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THE EFFECT OF SUBLETHAL COPPER CONCENTRATIONS ON SODIUM AND CHLORIDE FLUXES ACROSS THE GILLS IN GOLDFISH

by

© MICHAEL D. EAGLES

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "The Effect of Sublethal Copper Concentrations On Sodium and Chloride Fluxes Across the Gills in Goldfish" submitted by Michael D. Eagles, in partial fulfilment of the requirements for the degree of Master of Science.

W. C. Macleay
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Date Dec. 7. 1978

ABSTRACT

Tests were performed with mature goldfish, Carassius auratus, to determine the effect of 40 µg/l total copper on the fluxes of sodium and chloride ions across the gill. The 96 hour LC_{50} value was 60 µg/l total copper.

Exposure to copper, starting 12 hours prior to flux measurements, had no effect on sodium or chloride fluxes in normal, salt depleted, or blood sampled salt depleted fish. Sodium influx and efflux values and plasma sodium concentrations were reduced in the salt depleted group in which copper exposure started 2 days prior to flux measurements. Sodium netflux was unaffected, indicating that 2 day copper exposure reduced sodium permeability without affecting net uptake. Since 2 day copper exposure had no effect on plasma chloride concentrations or chloride netflux, it has been concluded that copper has no effect on chloride flux values.

Fish which had a 1 ml blood sample taken 48 hours prior to flux measurements showed significantly lower sodium influx and efflux compared with control fish but sodium and chloride netfluxes were not affected indicating an effect of blood sampling on sodium permeability.

Plasma sodium concentrations decreased during salt depletion and the resultant increased net uptake caused elevation in plasma sodium concentrations during flux measurements. However, fish which were exposed to copper during salt depletion had significantly lower plasma sodium concentrations than control salt depleted fish which indicates that copper augments the loss of sodium ions during salt depletion.

Plasma chloride concentrations in salt depleted fish were significantly lower after flux measurements than they were before flux

measurements. There was a net loss of chloride, across the gills, in the blood sampled fish and the 2 day copper exposed fish whereas in the other groups netflux was not significantly different from zero. with the loss of chloride ions in the urine, this may explain the decreased plasma chloride concentrations.

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INTRODUCTION

The mode of toxicity for copper is unknown. Copper accumulates in fish tissues during exposure to sublethal doses of 20-104 µg/l for 30 or more days, but does not accumulate during 4 day exposure to acute lethal concentrations of 200-400 µg/l (Brungs et al., 1973; McKim and Benoit, 1974). Copper concentrations in the gills and liver after 20 months exposure to 49-53 µg/l copper were 6.5 and 4.5 times higher than found in controls (Brungs et al., 1973). Brungs et al. (1973) also reported only 2 and 1 times higher concentrations in gills and liver, respectively, after 4 day exposure to acute lethal concentrations of 206 µg/l. Because copper accumulation was apparent over long periods of time McKim and Benoit (1974) concluded that the rate of copper turnover in the body of the fish was low. Since fish are able to tolerate 5 times as much copper in their tissues as found in fish dying from acute lethal exposure to copper, toxicity does not appear to be related to body uptake. Therefore it is possible that the mechanism of copper toxicity is by some effect on external membranes, such as the gill.

Effects of copper on sodium and chloride transport in vivo have not been examined, but in vivo experiments have provided indirect evidence for an effect of copper on active transport in fish. McKim et al. (1970), working on brook trout (Salvelinus fontinalis), and Christensen et al. (1972), working on brown bullhead (Ictalurus nebulosus), reported a decrease in plasma chloride concentrations following exposure to 25-100 µg/l copper for 6 to 30 days. Williams (personal communication), working with rainbow trout (Salmo gairdneri),

observed an initial decrease and subsequent recovery in plasma sodium and chloride concentrations during chronic exposure to sublethal concentrations of copper. This decrease in plasma ion concentrations may be due to a decreased influx or increased efflux of sodium and chloride ions during exposure to copper. The recovery that followed may be attributed to a delayed compensation, by the fish, for the initial salt loss.

Copper effects on ion transporting membranes have been studied only on in vitro frog skin. Ussing and Zerahn (1951) showed that 50 µg/l copper increased the potential difference across isolated frog skin, but did not affect the short circuit current (S.C.C.). They previously showed that net sodium transport was equal to the S.C.C. Since copper did not affect the S.C.C. they concluded that copper specifically inhibited passive chloride influx. Zadunaisky et al. (1963) observed that the S.C.C. was equal to the difference between net sodium and chloride transport and that 1270 µg/l copper, in the outside medium, produced an increase in both the potential difference and the S.C.C. They concluded that copper decreased passive chloride influx without affecting sodium influx, resulting in a S.C.C. equal to the net sodium transport. Ferreira (1970), also working with isolated frog skin, found that 6350 µg/l copper caused an increase in the S.C.C., sodium fluxes, and chloride fluxes. He concluded that copper, exposed to both sides of the frog skin, produced a higher permeability in the skin to sodium and chloride ions. Koefoed-Johnsen and Ussing (1958) and Koefoed-Johnsen et al. (1973) found that 635 µg/l copper produced an increased potential difference in isolated frog skin and concluded that this was due to a decrease in chloride permeability. These studies indicate that a large

range of copper concentrations affect the chloride permeability in frog skin. These copper concentrations are also large enough to produce mortality in fish.

The mechanism of sodium uptake, across the gills of freshwater fish, is thought to involve a one for one exchange between external sodium and internal hydrogen and/or ammonium ions (Krogh, 1937; Maetz and Garcia-Romeu, 1964; Kerstetter et al., 1970; Maetz, 1973). In most fish it is an ammonium/sodium exchange with a limited hydrogen/sodium exchange (Cuthbert and Maetz, 1972; Maetz, 1973). Chloride is taken up in exchange for bicarbonate ions (Krogh, 1937; Maetz and Garcia-Romeu, 1964; Kerstetter and Kirschner, 1972; De Renzis and Maetz, 1973). Both processes appear to be carrier-mediated through specific channels or pores in the transporting membrane (Kennedy et al., 1977; Lindemann, 1977).

The mechanism of sodium and chloride transport, across ion transporting epithelia, is similar in amphibians and freshwater fish (Motais and Garcia-Romeu, 1972). Since copper has a specific effect on ion transport in frog skin and on plasma concentrations in fish, the purpose of the present study was to determine whether sublethal concentrations of copper specifically altered sodium or chloride fluxes across the gills of a freshwater fish in vivo.

MATERIALS AND METHODS

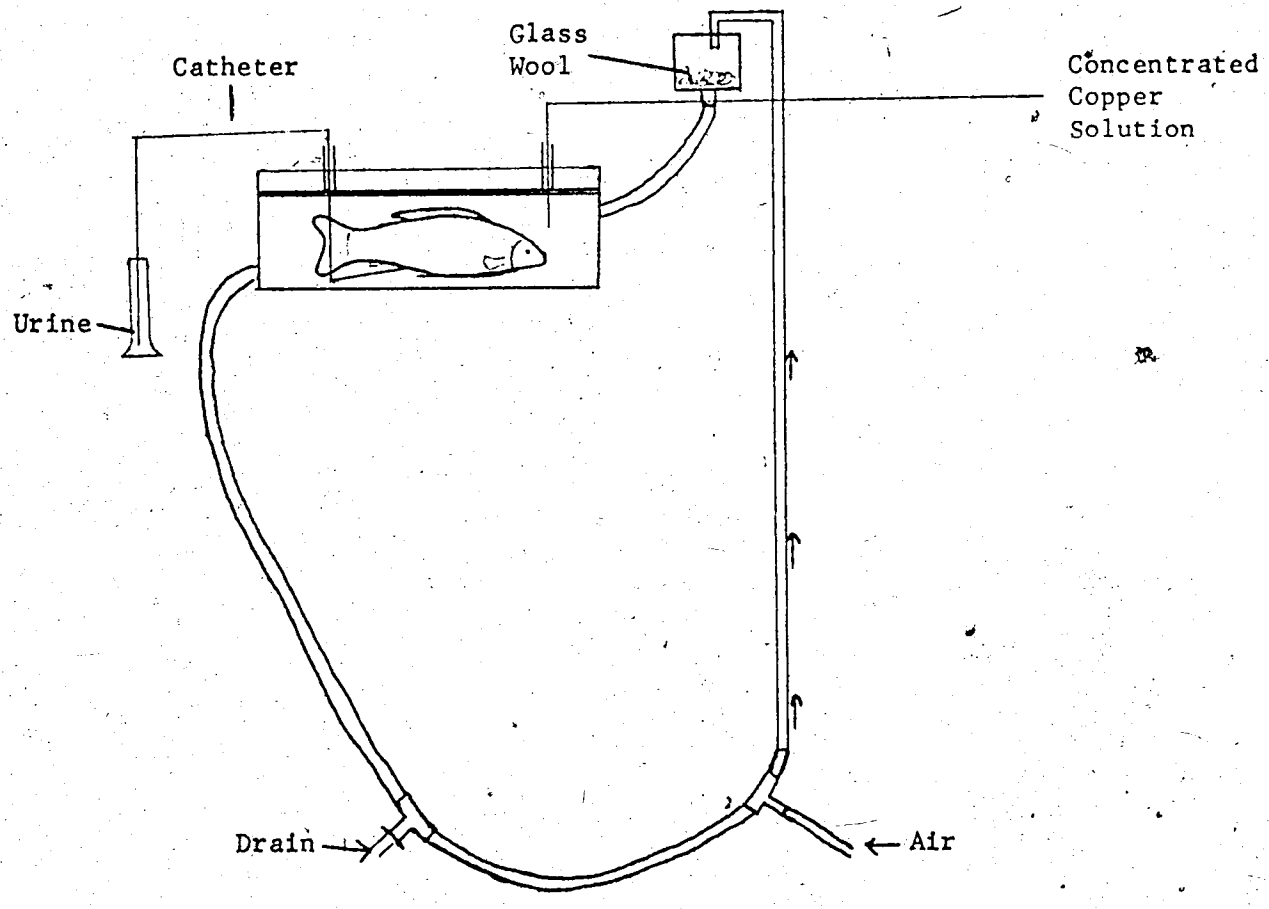
Care of Study Animals

Mature goldfish, (Carassius auratus), weighing 146.1 ± 54.7 grams (mean ± standard deviation) were obtained from Grassyforks Fisheries Co. (Martinsville, Indiana). The fish were shipped by air and on arrival were placed in holding tanks of dechlorinated tapwater at 25°C. They were kept under a natural photoperiod and were fed daily with Ewos fish food.

