivestad et al. (1976) pointed out that high hydrogen ion concentrations are relatively more toxic in waters with low concentrations of dissolved salts. Kwain (1975) showed a positive relationship between temperature and median lethal pH levels for rainbow trout fingerlings. Packer and Dunson (1972) and Swartz et al. (1978) determined that sulfuric acid, hydrochloric acid, and acid mine waste are not of equal toxicity.

As illustrated by the tabulation of acute and chronic pH exposure bioassays prepared by Spry et al. (1981), differences in test conditions make it difficult to generalize about the relative toxicity of acidic water to fishes. However, some important conclusions can be drawn. The effects of elevated hydrogen ion concentrations appear to be species-specific. For example, Grande et al. (1978) concluded that the relative order of tolerance to low environmental pH conditions for some salmonids is (from least tolerant to most tolerant): rainbow trout, Atlantic salmon, brown trout, and brook trout. Johansson et al. (1977) demonstrated that the early development of Atlantic salmon is more sensitive to hydrogen ion toxicity than is the early development of brown trout or brook trout. Intraspecific differences in tolerance have been demonstrated for brook trout (Robinson et al. 1976; Swartz et al. 1978) and brown trout (Edwards and Gjedrem 1979).

In addition to species specificity of tolerance to ambient acidity, the toxicity of acidic water varies among life history stages. Bioassay results (Menendez 1976; Daye and Garside 1977; Craig and Baksi 1977; Carrick 1979) reveal as a general trend that yolksac larvae are the least tolerant of acid stress followed by post-yolksac larvae, embryos, juveniles, and adults, in order of increasing tolerance.

Aluminum, leached from soils by acidic waters and carried into lakes and streams through watershed runoff, can reach high enough concentrations to be toxic to fish (Cronan and Schofield 1979). Schofield (1977) identified aluminum as the primary toxicant in snowmelt runoff into Little Moose Lake, New York, and discussed the complex interactions between aluminum and pH. Driscoll et al. (1980) determined that inorganic aluminum forms contribute most to fish toxicity, whereas organically complexed aluminum is of relatively low toxicity. Baker and Schofield (1982) further evaluated the effects of aluminum and hydrogen ions on early life history stages of white sucker (*Catostomus commersoni*) and brook trout. They concluded that the effects of aluminum vary for different life history stages; aluminum toxicity is dependent upon pH; and brook trout and white suckers are not equally sensitive to aluminum and hydrogen ions. Interestingly, they showed that aluminum actually enhanced survival of eggs at pH levels between 4.2 and 4.8. Damage and mucous clogging of gills due to aluminum exposure have been reported frequently (Freeman and Everhart 1971; Schofield 1977; Schofield and Trojnar 1980; Muniz and Leivestad 1980; Baker and Schofield 1982).

Physiological responses to ambient acidity

Studies of the physiological responses of fish to ambient acidity have usually involved acute exposures to very low pH. Spry et al. (1981) commented that these results may be of limited applicability in assessing chronic effects of acidification. Nevertheless, they do contribute to an understanding of some important physiological processes, especially ionoregulation and acid-base balance.

Leivestad and Muniz (1976) correlated the losses of sodium and chloride from the plasma of brown trout with acid stress in the Tovdal River of southern Norway and in tank experiments. Packer and Dunson (1970, 1972) attributed the decline in total body sodium of acid-exposed brook trout to decreased influx and increased efflux rates. Similar depletions in plasma sodium and chloride have been demonstrated with rainbow trout (McDonald and Wood 1981; Booth et al. 1982). McWilliams and Potts (1978) determined that high hydrogen ion concentrations may be associated with a shift in gill transepithelial potential from negative to positive, resulting in increased permeability of the gill epithelium to sodium. Furthermore, they reported that the presence of calcium in the water reduced the permeability of the gill epithelium to sodium. Spry et al. (1981) related this information to a field survey by Wright and Snekvik (1977) in which fish population status was positively correlated with lake calcium concentration.

McDonald et al. (1980) found that exposure of rainbow trout to high levels of hydrogen ions in soft water resulted in severe ionoregulatory dysfunction and minor acidosis. In contrast, exposure to ambient acid in hard water showed little effect on plasma ions and more serious acidosis. Lowered blood pH has been reported in several other studies of rainbow trout (Neville 1979; McDonald and Wood 1981; Booth et al. 1982), brook trout (Packer 1979), and carp (Cyprinus carpio) (Ultsch et al. 1981). Neville (1979) found increases in hemoglobin, hematocrit, and erythrocyte levels as additional effects of acid exposure on blood characteristics.

Daye and Garside (1976) studied the histopathology of brook trout surficial tissues in low pH waters. Their findings included hypertrophy of mucous cells in the gills, nares, and integument; and epithelial damage in the gills, corneae, integument, and esophagus. Fritz (1980) speculated that such injuries to corneal and olfactory tissues could affect salmonid imprinting, homing, and spawning. Integumental damage in prehatching embryos and gill epithelial alterations in alevins contributed to acid-induced mortalities of young Atlantic salmon (Daye and Garside 1980).

Falk and Dunson (1977) observed behavioral indications of stress in brook trout at pH 5.0 and 5.8. Johnson and Webster (1977) determined that spawning female brook trout avoided upwellings of pH 4.0 and 4.5. They suggested that this behavior could aid in increasing egg and larval survival in systems where such an avoidance response is possible.

Population extinction and reproductive failure

Occasional fish kills provide dramatic evidence of detrimental effects of acidification, and physiological studies of acute acid and metal toxicity add to our understanding of some relevant processes. However, the persistence of resources in impacted systems is of greater concern from a fisheries perspective.

In a survey of southern Norway, for example, the percentage of lakes devoid of trout populations increased gradually from 3.8% to 60.0% as lake pH decreased from 5.5 or more to 4.0 (Jensen and Snekvik 1972). A later study of 700 lakes in southern Norway (Wright and Snekvik 1977) revealed that about 40% of the lakes were devoid of fish and another 40% had sparse populations. Brown trout is the principal species of the region.

Fish population surveys of 50 Swedish lakes that ranged in pH from 4.40 to 7.45 were reported by Almer et al. (1974). The roach (*Leuciscus rutilus*) was missing from ten of the low pH lakes and reproductive failure was apparent below pH 5.5. Populations of perch (*Perca fluviatilis*), pike (*Esox lucius*), minnow (*Phoxinus phoxinus*), arctic char (*Salvelinus alpinus*), brown trout, ciscoe, and eel (*Anguilla vulgaris*) were also sparse or nonexistent.

Concern for possible acidification and metal deposition in the La Cloche Mountain Lakes of Ontario caused by a smelter prompted several studies of fish population status. Beamish and Harvey (1972) chronicled the gradual reductions and losses of populations of seven

species as the pH of Lumsden Lake dropped from 6.8 in September 1961 to 4.4 in August 1971. Beamish (1974) described the decline of populations in Ontario Society of Artists (O.S.A.) and Muriel Lakes. The average pH of O.S.A. Lake during the study was 4.5 and the average pH of Muriel Lake was 4.7. Beamish et al. (1975) studied the fish populations of George Lake, where the pH ranged from 4.8 to 5.3 between February 1972 and August 1973. They reported changes in the average size of age classes, reduction of population sizes, and disappearance of some species.

A survey of 217 high elevation lakes in the Adirondack Mountains of New York (Schofield 1976) showed that 51% of the lakes had pH values below 5.0. No fish were present in 90% of the low pH lakes. Schofield and Trojnar (1980) determined that in 53 acidified lakes of the Adirondacks, mean aluminum concentrations were inversely correlated with presence of stocked brook trout. The mean concentrations were 0.29 mg Al/liter for lakes in which trout were absent and 0.11 mg Al/liter for lakes in which trout were present.

Reproductive failure has been cited as a major contributor to the gradual extinction of fish populations in acidic waters (Jensen and Snekvik 1972; Beamish et al. 1975; Beamish 1976; Ryan and Harvey 1977, 1980). Of the many factors that determine reproductive success, investigators have specified mortality of early life history stages (Jensen and Snekvik 1972: Leivestad et al. 1976) and physiological disruptions during gametogenesis (Beamish et al. 1975; Lockhart and Lutz 1977) as the causes of reproductive failure due to acidic conditions.

As noted earlier, bioassays have established that early life history stages are relatively more sensitive to hydrogen ion than are juveniles and adult fish. Other studies have demonstrated impairment of adult reproductive physiology due to acid stress. Craig and Baksi (1977) showed reduced egg production and egg fertility in flagfish (Jordanella floridae) at low pH. Similarly, Lee and Gerking (1980) exposed adult desert pupfish (Cyprinodon nevadensis nevadensis) to acidic conditions and observed decreased egg production, egg laying, and egg viability. Ruby et al. (1977) determined that exposure to high levels of hydrogen ion of adult female flagfish inhibited the deposition of secondary yolk in the cytoplasm of oocytes. Beamish et al. (1975) and Lockhart and Lutz (1977) concluded that acidic water may impair plasma calcium regulation associated with vitellogenesis in reproductively maturing female fishes.

The goal of the research described here was to increase understanding of current and potential consequences of low environmental pH for the reproduction of salmonid fishes. This goal was addressed by studying final reproductive maturation and progeny survival of rainbow trout, a relatively acid-sensitive species among the salmonids (Grande et al. 1978), in a low pH environment. Plasma ion and sex hormone levels were evaluated as physiological indicators of reproductive stress in acid-exposed adult trout. Estradiol-17β is involved in vitellogenesis and calcium regulation in maturing female salmonids (Whitehead et al. 1978) and may serve as an indicator of reproductive stress early in the reproductive cycle. Similarly, the

androgens are the primary gametogenic hormones in male salmonids and they co-vary with estradiol-17β in females (Sower and Schreck 1982).