The fish were transferred to 10 gallon aquaria 9 days prior to flux measurements. The aquaria received a continuous flow of deionized water at room temperature (23 ± 0.5°C). During salt depletion and flux measurements the fish were not fed, thereby negating any salt intake from the food.

After 6 days of salt depletion, the fish were catheterized and placed in the experimental boxes used for flux measurements. These boxes, made of plexiglass, were covered and of two sizes. The first box was 5 x 30 x 13 cm and held 2 liters of water while the second was 5 x 25 x 10 cm and held 1.5 liters of water. Each box was connected to a closed tubing system which provided for aeration, filtration, and circulation of water through the box (Figure 1). The water passed through a glass wool filter which removed large amounts of mucus. The filter was changed every 24 hours and was completely removed during flux measurements to ensure that no isotope was bound to material retained by the filter (Figure 1).

Figure 1 Experimental apparatus used to measure sodium and chloride fluxes in goldfish.


Glass
Wool
Catheter
Concentrated
Copper
Solution
Urine
Drain
Air

Water Quality

To avoid fluctuating water quality during the experiments artificial water was prepared using reagent grade salts dissolved in distilled water to produce concentrations of 1 mM/l sodium chloride and 0.25 mM/l sodium carbonate. The carbonate was used to establish a pH of 7.3 ± 0.3 and a total hardness of 12 mg/l $CaCO_3$. Calcium was not used because it reduces sodium influx (Bornancin et al., 1972; Cuthbert and Maetz, 1972).

A 96 hour LC_{50} for copper was determined on mature and immature goldfish weighing 24.5 ± 7.9 grams (mean ± standard deviation). Although the fish were smaller than those used in later experiments and not all were mature, the LC_{50} value of 60 µg/l total copper did produce mortality among the larger fish (Appendix I). An initial total copper concentration of 40 µg/l was used for all experiments. This concentration stressed the fish without producing mortality during the flux measurements. Initial experiments showed a rapid decrease in dissolved copper concentrations in the water of the boxes in which flux measurements were made (Appendix II). Since the concentration of dissolved copper seems to be more important to toxicity in fish than total copper concentrations (Sprague, 1964), during flux measurements dissolved copper concentrations were kept stable at 17 µg/l by infusing a copper sulfate solution. The rate of infusion was 0.05 ml/hr of 4500 µg/l copper and dissolved copper concentrations in the water were measured every hour during initial experiments.

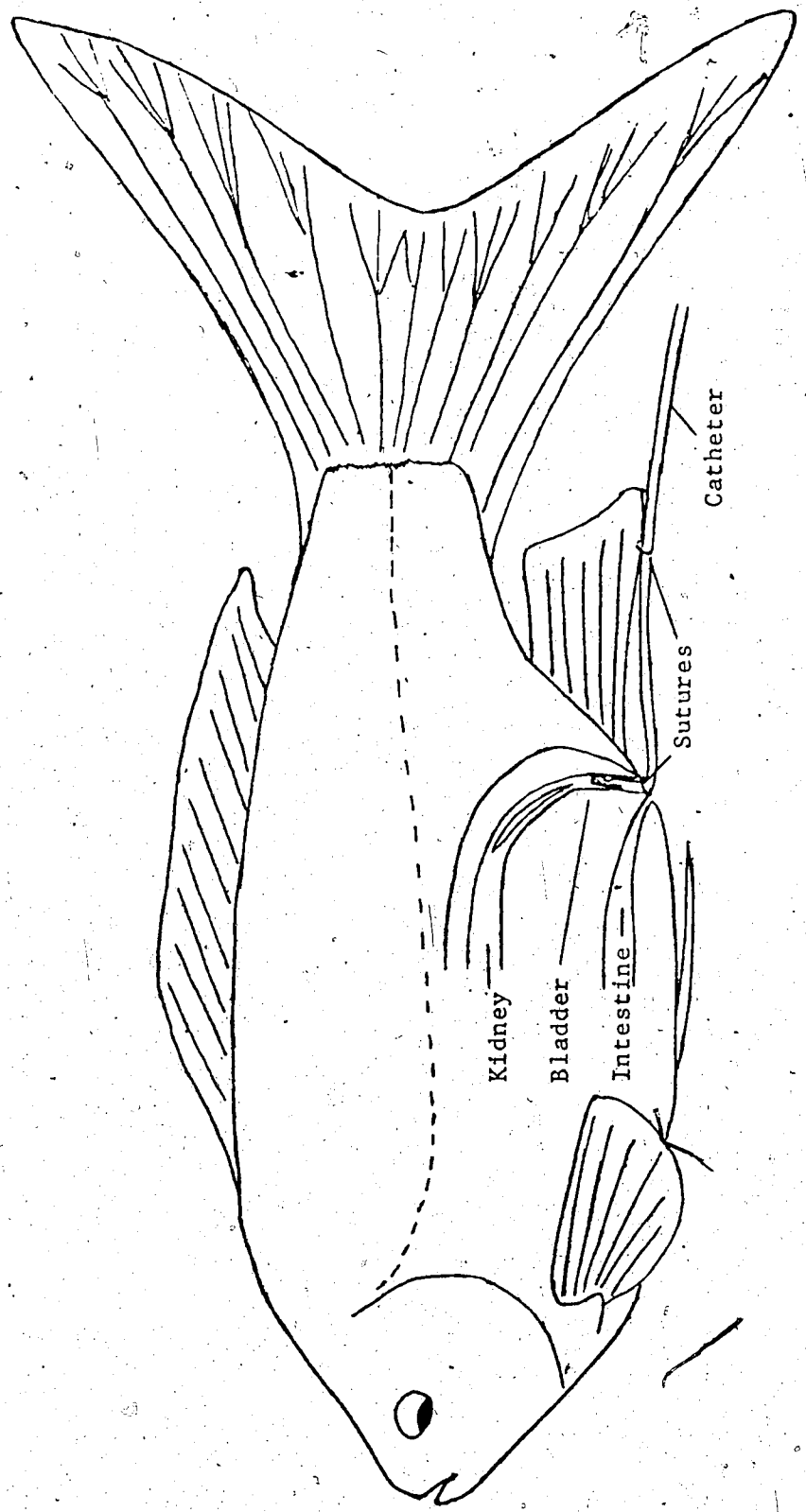
Operative Procedures

Prior to catheterization the fish were anaesthetized with 0.1 % tricaine methanesulphonate (MS222), dissolved in distilled water. Anesthesia was to the point of loss of equilibrium and opercular movement was barely detectable. The fish were then weighed, measured, and wrapped in wet paper towelling for catheterization.

Urinary catheters were produced from lengths of P.E. 50 or P.E. 90 tubing (Intrademic, Clay Adams Inc.), by perforating the wall of the tubing at one end, where it had been molded to conform to the shape of the bladder. Two sutures were used to hold the catheter in place (Figure 2). The first suture was positioned through the dorsal side of the rectum and around the urinary duct posterior to the bladder. This prevented leakage around the catheter. The second suture was used to attach the catheter to the anal fin, and it prevented the catheter from being pulled out of the bladder. The catheter was 70 cm long and extended out of the box into a graduated cylinder where urine was collected.

Blood samples were taken by using a 1 ml syringe with an ammonium heparinized 1 inch 22 gauge needle. The needle was inserted through the ventral surface of the fish into the caudal circulation, from which 1 ml of blood was taken. Following the operative procedure, the fish were placed in the experimental boxes to recover. Blood samples were taken on day 7, for all fish, and again on day 9 for half of the fish. The fish which were not blood sampled on day 9 were sampled after flux measurements on day 11.

Figure 2 Urinary system of the goldfish showing the position of the catheter.


Catheter
Sutures
Kidney
Bladder
Intestine

Experimental Protocol

After the fish were salt depleted and catheterized they were placed in the experimental boxes containing distilled water. After 24 hours the distilled water was replaced with the artificial water to be used for flux measurements and the fish were then held for an additional 12 hours before flux measurements were made (Figure 3). Thus, the fish were allowed 36 hours to recover from the stress of catheterization, handling, and the physical restrictions of the box. This should have been enough time for physiological processes to return to normal levels (Henderson and Chester-Jones, 1967; Houston and De Wilde, 1969; Hunn and Willford, 1970; Umminger and Gist, 1973). During the last half hour of this 36 hour period, 2 μC of ^{22}Na or 0.5 μC ^{36}Cl were added and allowed to disperse throughout the system. The ion fluxes were measured during the following 12 hour period. Initial and final samples of water were taken for sodium, chloride, and copper analysis and for determination of the activity of the isotope. Following flux measurements the experimental water was replaced by distilled water in which the fish were held for an additional 24 hours of salt depletion. Then artificial water was added and after 12 hours in the artificial water flux measurements were repeated. One half of the fish were exposed to copper during the first set of flux measurements and these fish acted as controls in the second set of flux measurements. The remainder of the fish which originally were controls were exposed to copper during the second set of flux measurements. This allowed each individual to act as its own control. There was no significant difference between the flux values for the controls between the two days, nor between copper exposed fish between the two days so values from both days were grouped together.

Figure 3 Experimental protocol for Group A. These fish were non blood sampled, salt depleted fish and one half of the group were exposed to copper starting 12 hours prior to flux measurements.

	Day												
	0	1	2	3	4	5	6	7	8	9	10	11
8:00am		Salt depletion in tanks containing distilled water								Flux Measurements		Flux Measurements
8:00pm	Salt depletion in tanks containing distilled water							Catheterized Put in boxes with distilled water	Artificial Water added Fish # 2,4, 6,8 were controls Fish # 1,3, 5,7 were exposed to 40 µg/l copper	Distilled Water added	Artificial Water added Fish # 2,4, 6,8 were exposed to 40 µg/l copper Fish # 1,3 5,7 were controls		

As well as the controls and 12 hour copper exposed groups, flux measurements were made on a second group of salt depleted fish to determine plasma ion concentrations in control and copper treated fish and to determine the effect of blood sampling on flux values (Figure 4). Flux measurements were also made using fish which were not salt depleted (Figure 5). Plasma ion concentrations and the effect of 12 hour exposure to copper were measured in these fish. To determine the effect of longer exposure to copper in salt depleted fish, a fourth group of fish were exposed to copper starting 2 days prior to flux measurements (Figure 6). The experimental protocol, for each group, is shown diagramatically in figures 2-6.

Analytical Procedures

Sodium concentrations in water, urine, and plasma were determined on duplicate samples by flame emission on a IL Flame Photometer (Model 143) using propane as fuel (Appendix III). Chloride concentrations in water, urine, and plasma were measured in duplicate by amperometric titrations with silver ions using a Buchler-Cotlove Model 4-2000 Chloridometer (Appendix III). Dissolved and total copper concentrations were determined, only on water samples, by absorption spectrophotometry, using the Jarrel-Ash Flame Emission-Atomic Absorption Spectrophotometer, with acetylene as a fuel. The method outlined by Traversy (1971) was used for preparing the samples for copper analysis (Appendix III). Ionic copper concentrations were determined using the Orion copper specific ion electrode.

The levels of radioactivity, for both isotopes, were measured in duplicate using a Nuclear Chicago Mark II Liquid Scintillation System

Figure 4 Experimental protocol for Group B. These fish were blood sampled, salt depleted fish of which one half of the group were exposed to copper, starting 12 hours prior to flux measurements.

	Day												
	0	1	2	3	4	5	6	7	8	9	10	11
8:00 am		Salt depletion in tanks containing distilled water								Flux Measurements		Flux Measurements
8:00 pm	Salt depletion in tanks containing distilled water							Catheterized Put in boxes with distilled water Fish # 1-8 Blood Sampled	Artificial Water Added Fish # 2,4, 6,8 were controls Fish # 1,3, 5,7 were exposed to 40 µg/l copper	Distilled Water Added Fish #1-4 Blood Sampled	Artificial Water Added Fish # 5 and 7 were controls Fish # 6 and 8 were exposed to 40 µg/l copper	Fish #5-8 Blood Sampled	

Figure 5 Experimental protocol for Group C. These fish were blood sampled, non salt depleted fish and one half of the group were exposed to copper, starting 12 hours prior to flux measurements.

	Day				
	1	2	3	4	5
8:00 am			Flux Measurements		Flux Measurements Fish # 5-8
8:00 pm	Catheterized Put in boxes with tapwater Fish # 1-8 Blood Sampled	Artificial Water added Fish # 2,4,6,8 were controls Fish # 1,3,5,7 were exposed to 40 µg/l copper	Distilled Water added Fish # 1-4 Blood Sampled	Artificial Water added Fish # 5 and 7 were controls Fish # 6 and 8 were exposed to 40 µg/l copper	Fish # 5-8 Blood Sampled

Figure 6 Experimental protocol for Group D. These fish were non blood sampled, salt depleted fish which had 2 day exposure to copper prior to flux measurements.

	Day								
	0	1	2	3	4	5	6	7	8
8:00 am	Salt depletion in tanks containing distilled water						Copper added (40 ug/l)	Catheterized Put in boxes with distilled water and 40 µg/l copper	Flux Measurements
8:00 pm	Salt depletion in tanks containing distilled water							Artificial Water and 40 µg/l copper added	Fish were Blood Sampled

(Appendix III). To determine the activity, 1 ml of sample was suspended in 10 ml of Aquasol (New England Nuclear) and activity was measured over 10 minutes. The activity was then corrected for the background counts.

Calculations

Influx and efflux values, expressed as μEq/hr/kg, for the blood sampled and non blood sampled controls showed a body weight dependent correlation which increased the variance within groups, thereby making comparison with other groups difficult. However, when flux values were expressed as μEq/hr/$kg^{0.67}$ the body weight dependent correlation was eliminated. This method of expressing flux values also eliminated all seasonal correlation indicating that seasonal variations were due to weight variations. Since transport in freshwater fish occurs mainly through specific cells in the gill it is more useful to describe flux on the basis of surface area rather than on a weight basis. The value of $kg^{0.67}$ was chosen because it best described the weight to surface area relationship of flux values for this experiment.

The influx was calculated as follows:

$$\text{Influx} = \frac{\text{CPM(initial)} - \text{CPM(final)} \times \text{Vol(l)} \times \text{External Conc.(initial)}}{\text{Surface area of the fish}(kg^{0.67}) \times \text{time(hr)} \times \text{CPM(initial)}}$$

Netflux was calculated from the formula:

$$\text{Netflux} = \frac{[\text{External Conc.(initial)} - \text{External Conc.(final)}] \times \text{Vol(l)}}{\text{Surface area of the fish}(kg^{0.67}) \times \text{time(hr)}}$$

Efflux was calculated as:

$$\text{Efflux} = \text{Influx} \quad \text{Netflux}$$

Urine excretion rates were calculated as:

$$\text{Urine Excretion Rate} = \text{Flow Rate} \times \text{Urine Concentration}$$

A Students t-test was used to determine the statistical significance of differences between controls and experimental groups where t was significant at a level of $p < 0.05$.

RESULTS

Sodium Influx

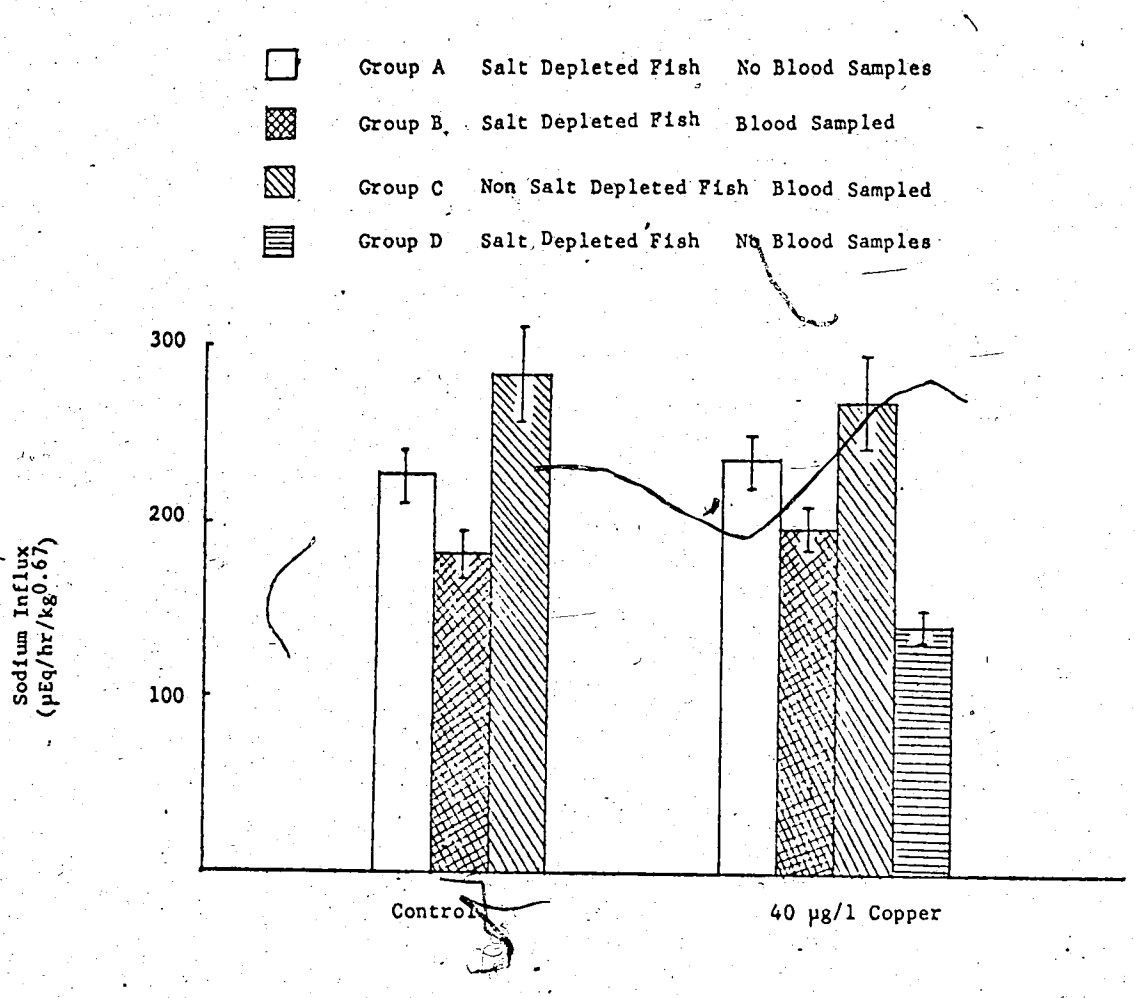
Sodium influx values for Group C (Blood sampled non salt depleted fish) controls were the highest of any of the groups at 284.9 ± 26.0 $\mu Eq/hr/kg^{0.67}$ (Figure 7 and Appendix IV). Group C fish exposed to copper for 12 hours prior to flux measurements, as well as during flux measurements, had a sodium influx value, 268.9 ± 26.4 $\mu Eq/hr/kg^{0.67}$, which was not significantly different from the Group C controls. The variance in this group was twice that of salt depleted fish indicating that uptake by non salt depleted fish was not as uniform as in salt depleted fish.