The performances of the gametes from the acid-exposed fish were compared to those from unexposed trout to demonstrate effects of high levels of hydrogen ion on oogenesis and spermatogenesis. Specifically, We were interested in the ontogenetic effects of low environmental pH on reproductive success and in evaluating the potential for maternal and paternal effects.

METHODS

Adult rainbow trout (age 2^+), averaging 57.4 ± 3.2 cm in length and 2.2 ± 0.5 kg in weight, were obtained in June 1982 from the Roaring River Hatchery (Oregon Department of Fish and Wildlife, Scio, Oregon) and transported to the Western Fish Toxicology Laboratory of the United States Environmental Protection Agency, Corvallis, Oregon. Blood samples were obtained (prior to October 5, 1982) by caudal puncture with a needle and ammonium-heparinized syringe, and gender of the fish was determined by the Ouchterlony Immunodiffusion Assay Technique through which vitellogenin is specifically detected in the plasma of female fish (Terry Owen, Helix Biotech, Ltd., Richmond, British Columbia, Canada). The fish were distributed on October 27, 1982 into eight 757-liter circular fiberglass tanks, with three females and four males in each tank. The tanks were set up outdoors to provide a natural photoperiod.

The inflowing well water was gradually softened by reverse osmosis (Purification Techniques, Inc., Brielle, New Jersey) from 23 mg/liter as $CaCO_3$ to 10 mg/liter over a period of five days. After an additional 3 days of acclimation, reagent grade sulfuric acid was introduced into the system over 5 days until pH levels of 4.5, 5.0, and 5.5 were reached in duplicate treatment tanks, while two control tanks (pH range 6.5 to 7.1) remained on softened water.

Total water hardness, determined at least twice daily by the EDTA titrimetric method (American Public Health Association et al. 1980), was 9.2 ± 1.2 mg/l as $CaCO_3$ during the experiment. A stock solution of

softened, vigorously aerated water was acidified with sulfuric acid and held at pH 4.0 ± 0.1 with a Beckman Model 942 pH Monitor (Beckman Instruments, Inc., Fullerton, California). The acidified stock solution was vigorously aerated to remove carbon dioxide that may have been generated by the addition of acid, and pumped to the head tank of a gravity-flow diluter. The acidic stock solution was diluted with softened well water to attain appropriate pH levels and delivered at a rate of 4.5 liters/minute to the tanks in the flow-through system. Treatment pH levels were automatically monitored and recorded every 3 hours and the diluter was manually adjusted as necessary in order to maintain exposure levels within ± 0.1 pH units of 4.5, 5.0, and 5.5. Water temperatures during the experiment ranged from 10.9 to 14.0 C and alkalinity, determined by Gran titration as described by Stumm and Morgan (1981), was 252 ± 11 μeq/l prior to addition of acid. Metal levels were below limits of detection by atomic absorption flame spectrophotometry.

Blood samples from each fish were drawn from the caudal artery with a needle and ammonium-heaparinized syringe under anesthesia (MS222, 50 mg/liter) and the plasma was stored at -20 C. Anesthetic solutions were titrated to treatment pH levels with 0.1 M NaOH to minimize stress. The first blood sample was taken following tank acclimation, just prior to the addition of acid to the system. The second sample was taken 7 days after treatment pH levels were reached, and the final sample was drawn at the time of spawning.

Fish were examined for reproductive maturity weekly, beginning with the fourth week of exposure. After 42 days of exposure (December 21, 1982), the ripe fish were anesthetized for blood sampling, killed with a blow to the head, and manually spawned. The gametes were collected separately from each fish and matings were conducted according to the following scheme, employing dry fertilization and 1-hour water hardening:

A) Unexposed females X unexposed males

Eggs from unexposed females were fertilized with sperm from unexposed males. Water hardening and rearing took place at each of the pH levels used in the prespawning exposure in order to assess the performance at low pH of the progeny of unexposed adults.

B) Acid-exposed females X unexposed males

Eggs from females exposed to each pH level were fertilized with sperm from unexposed males. Water hardening and rearing took place at the control pH level to determine the effects of acidic water on oogenesis.

C) Acid-exposed males X unexposed females

Eggs from unexposed females were fertilized with sperm from males from each pH exposure group. Water hardening and rearing took place at the control pH level to determine the effects of acidic water on spermatogenesis.

D) Acid-exposed females X acid-exposed males

Eggs from females exposed to each pH level were fertilized with sperm from males exposed to the same pH level. Water hardening and rearing took place at the respective parental exposure pH levels to assess reproductive performances when both parents and the progeny were exposed to low pH.

Aliquots of embryos from each fertilization group were placed in incubation cells within drawers of Heath incubators for rearing. The cells consisted of 4.6 cm sections of 6.4 cm diameter PVC pipe with plastic mesh screen attached across one end, forming a cup. Similar cups, constructed of 1.3 cm sections of 7.6 cm diameter PVC pipe, were inverted over each cell to confine the fish after hatching. The flow rate through the incubators was 2 liters/minute. The incubators were covered with black plastic sheets to exclude light. Dead eggs and larvae were counted and removed daily except as noted subsequently. A different incubator was used for each test pH level (4.5, 5.0, 5.5, and ambient). Within a treatment, cells of eggs were randomly distributed throughout the incubator.

Plasma estradiol-17β, androgen (testosterone and 11-ketotestosterone) and 17α-hydroxy-20β-dihydroprogesterone concentrations were determined by radioimmunoassay, following the general method of Korenman et al. (1974). Details of the procedure are described by Sower and Schreck (1982). Estradiol-17β was determined in 10 μl of plasma for males and 25 μl of plasma for females. Androgens were determined in 10 μl of plasma and

17α-hydroxy-20β-dihydroprogesterone was determined in 25 μl of plasma. Samples were extracted twice with diethyl ether and extraction efficiency was about 90% for all assays. Antiestradiol-17β and antitestosterone were provided by Dr. G. Niswender (Colorado State University, Fort Collins, Colorado) and anti-17α-hydroxy-20β-dihydroprogesterone was provided by Dr. A. P. Scott (Ministry of Agriculture and Fisheries, Lowestoft, Suffolk, United Kingdom). Antibody characteristics are given by Sower and Schreck (1982) and Scott et al. (1982). Plasma calcium concentrations were determined by atomic absorption flame emission spectrophotometry, and sodium concentrations were measured by flame photometry.

Progeny survival rates were statistically analyzed with contingency tables (Cochran and Cox 1957). Plasma calcium and sodium concentrations were evaluated by analysis of variance and Duncan's new multiple-range test for comparisons among means when appropriate (Steel and Torrie 1980).

RESULTS

Survival rates of the progeny of acid-exposed and unexposed trout appear in Table 1. On the eighth day of development, some eggs became infected with Saprolegnia fungi. Thus, mortalities through the last day before the occurrence of infection (7 days of development) were tallied, the infected eggs were removed, and the remaining eyed eggs were monitored for survival through hatching. Survival through yolksac absorption was then determined among the hatched fish.

The progeny of unexposed adults that were reared at pH 5.5 and 5.0 (groups B and C) had lower survival rates through 7 days and slightly lower hatching success than those reared in unacidified water (group A). No embryos reared at pH 4.5 (groups D and M) survived through 7 days.

Influences of acid-exposure on oogenesis are indicated by the reduced hatching percentages among the progeny of females that had been exposed to pH levels 5.5 and 4.5 (groups E and G). The progeny of females that had been exposed to pH 5.5 (group E) had lower survival from hatching through yolksac absorption than did controls (group A).

Impaired spermatogenesis at low pH is evident in the performances of the progeny of acid-exposed males (groups H, I, and J). The progeny of males exposed to pH 4.5 prior to spawning (group J) had lower survival through 7 days of development and through hatching than did the progeny of unexposed males (group A). The progeny of males exposed to pH levels 5.5 and 5.0 (groups H and I) had lower survival rates through hatching and yolksac absorption than did controls (group A).

Table 1. Survival rates of the progeny of acid-exposed and unexposed adult rainbow trout. Total numbers of eggs or larvae in each case are in parentheses.

	Parental prespawning exposure pH level		Progeny rearing pH level	Percent survival through 7 days	Percent of eyed eggs successfully hatched	Percent of larvae surviving to yolksac absorption	[d] Cumulative percent survival
	Female	Male					
A	control	control	control	91.3(689)	93.8(465)	86.2(436)	73.8
B	control	control	5.5	[a]64.2(749)	[a]89.2(381)	92.4(340)	52.9
C	control	control	5.0	[a]54.7(766)	[a]80.1(294)	81.5(238)	35.7
D	control	control	4.5	[a] 0 (761)	--	--	0
E	5.5	control	control	91.9(211)	[a]57.8(154)	[a]70.8(89)	37.6
F	5.0	control	control	[a]99.1(439)	91.1(426)	88.9(388)	80.3
G	4.5	control	control	93.1(189)	[a]26.4(148)	89.7(39)	22.0
H	control	5.5	control	93.8(689)	[a]80.8(386)	[a]79.5(312)	60.2
I	control	5.0	control	93.2(883)	[a]75.0(641)	[a]82.7(481)	57.8
J	control	4.5	control	[a]87.4(933)	[a]58.6(534)	86.6(313)	44.4
K	5.5	5.5	5.5	60.3(136)	[b]57.8(71)	[b]82.9(41)	28.9
L	5.0	5.0	5.0	[c]76.8(555)	84.0(374)	77.7(314)	50.1
M	4.5	4.5	4.5	0 (244)	--	--	0

[a] Different from group A at the corresponding stage of development, $P < 0.05$.

[b] Different from group B at the corresponding stage of development, $P < 0.05$.

[c] Different from group C at the corresponding stage of development, $P < 0.05$.

[d] Calculated as the product of the survival rates in the three preceding columns.

When both parents and the progeny were exposed to pH 5.5 (group K), hatching success and survival through yolksac absorption were reduced in comparison with the performances at pH 5.5 of the progeny of unexposed adults (group B). Females exposed to pH 5.0 produced gametes of high quality as reflected in the survival of their progeny in unacidified water and at pH 5.0 (groups F and L) through seven days of development in comparison with the performances of the progeny of unexposed females (groups A and C).

Calcium concentrations in the plasma of the adult trout are presented in Table 2. No clear indications of acid stress are apparent in the calcium levels of the females. Plasma calcium decreased in males that were exposed to pH 4.5 for 42 days ($P < 0.05$). Plasma sodium levels in the adults tended to decrease after 7 days of exposure to each treatment pH level (Table 2). Fish exposed to pH 4.5 for 42 days had sharply reduced sodium concentrations in comparison with unexposed fish ($P < 0.05$). Estradiol-17β, androgen, and 17α-hydroxy-20β-dihydroprogesterone levels in adult trout revealed no differences between treatments (Table 3). There was no apparent effect of the acid exposure on rate of sexual development since the fish all ovulated at the same time. When pressure was applied to the abdomen, eggs flowed easily from the ovipore. No eggs appeared to be atretic. In two of the females held at pH 4.5, approximately 10% of the eggs in the ovaries had not ovulated and were held tightly in the skein, although the remainder of the eggs were fully mature and ovulated. All males were ripe, having copious quantities of sperm at the time of spawning.

Hatching occurred synchronously among embryos reared in unacidified water and at pH levels 5.5 and 5.0. Three adult mortalities occurred at pH 4.5. One adult mortality occurred in unacidified water.

Table 2. Calcium and sodium concentrations (mean ± standard error) in plasma of acid-exposed adult rainbow trout.

Sex/Exposure pH	Prior to acidification (57 days prespawning)	7 days of acid exposure (35 days prespawning)	42 days of acid exposure (spawning)
		Calcium (milliequivalents/liter)	
Females			
4.5	7.6 ± 0.7	6.2 ± 0.5	5.5 ± 0.8
5.0	6.4 ± 0.5	7.0 ± 0.4	7.5 ± 1.5
5.5	5.8 ± 0.6	6.3 ± 0.6	5.7 ± 0.8
control	8.2 ± 0.5	8.7 ± 0.7	7.3 ± 1.6
Males			
4.5	5.5 ± 0.2	5.5 ± 0.3	[a]4.8 ± 0.1
5.0	5.4 ± 0.1	5.5 ± 0.2	5.3 ± 0.1
5.5	5.2 ± 0.1	5.2 ± 0.2	5.8 ± 0.3
control	5.3 ± 0.2	5.8 ± 0.2	5.2 ± 0.1
		Sodium (milliequivalents/liter)	
Male and Female			
4.5	153 ± 2	145 ± 4	[a]129 ± 4
5.0	153 ± 1	146 ± 3	147 ± 4
5.5	152 ± 3	143 ± 4	143 ± 6
control	153 ± 1	152 ± 1	154 ± 2

[a]Different from mean value in same exposure group prior to acidification and mean value in control group at spawning, $P < 0.05$.

Table 3. Estradiol-17β, androgen, and 17α-hydroxy-20β-dihydroprogesterone concentrations in plasma of acid-exposed adult rainbow trout.

Sex/Exposure pH	Prior to acidification (57 days prespawning)		7 days of acid exposure (35 days prespawning)		42 days of acid exposure (spawning)	
	mean	range	mean	range	mean	range
			Estradiol - 17 β (ng/ml)			
Females						
4.5	2.9	1.1-4.1	3.3	1.1-4-8	0.7	<0.1-1.5
5.0	3.0	1.0-5.5	4.0	1.7-5.4	0.7	0.3-0.8
5.5	3.4	1.4-7.6	3.7	2.4-5.5	0.3	0.1-0.5
control	2.9	1.2-5.6	3.4	1.3-5.8	0.6	<0.3-1.3
Males						
4.5	0.1	<0.1-0.2	0.1	<0.1-0.2	0.1	<0.1-0.1
5.0	0.1	<0.1-0.3	0.1	<0.1-0.3	0.1	<0.1-0.1
5.5	0.1	<0.1-0.2	<0.1	<0.1-0.1	<0.1	<0.1-0.1
control	0.1	<0.1-0.2	<0.1	<0.1-0.1	<0.1	<0.1-0.1
			Androgens (ng/ml)			
Females						
4.5	21	16-26	27	20-33	14	13-15
5.0	19	13-23	26	10-67	18	11-29
5.5	18	14-24	25	15-42	18	13-23
control	23	15-31	26	13-48	16	13-18
Males						
4.5	26	18-37	31	19-64	33	14-61
5.0	33	16-59	19	17-25	21	10-34
5.5	24	21-27	31	17-67	27	15-35
control	29	17-48	22	13-35	21	13-46
			17α-hydroxy-20β-dihydroprogesterone (ng/ml)			
Females						
4.5	<0.1	all <0.1	1.2	0.4-5.8	37	36.2-39.5
5.0	<0.1	<0.1-0.3	0.6	<0.1-1.0	26.7	6.8-50.9
5.5	0.1	<0.1-0.7	0.7	<0.1-2.4	32.1	21.1-43.1
control	0.2	<0.1-0.7	0.6	<0.1-2.9	24.6	15.3-33.9

DISCUSSION

Reproductive success of salmonids in acidic water is influenced by gamete quality and the developmental environment of the early life history stages. Exposure of adult rainbow trout to pH levels 5.5, 5.0, and 4.5 during final maturation affected gamete quality as demonstrated by the reduced survival in non-acidic water of the progeny of acid-exposed fish. Both oogenesis and spermatogenesis were impaired. Eggs from females that had been exposed to pH levels 5.5 and 4.5 prior to spawning had lower survival rates than did eggs from unexposed females, indicating detrimental effects of ambient acid during oogenesis. In addition, two of the five females in the pH 4.5 exposure group that survived through final maturation were incompletely ovulated and it is possible that the eggs that were ovulated at this level were not fully developed. In addition, two of the five females in the pH 4.5 exposure group that survived through final maturation were incompletely ovulated; in salmonid fishes, all eggs ovulate synchronously, suggesting an aberrant condition in the females with two distinct egg stages.

The high survival rates of the progeny of females exposed to pH 5.0 prior to spawning suggest that some physiological aspects of final reproductive maturation may actually be enhanced at this pH level. Physiological processes are sensitive to alterations in acid-base balance due to the pH-dependence of protein function (Spry et al. 1981). Thus, it is possible that oxygen exchange at the ovary or enzymatic reactions during ovulation, for example, are

facilitated at some pH levels, but the actual effects of acid observed at this level remain enigmatic. The response of eggs from females exposed to pH 5.0 prior to spawning appears not to be an experimental artifact. The proportions of survival of eggs from individual females within and among replicate pre-spawning exposure groups were compared and found to be similar in each case before they were pooled for comparisons between treatments. Thus, the performance of eggs from the females exposed to pH 5.0 cannot be explained by individual variability in adult sensitivity to acid-exposure during gametogenesis. This performance also cannot be accounted for by error in the acid delivery system, since there were no unusual responses by the males that were exposed to pH 5.0 prior to spawning, and plasma sodium levels in adults of both genders declined at this pH level. The enhancement of oogenesis at pH 5.0 should not be interpreted as indicating that reproductive success of salmonids will be increased by acid exposure at this pH level. Impaired spermatogenesis is apparent in the decreased survival rates of the progeny of male trout held at pH 5.5 and lower before spawning. Effects of acid exposure on gametogenesis have previously been demonstrated histologically and through determinations of egg production and fertility in flagfish at pH 6.0 and lower (Craig and Baksi 1977, 1978). Similarly, Lee and Gerking (1980) showed reductions in egg quality and quantity in desert pupfish below pH 7.0. The present study provides evidence that salmonid reproductive physiology is sensitive to environmental acidity as well. Examination of the cumulative percent survival of the offspring of acid-exposed fish suggests that oogenesis is affected more seriously than is

spermatogenesis at pH levels 5.5 and 4.5 in rainbow trout, while the apparent enhancement of oogenesis at pH 5.0 remains unexplained.

Direct effects of environmental acidity on early life history stages are shown by the increased mortality at pH 5.5, 5.0, and 4.5 of the progeny of unexposed trout. No embryos that were exposed to pH 4.5 survived to the eyed stage. Embryos that were exposed to pH levels 5.5 and 5.0 were most sensitive to the acid during the first 7 days of development and during hatching. These sensitive periods have been identified for toxicants in general (Rosenthal and Alderdice 1976).

Plasma calcium levels are known to increase in conjunction with vitellogenesis in many teleosts, including salmonids (Bromage et al. 1982). Vitellogenesis is generally completed prior to the final 6 weeks of reproductive maturation and calcium levels decline gradually during this period in trout (Whitehead et al. 1978). Plasma calcium levels in the acid-exposed female trout tended to decrease as expected, but variability among individuals precluded any clear indication of reproductive stress based on calcium regulatory failure. Observations of low female to male plasma calcium ratios among mature fish in acid-stressed populations were ascribed to disruption of calcium regulation associated with vitellogenesis (Beamish et al. 1975; Lockhart and Lutz 1977). However, these observations occurred after vitellogenesis was probably completed. Perhaps episodic exposure to low pH earlier in the reproductive cycle or chronic exposure would result in discernible calcium regulatory dysfunction. Indeed, plasma calcium levels in male trout tend to be relatively invariant

(Whitehead et al. 1978), but they were reduced after exposure to pH 4.5 for 42 days.