Salt depletion significantly decreased sodium influx values for Group B (Blood sampled salt depleted fish) controls compared with Group C controls (Figure 7 and Appendix IV). There was no significant difference between Group B controls (180.6 ± 13.4 $\mu Eq/hr/kg^{0.67}$) and Group B copper exposed (195.8 ± 12.5 $\mu Eq/hr/kg^{0.67}$) fish.

There was no significant difference in sodium influx values between Group A (Non blood sampled, salt depleted fish) controls (225.6 ± 13.9 $\mu Eq/hr/kg^{0.67}$) and Group A copper exposed fish (235.3 ± 14.3 $\mu Eq/hr/kg^{0.67}$) (Figure 7 and Appendix IV). Influx values for Group A control fish were significantly lower than values for Group C controls and significantly higher than Group B controls. It appears that blood sampling decreases sodium influx and short term exposure to sublethal copper concentrations had no effect on sodium influx.

Group D (Non blood sampled, salt depleted fish with 2 day copper exposure) influx values, 140.7 ± 8.8 $\mu Eq/hr/kg^{0.67}$, were significantly

Figure 7 Sodium influx in goldfish under various experimental conditions. The number of control samples for each group were: 33 for A, 17 for B, and 19 for C. The number of fish in the groups exposed to copper were: 35 for A, 16 for B, 20 for C, and 23 for D. The vertical bars represent ± SEM.


Group A Salt Depleted Fish No Blood Samples
Group B Salt Depleted Fish Blood Sampled
Group C Non Salt Depleted Fish Blood Sampled
Group D Salt Depleted Fish No Blood Samples
300
200
100
Sodium Influx
(μEq/hr/kg0.67)
Control
40 μg/l Copper

lower than in the Group A controls (Figure 7 and Appendix IV). These results indicate that copper has a delayed effect on sodium influx.

Sodium Efflux

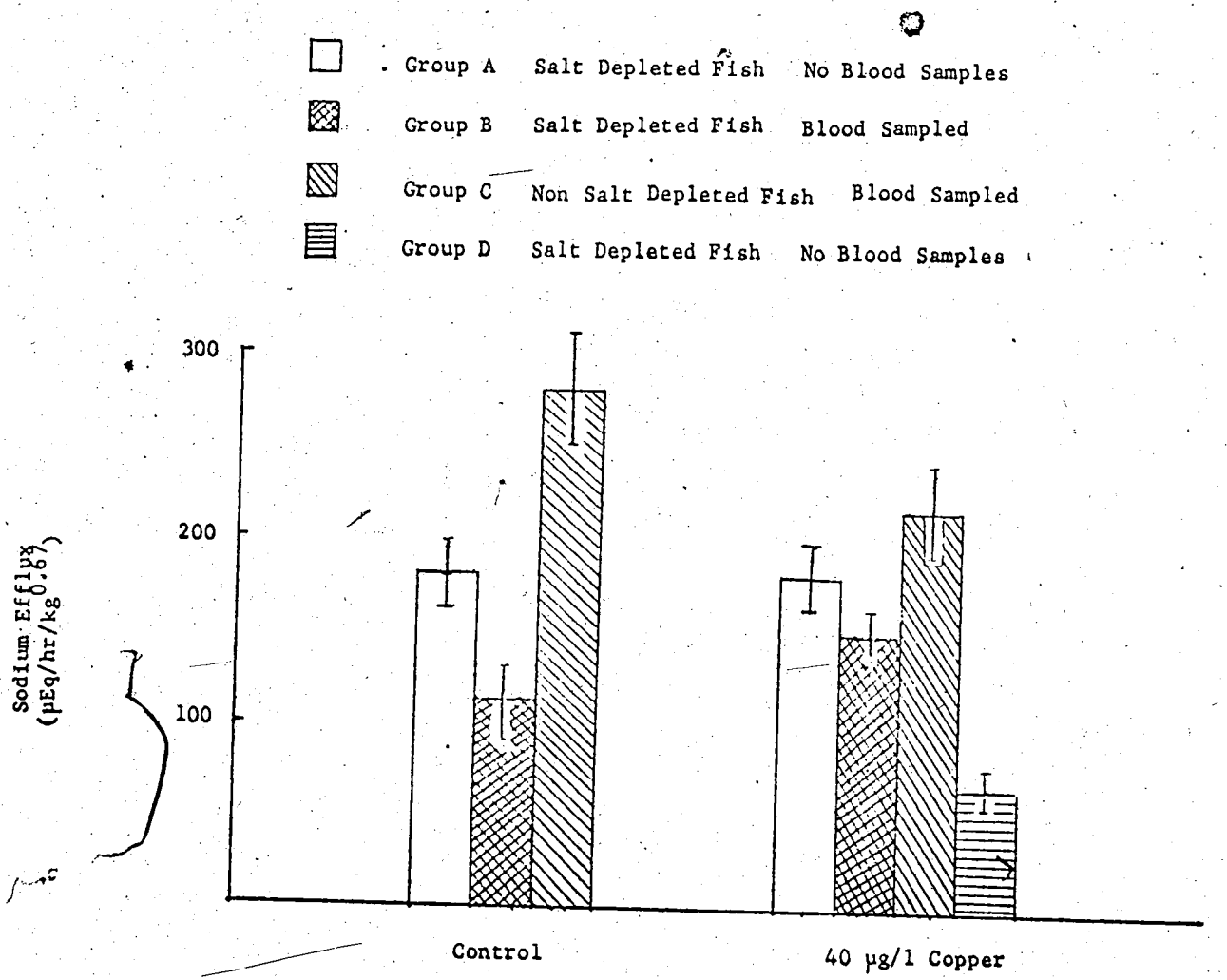
Efflux values for sodium followed the same general pattern as influx values, indicating a direct relationship between influx and efflux. Group C controls had the highest efflux rate, 277.2 ± 31.4 $\mu Eq/hr/kg^{0.67}$, which was not significantly different from the Group C copper exposed fish, 220.6 ± 25.7 $\mu Eq/hr/kg^{0.67}$ (Figure 8 and Appendix IV). Twelve hour copper exposure had no effect on sodium efflux in the other groups as well. This is indicated by the lack of a significant difference between Group A controls (181.9 ± 18.1 $\mu Eq/hr/kg^{0.67}$) and Group A copper exposed fish (182.8 ± 15.9 $\mu Eq/hr/kg^{0.67}$) or Group B controls (111.7 ± 19.1 $\mu Eq/hr/kg^{0.67}$) and copper exposed fish (149.1 ± 18.5 $\mu Eq/hr/kg^{0.67}$) (Figure 8 and Appendix IV).

The sodium efflux from Group B control fish was significantly lower than that of Group C controls indicating that salt depletion reduced salt loss through the gills. Blood sampling significantly decreased sodium efflux values for Group B fish compared with Group A controls. The lowest efflux values were observed in Group D fish, 66.7 ± 9.3 $\mu Eq/hr/kg^{0.67}$, and this efflux rate was significantly lower than that of Group A controls indicating an inhibitory effect on sodium efflux of 2 day copper exposure.

Sodium Netflux

Netflux values for sodium in Group C controls, 7.8 ± 23.0 $\mu Eq/hr/kg^{0.67}$, were the lowest of all the groups and this value was not significantly different from zero. From this observation, it was concluded that homeostasis of internal sodium concentrations had been

Figure 8 Sodium efflux in goldfish under various experimental conditions. The number of control samples for each group were: 33 for A, 17 for B, and 19 for C. The number of fish in the copper exposed groups were: 35 for A, 16 for B, 20 for C, and 23 for D. The vertical bars represent ± SEM.


Group A Salt Depleted Fish No Blood Samples
Group B Salt Depleted Fish Blood Sampled
Group C Non Salt Depleted Fish Blood Sampled
Group D Salt Depleted Fish No Blood Samples
Sodium Efflux
(μEq/hr/kg0.67)
300
200
100
Control
40 μg/l Copper

achieved in this group. Sodium netflux for Group C copper exposed fish, 48.3 ± 23.5 μEq/hr/kg$^{0.67}$, was not significantly different from the Group C controls (Figure 9 and Appendix IV).

Group B control netflux values, 68.9 ± 20.6 μEq/hr/kg$^{0.67}$, were not significantly different from the netflux values of Group B copper exposed fish, 46.8 ± 13.5 μEq/hr/kg$^{0.67}$ (Figure 9 and Appendix IV). Sodium netflux of Group B controls was significantly higher than Group C controls. This indicates that salt depletion stimulated net sodium uptake.

Blood sampling salt depleted fish did not affect sodium netflux since net sodium fluxes were not significantly different in Group A and Group B fish. Exposure to 40 μg/l copper for 12 or 48 hours had no effect on sodium netflux since Group D netflux values, 74.4 ± 8.0 μEq/hr/kg$^{0.67}$, were not significantly different from Group A controls (Figure 9 and Appendix IV).

Chloride Influx and Efflux

Chloride influx and efflux values were obtained only in Group A (Figure 10 and 11 and Appendix V). Comparison of influx values of controls, 160.2 ± 17.7 μEq/hr/kg$^{0.67}$, and copper exposed fish, 178.4 ± 14.3 μEq/hr/kg$^{0.67}$, showed no significant difference. Similarly there was no significant difference in efflux values between controls, 158.5 ± 16.6 μEq/hr/kg$^{0.67}$, and copper exposed fish, 169.1 ± 20.5 μEq/hr/kg$^{0.67}$. This indicates that short term exposure to copper had no effect on chloride influx and efflux.

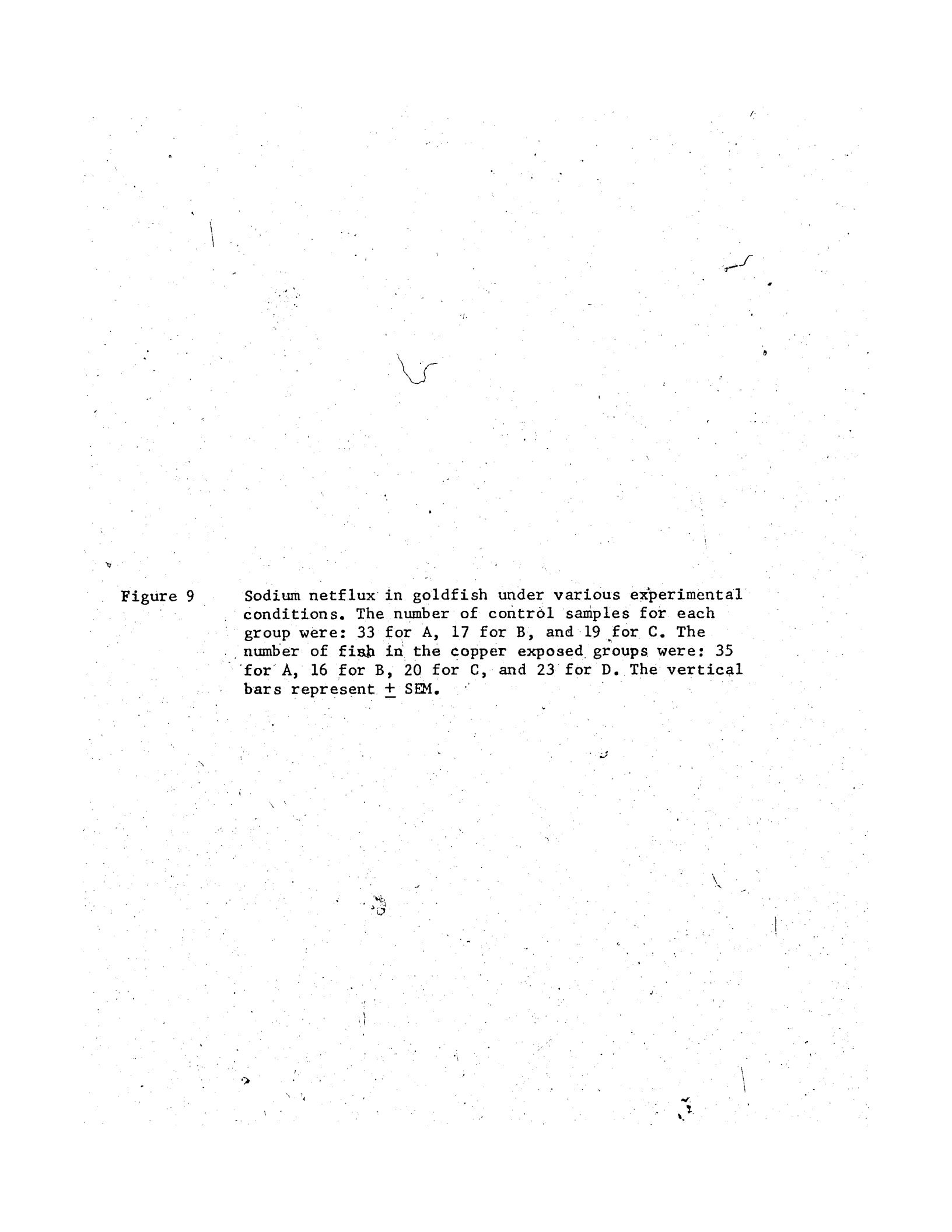


Figure 9 Sodium netflux in goldfish under various experimental conditions. The number of control samples for each group were: 33 for A, 17 for B, and 19 for C. The number of fish in the copper exposed groups were: 35 for A, 16 for B, 20 for C, and 23 for D. The vertical bars represent $\pm$ SEM.

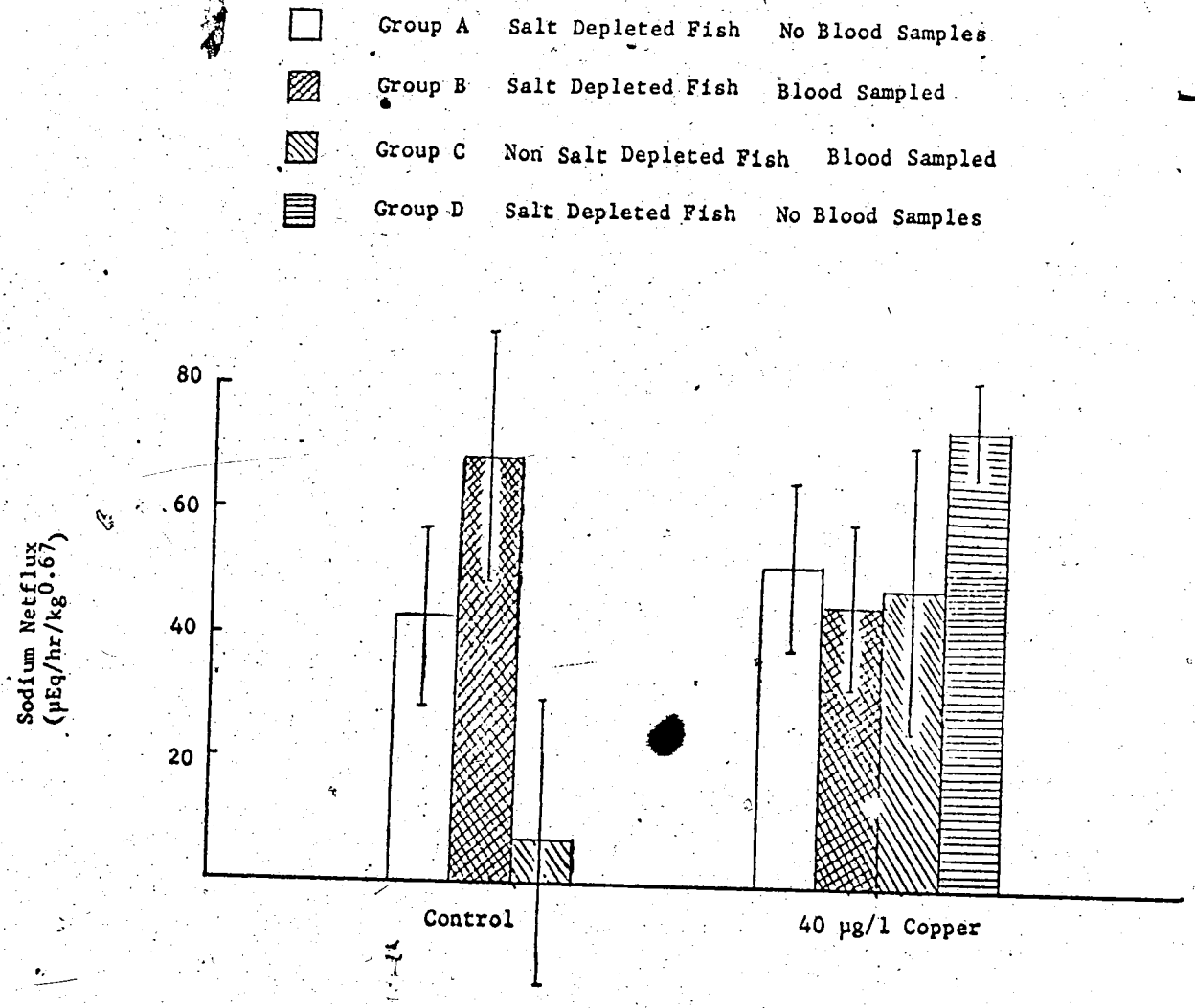

Group A Salt Depleted Fish No Blood Samples
Group B Salt Depleted Fish Blood Sampled
Group C Non Salt Depleted Fish Blood Sampled
Group D Salt Depleted Fish No Blood Samples
Sodium Netflux (μEq/hr/kg0.67)
80
60
40
20
Control
40 μg/l Copper

Figure 10 Chloride influx in non blood sampled, salt depleted fish. Control values were based on 29 fish while the copper exposed values were based on 30 fish. The vertical bars represent $\pm$ SEM.