The low plasma sodium levels in adult trout exposed to pH 4.5 for 6 weeks are consistent with current theory on the effects of acidic conditions on ionoregulation. Packer and Dunson (1970) showed that low pH conditions decrease sodium influx rates and increase efflux rates. Ambient acidity is believed to increase the permeability of the gill epithelium to sodium, accounting for the increased efflux rates (McWilliams and Potts 1978). McDonald et al. (1980) determined that ionoregulation and acid-base balance in acid-exposed rainbow trout are influenced by water hardness. Acute acid exposure in soft water produces severe ionoregulatory dysfunction, but only minor acidosis. However, in hard water, ionoregulation is slightly affected and profound acidosis may occur. Sublethal effects on sodium regulation of adult trout at pH 4.5 may have little meaning in the reproductive process, since no embryos survived to the eyed stage at this pH level.

Sex hormone concentrations did not reveal any gross physiological abnormalities due to acid exposure. Hormone profiles in the acid-exposed fish followed patterns typical for this species (Scott and Sumpter 1983). Estradiol-17β decreased just prior to spawning in the females and remained low throughout final maturation in the males. Androgen levels gradually declined in both males and females. 17α-hydroxy-20β-dihydroprogesterone concentrations were low in the females until ovulation, when they rose dramatically. Because these hormone levels are dynamically regulated and highly variable between

individuals (Schreck et al. 1972; Hille 1982), they are unlikely to provide useful warnings of reproductive impairment at sublethal pH levels.

Hatching was synchronous among the embryos reared at pH levels 5.5 and 5.0 and in unacidified water. Delayed hatching has been noted in Atlantic salmon and rainbow trout embryos that were exposed to low pH after they had reached the eyed stage (Peterson et al. 1980; Nelson 1982). Atlantic salmon embryos that were continuously exposed to low pH from fertilization through hatching, however, were not delayed (Peterson et al. 1980). Delays that have occurred in some studies may be due to inhibition of the hatching enzyme, chorionase, or to reduced activity of embryos just before hatching (Peterson et al. 1980; Peterson and Martin-Robichaud 1983).

Lethargic behavior was characteristic of rainbow trout yolksac larvae at pH levels 5.5 and 5.0. Rombough (1982) observed similar behavior in buttoned-up pink and chum salmon at sublethal pH levels. In an unprotected environment, such behavior might have important implications for the survival of young fish. For instance, salmonid larvae that remain inactive in the gravel may be vulnerable to predation by benthic invertebrates or other fish species, like some sculpins which are known to prey upon salmonid eggs and fry (Scott and Crossman 1973). This vulnerability to predation would depend, of course, on the tolerance of the potential predators for acidic conditions as well.

Salmonid reproductive success in acidic surface waters is determined by an array of water quality factors and biological responses. The experiment reported here demonstrates that salmonid reproduction is endangered at pH levels 5.5 and lower through effects on gamete quality and direct exposure of developing embryos and fry to acidic conditions. However, several other considerations are necessary for approaching a broad understanding of impacts of low environmental pH on salmonid reproduction.

One such consideration is the possible influence on reproductive success of acute or chronic exposure of adult fish to acidic water earlier in the reproductive cycle. In this study, continuous exposure to low environmental pH for 6 weeks prior to spawning affected gamete quality. Yet, ovarian maturation generally commences as early as 6 months before spawning in rainbow trout (Whitehead et al. 1978). Acidic conditions throughout this period of maturation or acute episodic exposures could exert additional influences on gamete quality. For example, disruptions of calcium balance in maturing female fishes as described by Beamish et al. (1975) may become important if acid-exposure occurs at the onset or peak of vitellogenetic activity, but were not observed as a consequence of acid-exposure during final maturation when vitellogenesis had probably been completed.

Studies of salmonid reproduction at low pH under controlled conditions may show physiological effects that are reflected in gamete quality, but they do not address potential effects on spawning behavior. Female brook trout avoided areas at pH 4.0 and 4.5 in

selecting sites for redd construction (Johnson and Webster 1977). Regardless of gamete quality, if female rainbow trout are unable to locate suitable spawning habitat, reproductive failure may occur. As yet, pH levels at which spawning behavior would be inhibited have not been investigated for rainbow trout.

This study partitions out the direct effects of acidic water on gamete quality and progeny survival in salmonids. As such, these data are useful for assessing impacts on salmonid reproduction in systems in which a high concentration of hydrogen ions is the dominant toxic factor. However, another factor that may be important in some acidic systems is metal toxicity. Aluminum, leached from soils by acidic water and transported into surface waters, may reach concentrations that are toxic to fish (Cronan and Schofield 1979). It cannot be assumed that aluminum toxicity and hydrogen-ion toxicity will simply be additive. Baker and Schofield (1982) found that the presence of aluminum was antagonistic to hydrogen-ion toxicity between pH 4.2 and 4.8. Interactions between acids and metals could conceibably alter gamete quality as well as survival of early life history stages.

Acidic waters of pH 5.5 and lower pose a threat to salmonid reproduction and, therefore, valuable fishery resources may be endangered. Prudent evaluations of current impacts or predictions of future impacts of low environmental pH on fish populations must recognize that many physiological and behavioral aspects of fish reproduction may be affected by low pH conditions and high metal concentrations. Caution should be taken to avoid overly simplistic models and techniques for assessment purposes.

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APPENDIX 1

Effect of Prior Environmental Acidification on Physiological Stress Responses of Juvenile Rainbow Trout to Acute Handling Stress

ABSTRACT

When juvenile rainbow trout, Salmo gairdneri, were exposed to acidic conditions for 5 d, plasma glucose, plasma sodium and hematocrit levels were significantly altered. At the lowest pH (4.7), plasma glucose increased from 88 to 258 mg/dL and plasma sodium decreased from 154 to 130 mmol/L by day 5, and hematocrit generally remained higher than control levels throughout the exposure. Plasma cortisol was affected only slightly during initial hours of exposure to test pH's of 5.7, 5.2, and 4.7. However, when the fish were subsequently subjected to a 30-s handling stress, the post-stress plasma cortisol level rose to a peak of 338 ng/mL at pH 4.7 as compared with 136 ng/mL in fish held at ambient pH (6.6). Effects on plasma glucose or sodium levels from the handling stress were masked by the continuous response to the chronic acid exposure. As judged by the corticosteroid response, the results indicate that acid-stressed fish were more sensitive to additional handling stress, even though they appeared to be physiologically normal after 5 d. Plasma glucose and sodium appeared to be better indicators than plasma cortisol of chronic acid stress alone, but the greater cortisol response to a handling stress at the low pH suggested that this may be a useful method of detecting increased interrenal activity during early stages of environmental acidification.

INTRODUCTION

The detrimental effects of acidic precipitation on fisheries resources is a global problem of which biologists are now well aware (see reviews by Haines 1981; Haines and Johnson 1982). In addition to direct effects upon survival and reproductive success, the sublethal effects of acid stress upon physiological mechanisms in fish are becoming increasingly better understood (see reviews by Fromm 1980; Spry et al. 1981; Wood and McDonald 1982). By comparison, relatively little work has addressed the potentially compounding effects of chronic acid exposure and additional stresses, such as physical disturbance or exercise, on physiological responses in fish. Graham et al. (1982) reported that post-exercise changes in a number of hematological and other physiological conditions--for example, plasma ion concentrations--were more severe in acid-exposed rainbow trout, Salmo gairdneri, than in control fish, when held in soft water. Graham and Wood (1981) had earlier found that severe exercise significantly increased the toxicity of H_2SO_4 to rainbow trout.

The suitability of both plasma cortisol and glucose as indicators of certain kinds of stress in fish is now well established (see reviews by Donaldson 1981; Schreck 1981; Wedemeyer and McLeay 1981). Recently, Brown et al. (1984) found increases in plasma cortisol and glucose in rainbow trout, as well as changes in interrenal histology and plasma thyroid hormones, during a 21-d chronic exposure to acid. To evaluate the possibility that responses to other subsequent stresses are modified in fish stressed by acidification, we assessed the changes in cortisol and glucose in rainbow trout resulting from an acute handling stress

following a chronic exposure to low environmental pH. We also determined plasma sodium and potassium, and hematocrit as indicators of possible impairment of physiological performance after exposure of fish to acid.

METHODS AND MATERIALS

Experimental Design

Juvenile rainbow trout (Willamette R. stock) used in the experiment weighed 51.7 ±1.9 g (mean ±SE) and had a fork length of 17.4 ±0.2 cm. The fish were reared and maintained in ambient flow-through well water at the Western Fish Toxicology Station, Environmental Protection Agency, Corvallis, Oregon. Twelve days prior to the experiment (in April 1983), the fish were transferred for acclimation to covered, outdoor 1.2-m-diameter circular tanks, each containing 350 L and receiving 4 L/min aerated, treated (soft) well water with ambient temperature of 17-19° C; pH was 6.6, alkalinity 12.6 mg/L as $CaCO_3$, total calcium 1.5 to 2.3 mg/L, and total hardness between 7.3 and 12 mg/L as $CaCO_3$. Soft-water conditions were established and maintained by a reverse osmosis system (Purification Techniques Inc., Brielle, New Jersey). A total of 120 fish per tank were held at a density of 18 g/L under a natural photoperiod and were fed daily to satiation with Oregon Moist Pellets up to and including the day before, but not during, the experiment.