Group A
Salt Depleted Fish
No Blood Samples

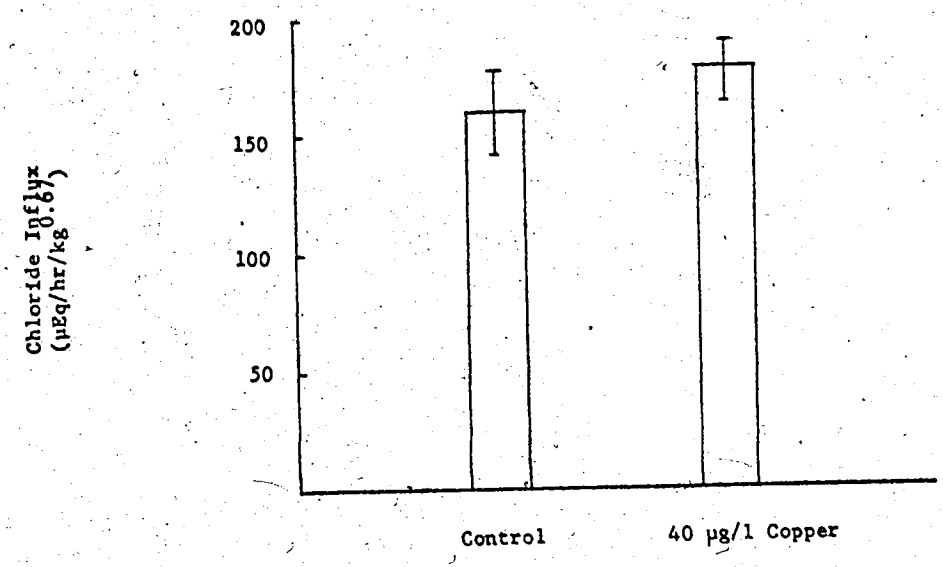
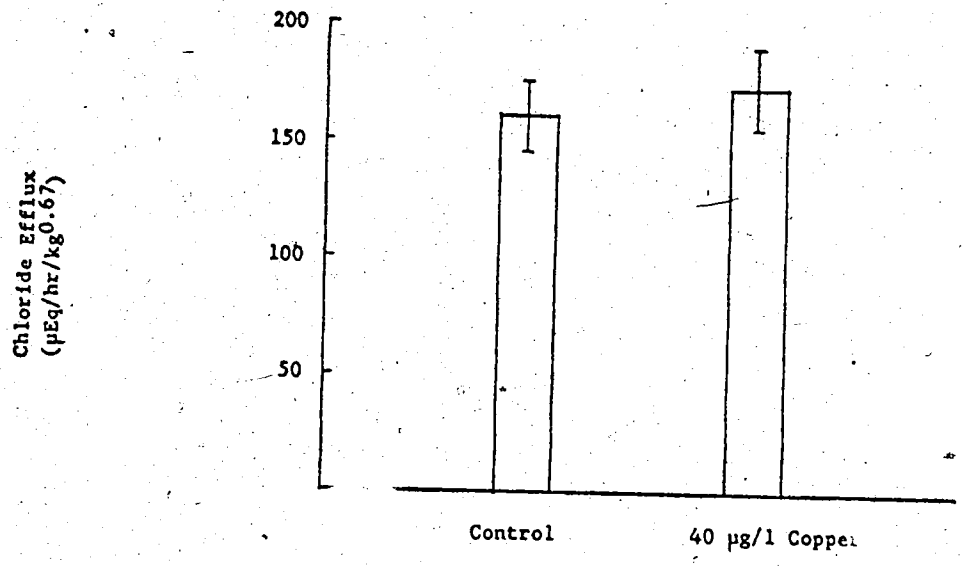


Figure 11 Chloride efflux in non blood sampled, salt depleted goldfish. Control values are based on 29 fish while the copper exposed values are based on 30 fish. The vertical bars represent ± SEM.

Group A
Salt Depleted Fish
No Blood Samples



Chloride Netflux

Chloride netflux values were determined for all groups (Figure 12 and Appendix V). There was no significant difference in chloride netflux values within or between any of the four groups. Although all groups showed some net chloride flux only in Group B copper exposed fish and Group D fish were chloride netfluxes significantly different from zero. In both cases, Group B (-38.1 ± 13.6 $\mu Eq/hr/kg^{0.67}$) and Group D (-23.9 ± 3.6 $\mu Eq/hr/kg^{0.67}$), there was a net loss of chloride from the body. These results indicate that copper exposure, blood sampling, and salt depletion had no effect on chloride netflux.

Plasma Concentrations

Plasma sodium concentrations for salt depleted fish, 102.6 ± 5.2 mEq/l, were significantly lower than plasma sodium concentrations in the non salt depleted fish, 140.7 ± 6.4 mEq/l (Figure 13 and Appendix VI). There was no significant difference between plasma sodium concentrations for the salt depleted fish before flux measurements and the controls and 12 hour copper exposed fish after flux measurements (Figure 13 and Appendix VI). Plasma sodium concentrations for the salt depleted fish before flux measurements were significantly higher than plasma sodium concentrations after flux measurements for fish with 2 day exposure to copper. Since the 2 day copper exposed group had a high net uptake of sodium it was concluded that copper exposure during salt depletion stimulated loss of plasma sodium ions.

Plasma chloride concentrations showed no significant difference between salt depleted, 84.5 ± 3.5 mEq/l, and non salt depleted fish, 89.8 ± 3.8 mEq/l (Figure 14 and Appendix VI). However, plasma chloride

Figure 12 Chloride netflux in goldfish under various experimental conditions. The number of control samples for each group were: 29 for A, 18 for B, and 20 for C. The number of fish in the copper exposed groups were: 30 for A, 17 for B, 22 for C, and 23 for D. The vertical bars represent $\pm$ SEM.

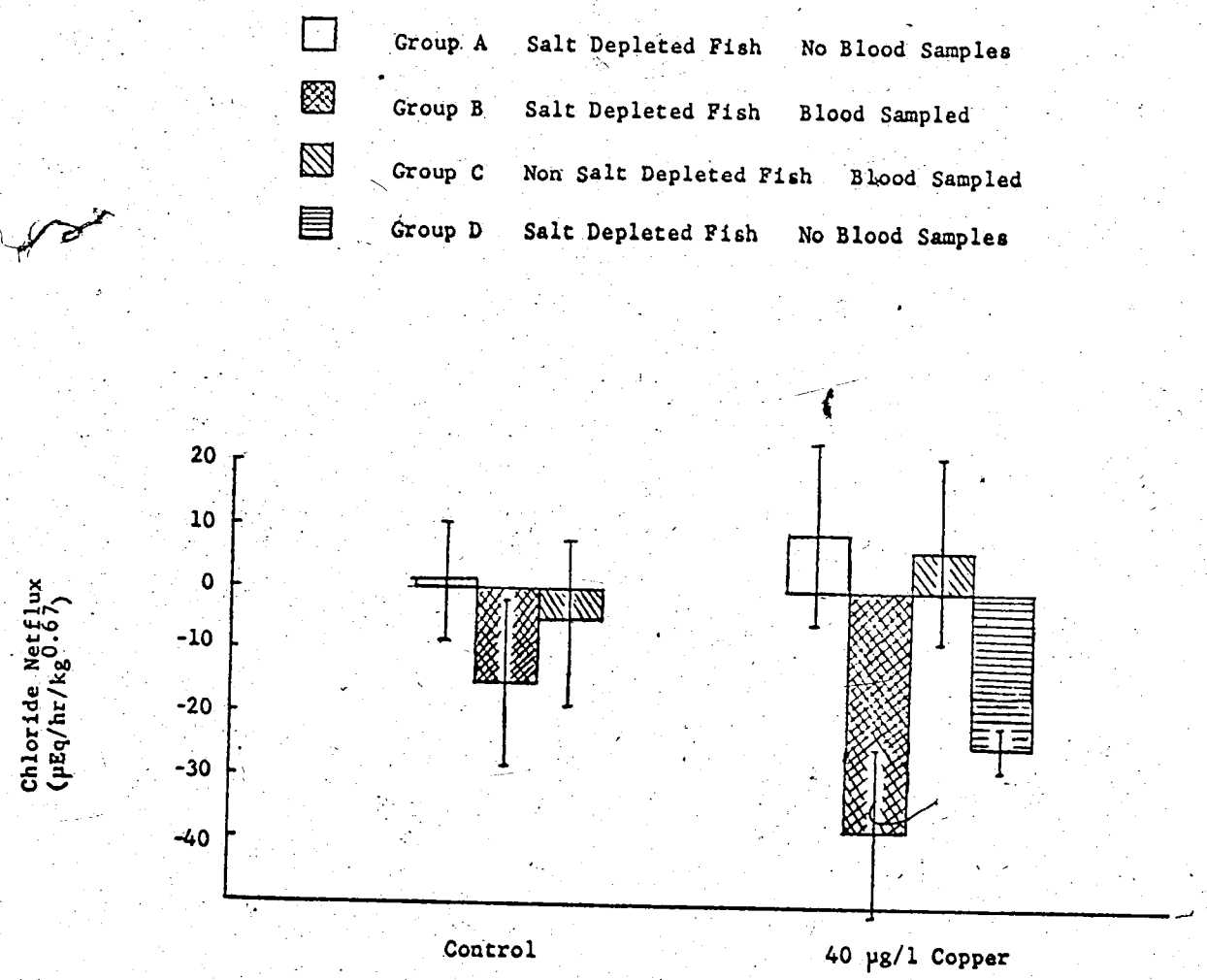

Group A Salt Depleted Fish No Blood Samples
Group B Salt Depleted Fish Blood Sampled
Group C Non Salt Depleted Fish Blood Sampled
Group D Salt Depleted Fish No Blood Samples
Chloride Net flux (μEq/hr/kg0.67)
20
10
0
-10
-20
-30
-40
Control
40 μg/l Copper

Figure 13 Plasma sodium concentrations in goldfish prior to and after flux measurements. Values for non salt depleted fish were based on 15 fish, salt depleted fish values were based on 24 fish, values for controls and 12 hour copper exposure, after flux measurements, were based on 6 fish, and values for the 2 day copper exposed fish, after flux measurements, were based on 23 fish. The vertical bars represent $\pm$ SEM.

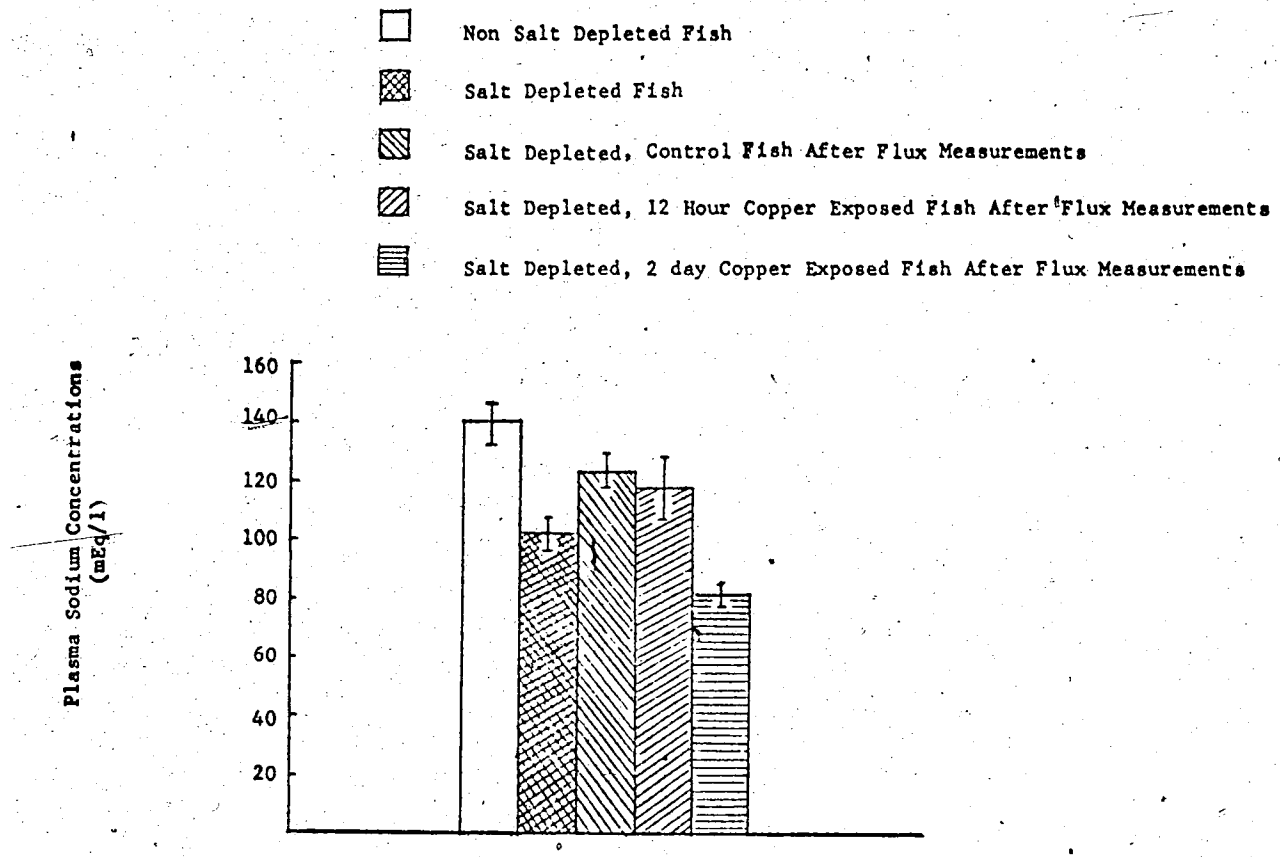
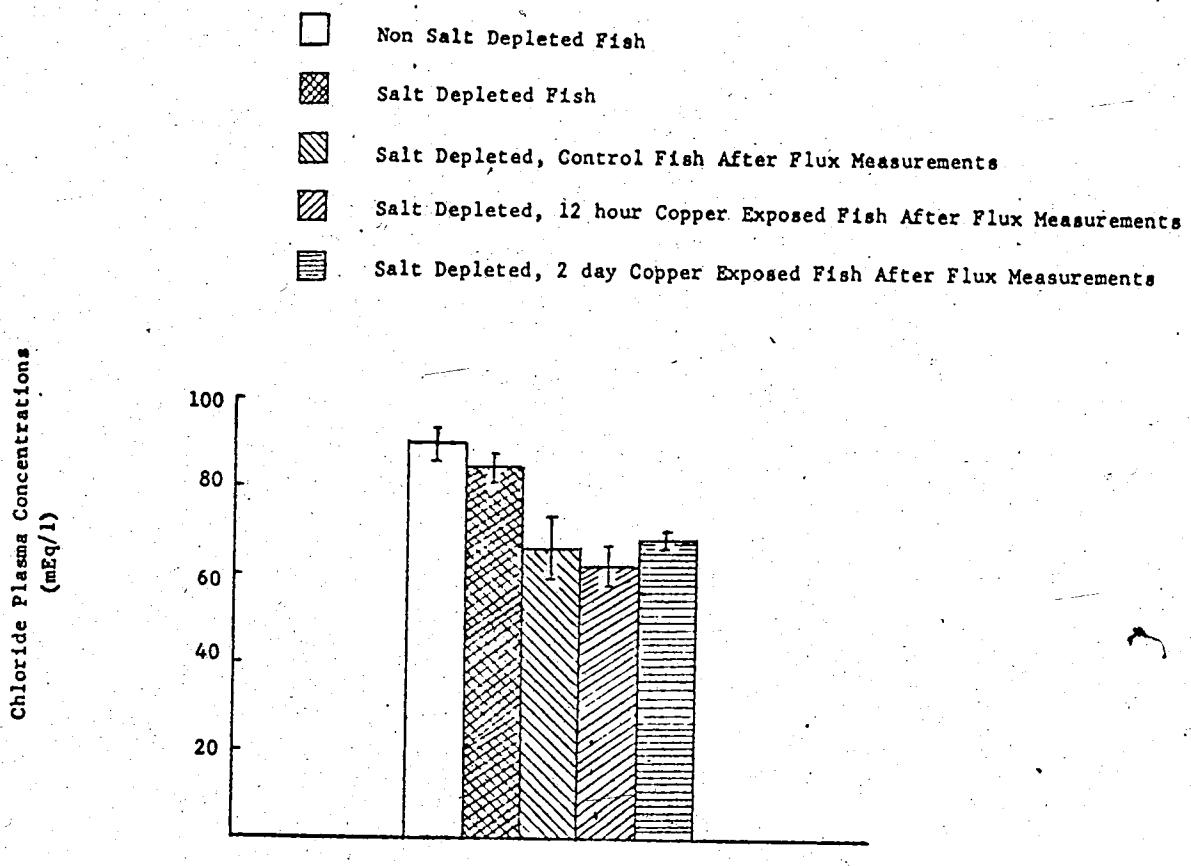

Non Salt Depleted Fish
Salt Depleted Fish
Salt Depleted, Control Fish After Flux Measurements
Salt Depleted, 12 Hour Copper Exposed Fish After Flux Measurements
Salt Depleted, 2 day Copper Exposed Fish After Flux Measurements
Plasma Sodium Concentrations (mEq/l)
160
140
120
100
80
60
40
20

Figure 14 Plasma chloride concentrations in goldfish prior to and after flux measurements. Values for non salt depleted fish were based on 15 samples, salt depleted fish values were based on 24 fish, values for controls and 12 hour copper exposure, after flux measurements, were based on 6 samples, and values for the 2 day copper exposed fish, after flux measurements, were based on 23 fish. The vertical bars represent $\pm$ SEM.