For the experiment, aerated soft water was acidified with concentrated reagent-grade H_2SO_4 and held at pH 4.0 (±0.1) with a Beckman Model 942 pH Monitor (Beckman Instruments, Fullerton, California). The acidified stock solution was vigorously reaerated to remove CO_2,

pumped to a head tank of a gravity-flow diluter, and then diluted with softened well water to attain each experimental pH in separate mixing chambers that flowed directly into the experimental tanks. Treatment pH levels were automatically monitored and recorded every 3 h, and the diluter was manually adjusted whenever necessary to maintain exposure levels within ±0.1 pH unit.

Fish were subjected to acidic conditions by changing the inflow supply over to the acid-blended water through a single valve at the start of the exposure without disturbing the experimental tanks. From the ambient pH of 6.6, final pH levels of 5.7, 5.2, and 4.7 were reached in 20 h, and were within 0.3 pH unit of the final pH at 6 h and within 0.1-0.2 pH unit at 10 h. Temperature remained between 17 and 19° C throughout the experiment. Duplicate tanks of fish for each pH, including ambient, were established for sampling; an additional two tanks with fish at ambient pH were left undisturbed as controls for the handling phase of the experiment.

At 2, 4, 6, 10 and 20 h, and at 2, 3 and 5 d after the initial changeover to the acidic water, five or six fish were quickly removed with a hand net from each tank for blood sampling according to the protocol described in the next section. After 5 d, the fish were subjected to an acute handling stress by first capturing them with hand nets and then holding them, as a group, out of the water in a bag seine for 30 s and returning them to the tank; the 5-day acid exposure samples also served as initial values for this phase of the experiment. At 1, 3, 6, 12, 24 and 48 h after handling, five or six fish were obtained from each tank for sampling. As control, samples were simultaneously obtained from the previously undisturbed fish in the two additional tanks at ambient pH.

Sampling and Analysis

Sample fish removed from the tanks were rapidly anesthetized in a 200-mg/L concentration of tricaine methanesulfonate (MS-222); total time for removal and complete immobilization was < 1 min. Individual fish were then taken serially from the anesthetic for blood sampling; total time per tank was < 10 min. This method has been successfully used for cortisol analysis in juvenile rainbow trout (Barton et al. 1980; Barton and Peter 1982) and was verified for use under our present conditions before this study. After severing the caudal peduncle, blood was obtained from the caudal vasculature with an ammonium-heparinized 0.25 mL Natelson capillary tube. Plasma was separated by centrifugation and stored at -20 C for future assay. Additional blood was also collected in heparinized microhematocrit tubes for immediate hematocrit determination.

Plasma cortisol was determined by the ^{3}H-radioimmunoassay described by Foster and Dunn (1974) and modified in our laboratory (Redding et al. 1984b) for use with salmonid plasma; inter- and intra-assay coefficients of variation (CV) have consistently been ca. 5%. We assayed plasma glucose by an ortho-toluidine colorometric procedure modified from Wedemeyer and Yasutake (1977) using Sigma® pre-mixed reagent; prior to this study, inter- and intra-assay CV were found to be 7% and 3%, respectively. Plasma sodium (as Na^{+}) and potassium (as K^{+}) were measured with a self-calibrating NOVA 1 Sodium/Potassium Analyzer (Nova Biomedical, Newton, Massachusetts); according to manufacturer specifications, the instrument has inter- and intra-assay CV of <2.0% and <1.0%, respectively, with pooled serum. We determined hematocrit as percent packed cell volume (% PCV) by direct measurement after centrifugation, using a CRITOCAP™ tube reader.

Since duplicated results were in agreement, data from each pair of tanks for each time and treatment were pooled. One-way analyses of variance (ANOVA) were conducted both within treatments through time and among treatments for specific times. Homogeneity of variance was assessed using Bartlett's test (Snedecor and Cochran 1967) and, where appropriate (i.e. for cortisol and glucose), data were transformed to logarithmic values for recalculation of ANOVA. Significant differences among means were then determined using Duncan's new multiple-range test at the 5% level (Steel and Torrie 1980). For ease of interpretation, arithmetic means and standard errors are presented.

RESULTS

Responses to Reduced Environmental pH

Plasma cortisol from fish held at pH 4.7 appeared to be elevated during the course of the 5-d exposure compared with that from fish maintained at the ambient pH of 6.6 (Table 1). Similarly, cortisol levels were also slightly higher in fish held at pH 5.2 and 5.7 than in those from the ambient pH throughout this period; significant differences from ambient were apparent during initial stages of acidification at 2, 4, 6 and 20 h for all three test pH's (Table 1). At 10 h, cortisol was higher than at other times in the ambient pH group, and was not different from the test groups. A significant elevation in plasma glucose was evident at 2 d in the pH 4.7 group and was ca. 300% higher, at 258 mg/dL, than in the ambient pH group at the end of the 5-d exposure (Table 1).

Plasma sodium was significantly depressed in fish exposed to pH 4.7 after 5 d, being 13% lower at 130 mmol/L, than the pH 6.6 group (Table 1). Plasma potassium (data not shown) generally showed a declining trend at all pH's from levels of around 4 to 5 mmol/L at the beginning of the experiment to around 1 to 2 mmol/L at the end of the experiment 7 d later. No differences from ambient pH in plasma potassium attributable to either the chronic acidity or to the subsequent handling stress were apparent.

Hematocrits tended to be higher in the acid-stressed fish and were significantly so at 6 and 20 h, and at 3 and 5 d (Table 1). However, it is not known if the differences evident at 3 and 5 d were due to the increased environmental acidity, or to the significant decline in hematocrit observed in the ambient pH group, possibly caused by some other factor.

Responses to Additional Handling Stress

All groups demonstrated a significant elevation ($P<0.01$) in plasma cortisol after being subjected to the acute handling stress (Fig. 1). However, cortisol levels was ca. 250% higher at 1 h in fish from pH 4.7 than it was in fish from the ambient pH, being 338 and 136 ng/mL, respectively. Plasma cortisol also tended to remain higher in all three acid-treated groups than in the ambient pH group for subsequent samples, with significant differences occurring at all sample times following handling (Fig. 1). Two fish died between 3 and 6 h after handling at pH 4.7.

After handling, there were significant transient increases in plasma glucose between 1 and 12 h (inclusive) in the pH 6.6 ($P<0.01$) and 5.7 ($P<0.05$) groups (Fig. 2). However, in the pH 5.2 and 4.7

groups, transient changes in plasma glucose due to the additional handling did not differ significantly from the elevated background levels caused by the chronic acid exposure. In the pH 4.7 group, plasma glucose, which was already elevated at the onset of handling, continued to increase and reached a maximum of 335 mg/dL at 48 h after handling (Fig. 2). Plasma glucose in the pH 5.2 group also remained elevated above that in the ambient pH group at 48 h post-handling (Fig. 2).

All groups, including ambient pH, showed transient decreases in plasma sodium after handling (Fig. 3); temporal changes were significant in pH groups 6.6 ($P<0.05$), 5.7 ($P<0.01$) and 4.7 ($P<0.05$). Plasma sodium in fish at pH 4.7, already depressed just before handling, continued to decline significantly to the low level of 114 mmol/L observed at 48 h post-handling (Fig. 3).

A transient increase in hematocrit to 57% in the pH 4.7 group 3 h after handling was significantly greater than the hematocrit of 45% observed in the ambient pH group at the same time (Fig. 4). Although hematocrit generally tended to be higher in lower pH groups after handling, differences among treatments at other time intervals were not significant. There was also a significant increase in hematocrit over the 48-h post-handling period in the unhandled control group held at ambient pH (Fig. 4).

DISCUSSION

Acid-stressed fish appear to be more sensitive to additional handling stress than unstressed fish. When the rainbow trout in our experiment were subjected to the 30-s handling stress, the effect of prior acidification on interrenal activity was clearly apparent in the

higher plasma cortisol response to handling at the low pH. However, any possible effects on glucose or sodium levels from handlng were masked by the continuous response to the chronic acid exposure. From the acid exposure alone, plasma glucose, plasma sodium and hematocrit levels were significantly altered after 5 d, particularly at pH 4.7, but plasma cortisol was not.

There was a slight acute corticosteroid response to the acid conditions during the initial 20 h of exposure, which appeared to be recovered by 2 d. Mudge et al. (1977) also reported a transient rise in plasma cortisol in brook trout, Salvelinus fontinalis, at 1-3 h during exposure to pH 4.0, with titers returning to control levels within 24 h. Our findings of apparently recovered, or resting, plasma cortisol levels after 2-5 d of acid exposure are consistent with those of Brown et al. (1984) for the early phase of chronic acid exposure, as they did not show changes in plasma cortisol in rainbow trout during environmental acidification until after 8 d; in their study, sampling began after 1 d. Moreover, Lee et al. (1983) showed no significant change in plasma cortisol of rainbow trout after 2- or 3-wk exposures to pH's as low as 4.2, although interim samples were not taken. The small transitory increase in plasma cortisol at 10 h in the ambient pH group may have resulted from increased activity around the tanks or from repeated sampling, although effects of these factors were not evident in the handling phase of this experiment, nor in other investigations (Barton et al. 1980; B.A. Barton anb C. B. Schreck, unpubl. data).

When our fish were subjected to the 30-s handling stress after 5 d of acidification, the plasma cortisol response of the pH 4.7 group was about double that of the other pH groups including the ambient pH. This result supports that of Brown et al. (1984), who concluded that chronic acid exposure stimulated increased activity of interrenal cells, as demonstrated by interrenal hyperplasia and increased size of nuclei after 4 d. By comparison, Mudge et al. (1977) found evidence of decreased cell and nuclear size, suggesting that activity of interrenal tissue decreased after acid exposure. However, it should be noted that their histological investigation was restricted to measurements taken after 3- to 24-h exposure periods only. On the basis of the findings of Brown et al. (1984) of increased interrenal activity and our results from the subsequent handling stress, we speculate that clearance rate of cortisol may also have increased during chronic acidification to compensate for the increased interrenal output, as a measure directed toward maintaining homeostasis. For example, an increase in plasma corticosteroid clearance rate in response to a chronic environmental change was observed by Redding et al. (1984a), who subjected freshwater-adapted juvenile coho salmon, Oncorhynchus kisutch, to continuous salinity. Thus, the hypothalamic-pituitary-interrenal axis may have been stimulated in response to chronic acid stress, but the differences in response--i.e., at pH 4.7 as compared to ambient--were not apparent until the fish experienced an additional rapid plasma corticosteroid increase, caused by the 30-s handling stress. The pattern of rapid plasma cortisol elevation, followed by recovery, in response to the handling stress was characteristic of what has been previously shown for juvenile salmonids (Strange and Schreck 1978; Barton et al. 1980;

Pickering et al. 1982; B. A. Barton and C. B. Schreck 1983, unpublished results); this subject has been reviewed extensively by Donaldson (1981) and Schreck (1981).

Our results demonstrating an increase in plasma glucose after 2-d exposure to pH 4.7 corroborate those of Brown et al. (1984) who found significant rises in plasma glucose of rainbow trout after 4-d exposures to both pH 4.7 and 5.2. Similarly, Lee et al. (1983) reported that plasma glucose levels were higher in rainbow trout exposed for 2 wk to pH 4.2 and pH 4.8, than in those held at higher pH's. When fish were given the handling stress after the acid exposure, plasma glucose levels in the ambient and pH 5.7 groups showed response-recovery patterns typical of salmonids subjected to an acute physical disturbance (Nakano and Tomlinson 1967; Wedemeyer 1972; Wydoski et al. 1976; Casillas and Smith 1977; Perrier et al. 1978; Pickering et al. 1982; B.A. Barton and C.B. Schreck 1983, unpublished results). However, at the two lower pH's, handling responses were masked by the higher background glucose levels that resulted from acid exposure; this was particularly evident at pH 4.7. At 6-7 d of acidification at pH 4.7, plasma glucose was elevated to 300-350 mg/dL, which is similar to the level Brown et al. (1984) reported after a 21-d exposure to this pH.

We suspect that the comparatively high glucose elevation observed at 6-7 d in response to the acid stress in our experiment was a combination of: the relatively soft ambient water, the aggravating effect of the additional handling at 5 d, and the relatively high ambient temperature used in our experiment. Recent evidence with juvenile chinook salmon, Oncorhynchus tshawytscha, (B. A. Barton and C.B. Schreck 1984, unpublished results) has clearly shown that the

glucose response to a similar 30-s handling stress was three times greater in fish acclimated to 21° C than in those acclimated to 12 or 7° C, and was very similar to that found for the ambient pH group in this experiment. The apparently more rapid response of plasma glucose to the acid stress alone in our fish than in those of Brown et al. (1984), may also have been because of the relatively soft water used in our study. For example, Graham et al. (1982) found that physiological responses were more severe in acid-exposed trout subjected to strenuous exercise when held in soft water rather than hard water, and post-exercise mortality doubled.

Leach and Taylor (1980) provided evidence to indicate that increased endogenous cortisol may have a functional role in sustaining elevated glucose levels in response to stress. Possibly, increased interrenal activity resulting from acid exposure, demonstrated histologically by Brown et al. (1984) and in our experiment by the secondary handling stress challenge, may be responsible for an increased cortisol output to maintain high levels of blood glucose as a readily available energy source. Mobilization of energy reserves would presumably be necessary to allow the fish to cope with the effects of chronic acid stress, such as by increasing metabolic or ionoregulatory activity.

The decline in plasma sodium concentration in fish exposed to pH 4.7 for 5 d was similar to the decline observed at pH 4.5 in the adult rainbow trout as described and discussed earlier in the main body of this report. In that investigation, plasma sodium decreased from 153 to 145 mmol/L after 7 d exposure of pH 4.5. Reduced plasma sodium as a result of acid exposure has also been reported by others in juvenile

salmonids (Lee et al. 1983; Saunders et al. 1983; see reviews by Fromm 1980; Wood and McDonald 1982). Decreased sodium influx rates and increased efflux rates at low pH were previously demonstrated in brook trout by Packer and Dunson (1970). Furthermore, ionoregulatory dysfunction in rainbow trout exposed to acidic conditions was exacerbated in soft water (McDonald et al. 1980).

The perturbations in plasma sodium within the first 24 h after handling in pH 6.6 and 5.7 water are consistent with those which Redding and Schreck (1983) observed for juvenile coho salmon chronically stressed by confinement in fresh water. These results support the notion that under normal conditions, there is a short-lived reduction in ionoregulatory ability when fish are subjected to physical disturbances. The results differ from those of Graham et al. (1982), who observed an elevation in plasma sodium of rainbow trout after 6 min strenuous exercise at pH 7.5 in both hard and soft water. Graham et al. (1982) attributed an increase in blood constituents to high intracellular lactate loading, causing water to move across the osmotic gradient with a resultant decrease in blood volume. Although there was an indication of a slight increase in plasma sodium 1 h after handling in the ambient and higher pH groups, we postulate that the handling stress used in our experiment was insufficient to sustain through time the lactate response normally associated with severe exercise. For example, we found that in juvenile chinook salmon subjected to a 30-s handling stress similar to that used in the present study, there was a transient rise in plasma lactate but the fish recovered within 3 h (B. A. Barton and C. B. Schreck 1983, unpublished results). By comparison, Graham et al. (1982) reported that lactate levels peaked at 2 h and took 8 h or more to recover.

The continuous decline in plasma sodium at pH 4.7, like that of plasma glucose, masked any transitory changes that may have resulted from the additional handling stress. Although less obvious, this decreasing trend was probably responsible for the lack of significance of the handling response at pH 5.2 as well.

The tendency towards increased hematocrit in the acid-exposed fish is in agreement with earlier studies (Dively et al. 1977; Neville 1979; Milligan and Wood 1982; Saunders et al. 1983), and Milligan and Wood (1982) attributed the increase to erythrocyte swelling. Furthermore, they found a redistribution of body water, resulting in decreased plasma volume with no change in total body water. Handling stress has also been shown to increase hematocrit (Fletcher 1975; Soivio and Oikari 1976) and erythrocytic swelling (Soivio et al. 1977) in fish. In the handling phase of our experiment, only the combined stress of handling and acidity at pH 4.7 was sufficient to cause a significant increase in hematocrit above the pre-handling level.

As a management strategy, fish stocking will probably be used more extensively in the future to provide short-term fisheries in waters which no longer support natural reproduction because of reduced pH. Since we have shown that acid-stressed fish could have an increased sensitivity to additional stresses, particularly at pH's below 5, fish stocked in acid waters may appear to be physiologically normal (e.g. cortisol titer) within a few days after planting, when in fact they are not. The observed mortality in the pH 4.7 group, 3 to 6 h after handling, supports that view. Thus as a management consideration, extra attention should be given to ensure survival of fish stocked in acidic waters, especially if the fish are likely to encounter other

stressful conditions in the new environment. As physiological stress indicators, our results show that both plasma glucose and plasma sodium appeared to be more suitable for chronic stress than plasma cortisol; this observation for glucose agrees with the conclusion of Brown et al. (1984). However, a secondary acute stress challenge, such as the 30-s handling stress employed in our study, may prove to be a useful method for determining possible increases in interrenal activity at an early stage of environmental acidification as an alternative to histological examination.

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Mention of trade names or manufacturers does not imply U.S. Government endorsement of these commercial products.

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55

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Table 1. Mean plasma cortisol (ng/mL, n=10), plasma glucose (mg/dL, n=8), plasma sodium (mmol/L, n=8) and hematocrit (% PCV, n=8-13) values ±SE in juvenile rainbow trout after continuous exposure to reduced environmental pH. Sample sizes (n) represent pooled data from duplicate treatments. Values marked with an asterisk (*) indicate a significant difference from the ambient pH (6.6) at that time point (Duncan's new multiple-range test at 5%).

Time		pH 6.6	pH 5.7	pH 5.2	pH 4.7
2 h	Cortisol	16 ±5	39 ±15*	44 ±15*	27 ±9*
	Glucose	86 ±7	87 ±6	94 ±13	88 ±14
	Sodium	151 ±3	148 ±3	154 ±2	154 ±2
	Hematocrit	46 ±1	48 ±2	48 ±2	50 ±1
4 h	Cortisol	6 ±3	49 ±15*	44 ±10*	50 ±34*
	Glucose	92 ±6	80 ±3	95 ±11	80 ±3
	Sodium	149 ±4	148 ±4	153 ±2	154 ±1
	Hematocrit	48 ±1	47 ±1	49 ±1	51 ±1
6 h	Cortisol	10 ±4	44 ±10*	32 ±9*	33 ±10*
	Glucose	99 ±11	91 ±6	84 ±6	80 ±4
	Sodium	152 ±2	152 ±2	152 ±2	155 ±2
	Hematocrit	47 ±1	50 ±1	50 ±2	53 ±1 *
10 h	Cortisol	34 ±6	29 ±10	58 ±12	55 ±19
	Glucose	93 ±10	90 ±9	99 ±14	92 ±5
	Sodium	149 ±1	149 ±2	153 ±1	151 ±2
	Hematocrit	49 ±2	50 ±1	50 ±2	51 ±1
20 h	Cortisol	16 ±5	47 ±11 *	48 ±6 *	49 ±12*
	Glucose	74 ±3	91 ±12	109 ±13	89 ±6
	Sodium	149 ±2	147 ±2	152 ±1	143 ±2 *
	Hematocrit	48 ±1	50 ±1	53 ±1 *	53 ±2 *
2 d	Cortisol	21 ±9	10 ±3	8 ±2	26 ±8
	Glucose	77 ±6	92 ±10	101 ±7	166 ±21*
	Sodium	144 ±1	142 ±1	139 ±3	138 ±2
	Hematocrit	48 ±2	49 ±1	50 ±1	53 ±2
3 d	Cortisol	15 ±6	16 ±8	12 ±4	30 ±7
	Glucose	87 ±9	94 ±8	110 ±19	154 ±19*
	Sodium	147 ±2	149 ±2	150 ±2	143 ±4 *
	Hematocrit	43 ±1	50 ±2 *	50 ±1 *	52 ±2 *
5 d	Cortisol	9 ±3	9 ±7	11 ±3	23 ±9
	Glucose	85 ±10	79 ±5	143 ±30	258 ±42*
	Sodium	149 ±2	145 ±3	143 ±4	130 ±3 *
	Hematocrit	42 ±1	47 ±1 *	46 ±1 *	49 ±1 *

Figure 1. Responses of plasma cortisol (ng/mL ±SE) in juvenile rainbow trout subjected to a 30-s handling stress after 5 d during continuous exposure to pH's of 5.7, 5.2 or 4.7, or ambient pH of 6.6. Sample sizes of n=10 represent pooled data from duplicate treatments. Values marked with an asterisk (*) indicate a significant difference from the ambient pH at that time point (Duncan's new multiple-range test at 5%). Open squares are values for unstressed controls at ambient pH.

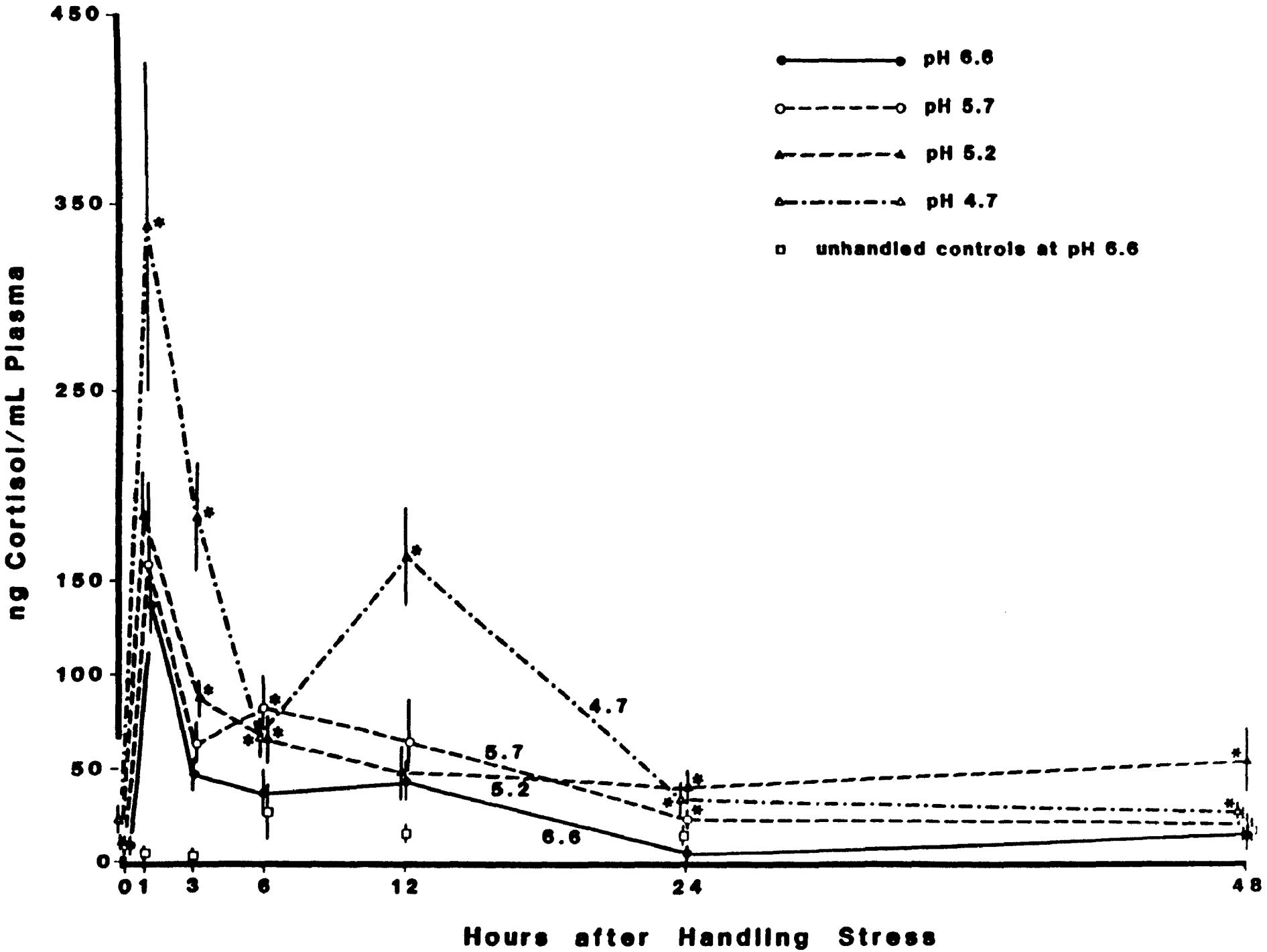

pH 6.6
pH 5.7
pH 5.2
pH 4.7
unhandled controls at pH 6.6
ng Cortisol/mL Plasma
450
350
250
150
100
50
0
0 1 3 6 12 24 48
Hours after Handling Stress
4.7
5.7
5.2
6.6

Figure 2. Responses of plasma glucose (mg/dL ±SE) in juvenile rainbow trout subjected to a 30-s handling stress after 5 d during continuous exposure to pH's of 5.7, 5.2 or 4.7, or ambient pH of 6.6. Sample sizes of n=8 represent pooled data from duplicate treatments. Values marked with an asterisk (*) indicate a significant difference from the ambient pH at that time point (Duncan's new multiple-range test at 5%). Open squares are values for unstressed controls at ambient pH. (See Fig. 1 for legend.)

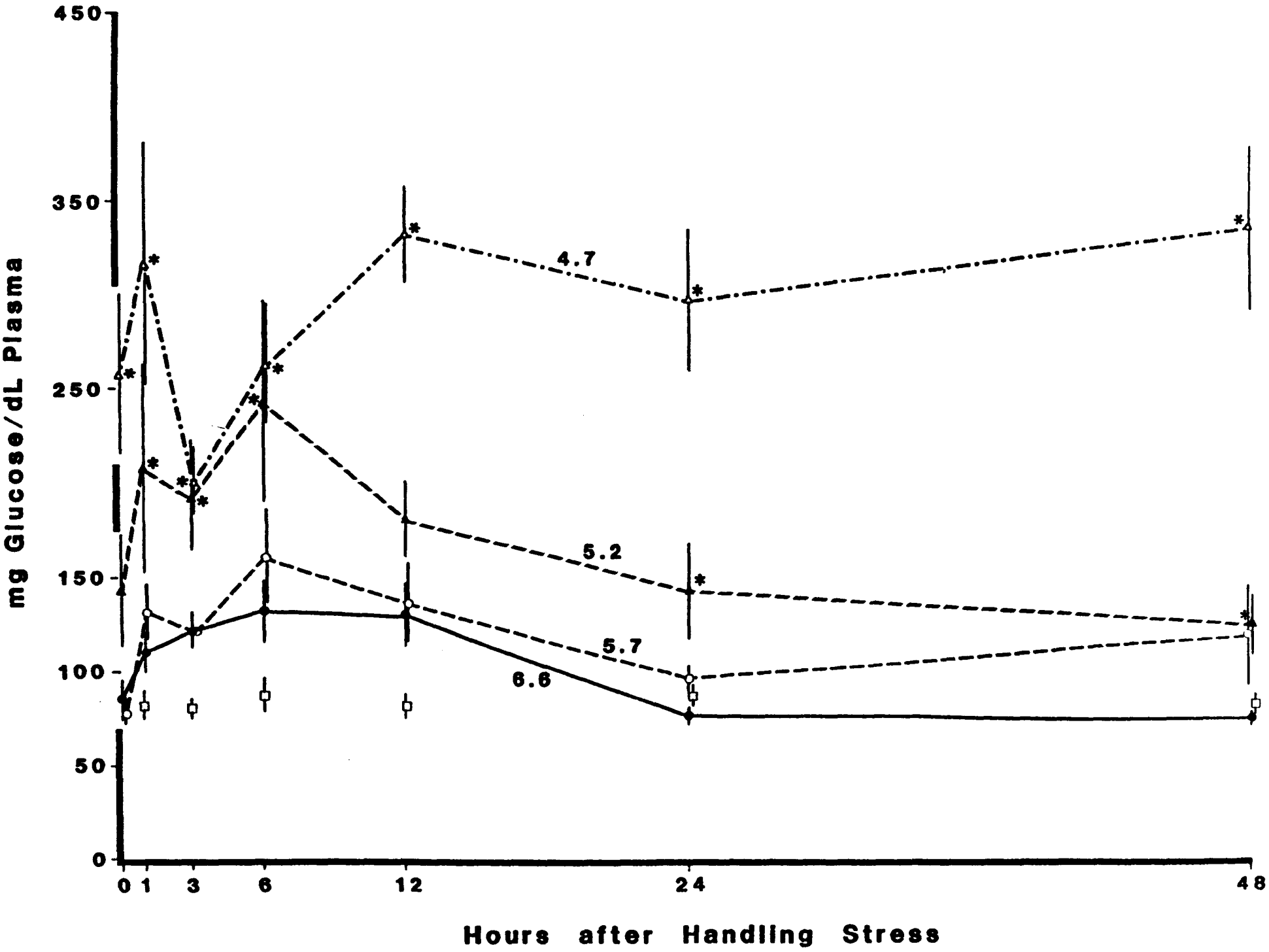

450
350
250
150
100
50
0
mg Glucose/dL Plasma
4.7
5.2
5.7
6.6
0 1 3 6 12 24 48
Hours after Handling Stress

Figure 3. Responses of plasma sodium (mmol/L ±SE) in juvenile rainbow trout subjected to a 30-s handling stress after 5 d during continuous exposure to pH's of 5.7, 5.2 or 4.7, or ambient pH of 6.6. Sample sizes of n=8 represent pooled data from duplicate treatments. Values marked with an asterisk (*) indicate a significant difference from the ambient pH at that time point (Duncan's new multiple-range test at 5%). Open squares are values for unstressed controls at ambient pH. (See Fig. 1 for legend.)

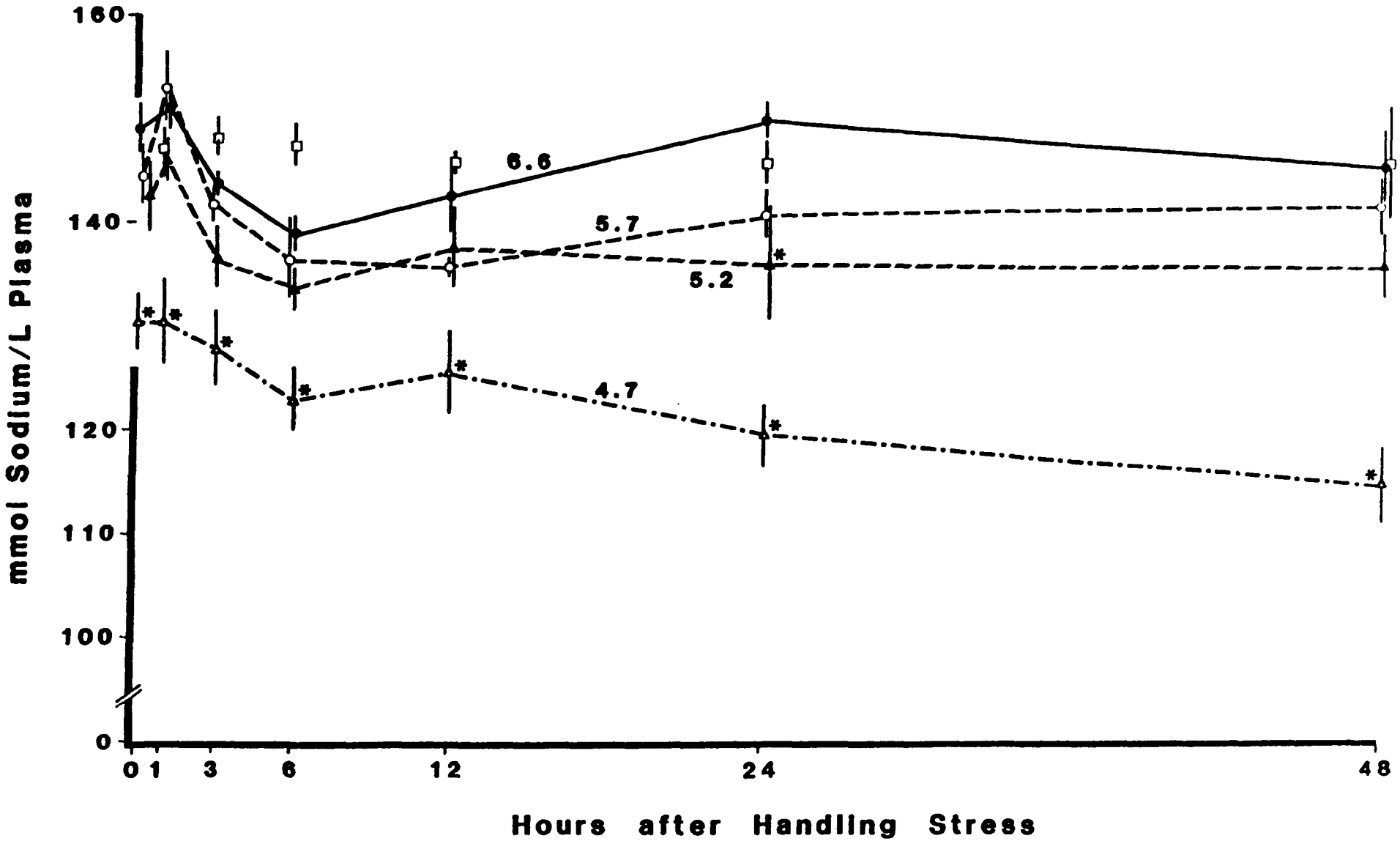
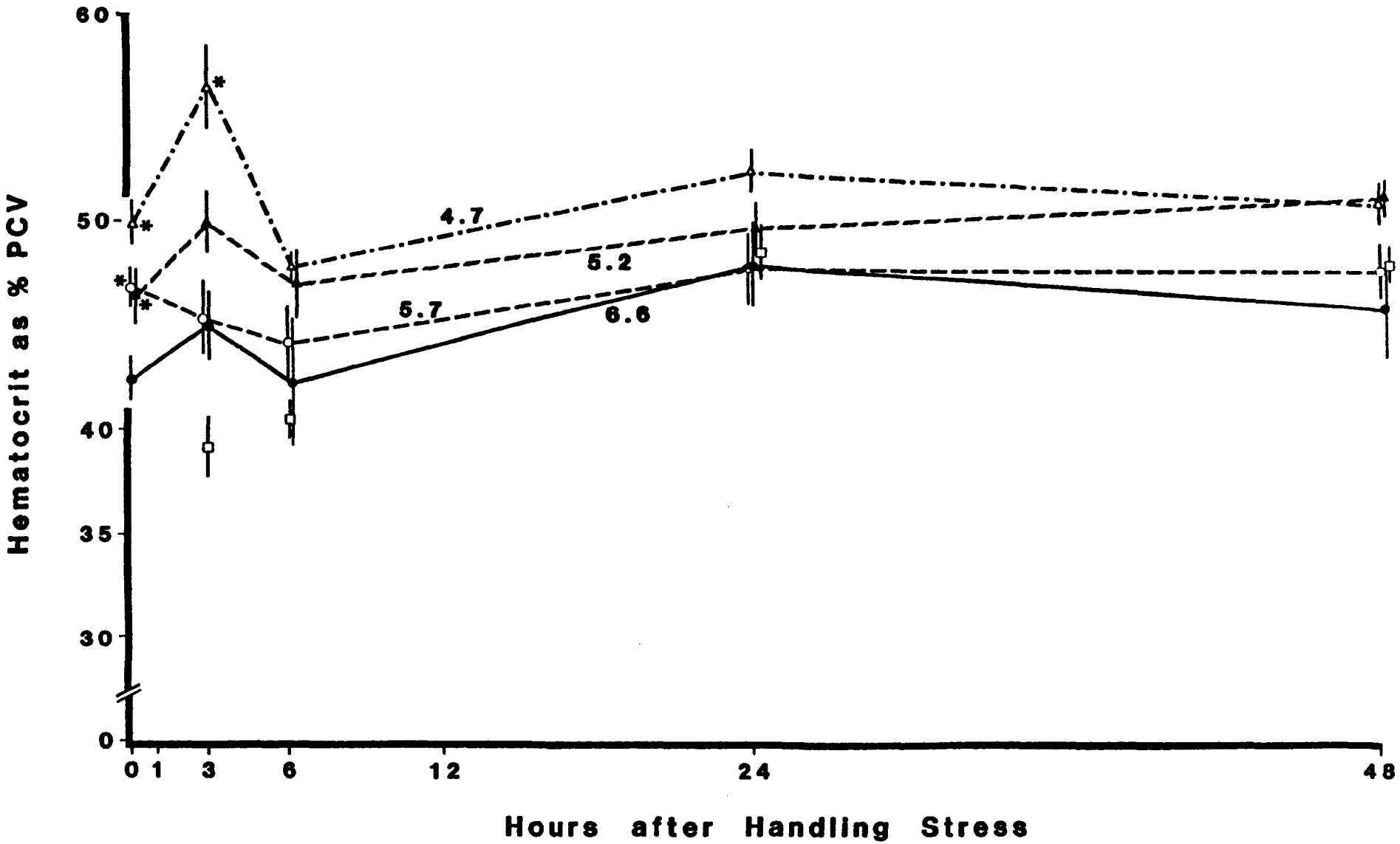

160
140
120
110
100
0
mmol Sodium/L Plasma
6.6
5.7
5.2
4.7
0 1 3 6 12 24 48
Hours after Handling Stress

Figure 4. Responses of hematocrit (% PCV ±SE) in juvenile rainbow trout subjected to a 30-s handling stress after 5 d during continuous exposure to pH's of 5.7, 5.2 or 4.7, or ambient pH of 6.6. Sample sizes of n=6-13 represent pooled data from duplicate treatments. Values marked with an asterisk (*) indicate a significant difference from the ambient pH at that time point (Duncan's new multiple-range test at 5%). Open squares are values for unstressed controls at ambient pH. (See Fig. 1 for legend.)


Hematocrit as % PCV
60
50
40
35
30
0
4.7
5.2
5.7
6.6
0 1 3 6 12 24 48
Hours after Handling Stress