Non Salt Depleted Fish
Salt Depleted Fish
Salt Depleted, Control Fish After Flux Measurements
Salt Depleted, 12 hour Copper Exposed Fish After Flux Measurements
Salt Depleted, 2 day Copper Exposed Fish After Flux Measurements
Chloride Plasma Concentrations (mEq/l)
100
80
60
40
20

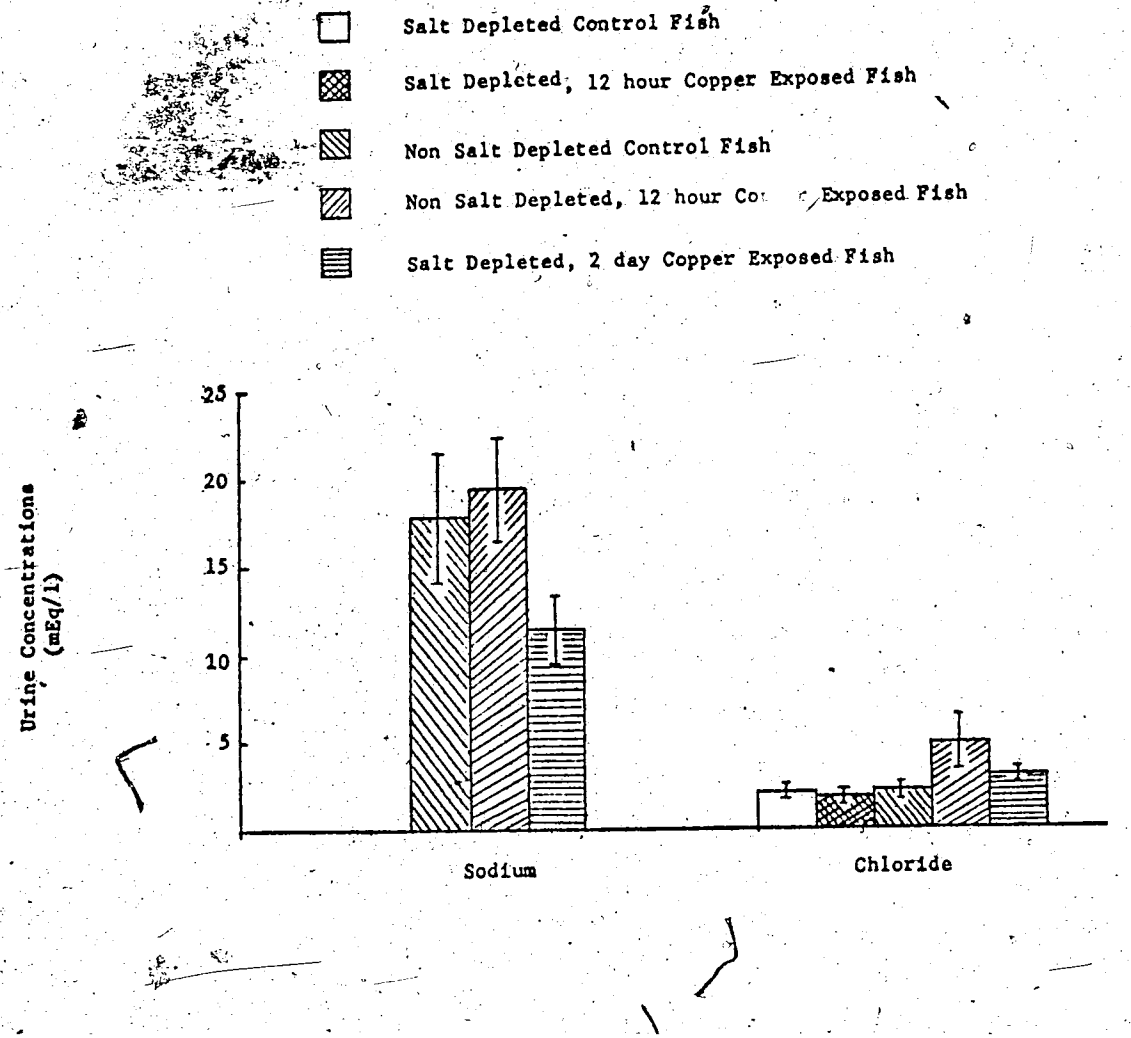
concentrations after flux measurements were significantly lower for both the controls, 65.6 ± 6.9 mEq/l, and those exposed to copper for 12 hours before flux measurements, 61.8 ± 5.2 mEq/l. There was no significant difference in plasma chloride concentrations, after flux measurements, between any of the groups studied (Figure 14 and Appendix VI). The lowered plasma chloride concentrations would be expected, since there was a loss of chloride ions in the urine and netflux of chloride, across the gills is not significantly different from zero or shows a net loss from the body.

Urine Concentrations

Urine sodium concentrations and excretion rates were determined in non salt depleted fish and salt depleted, 2 day copper exposed fish (Figures 15 and 16 and Appendix VI). Sodium concentrations for the 2 day copper exposed fish, 11.45 ± 1.94 mEq/l, were significantly lower than sodium concentrations in non salt depleted fish, 17.75 ± 3.61 mEq/l. Sodium excretion rates were not significantly different in the salt depleted, 2 day copper exposed fish, 0.86 ± 0.19 mEq/kg/day, and non salt depleted fish, 1.01 ± 0.19 mEq/kg/day.

Urine chloride concentrations and excretion rates showed no significant difference between any group whether they were non salt depleted, controls, 12 hour copper exposed, or 2 day copper exposed (Figures 15 and 16 and Appendix VI). It can be concluded that the varying conditions had no effect on chloride loss through the urine.

Figure 15 Sodium and chloride urine concentrations for non salt depleted and salt depleted goldfish under various experimental conditions. Sodium concentrations for non salt depleted control group were based on 14 fish, non salt depleted 12 hour copper exposed sodium values were based on 10 fish while sodium values for salt depleted fish with 2 day copper exposure were based on 15 fish. Non salt depleted control chloride values were based on 16 fish, non salt depleted 12 hour copper exposed chloride values were based on 14 samples, salt depleted control chloride values were based on 4 fish, salt depleted 12 hour copper exposed values were based on 3 fish and the chloride values for salt depleted fish with 2 day copper exposure were based on 12 fish. The vertical bars represent $\pm$ SEM.


Salt Depleted Control Fish
Salt Depleted, 12 hour Copper Exposed Fish
Non Salt Depleted Control Fish
Non Salt Depleted, 12 hour Co Exposed Fish
Salt Depleted, 2 day Copper Exposed Fish
Urine Concentrations (mEq/l)
25
20
15
10
5
Sodium
Chloride

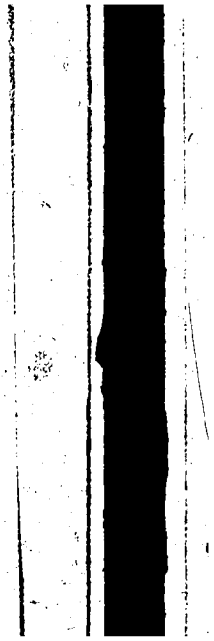
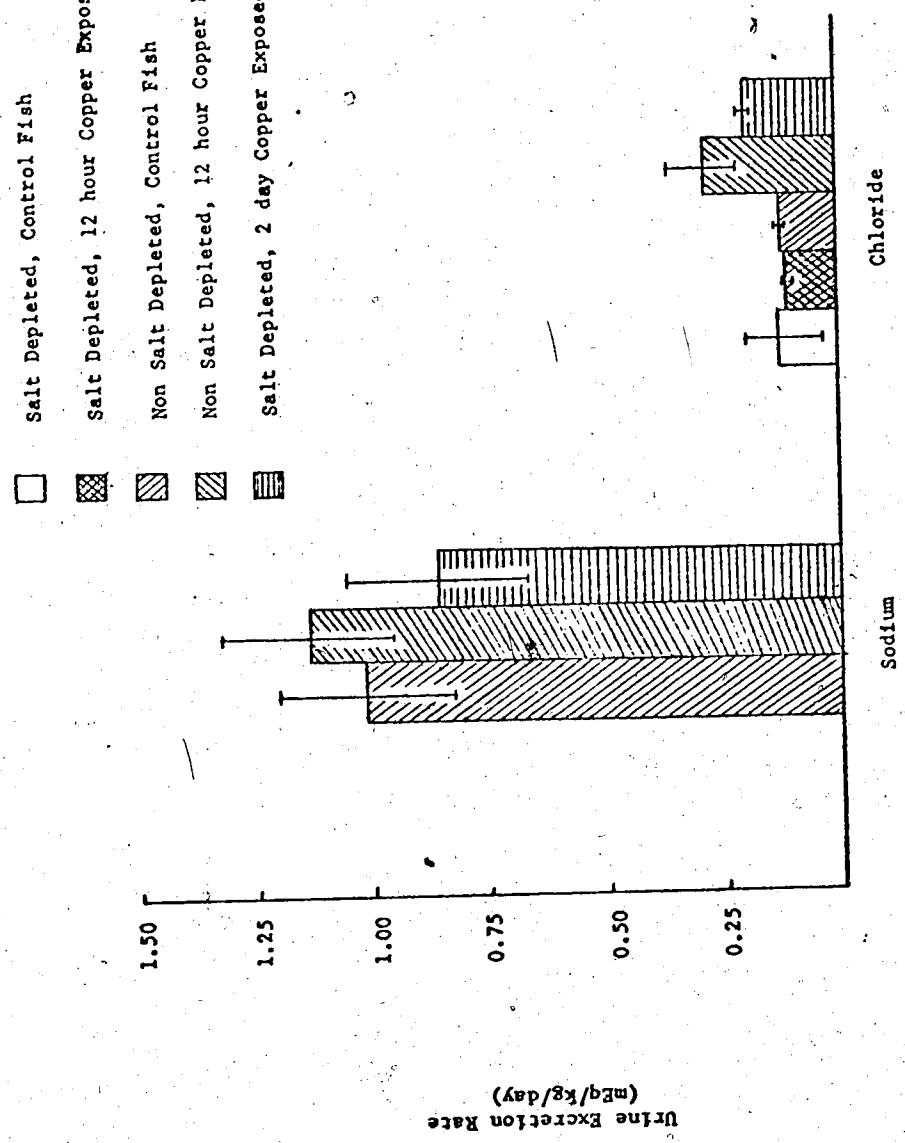


Figure 16 — Urinary sodium and chloride excretion rates for goldfish under various conditions. Non salt depleted control values for sodium were based on 14 fish, non salt depleted 12 hour copper exposed values for sodium were based on 10 fish while sodium values for salt depleted fish with 2 day exposure to copper were based on 15 fish. Non salt depleted control chloride values were based on 16 fish, non salt depleted 12 hour copper exposed values for chloride were based on 14 samples, salt depleted control values for chloride were based on 4 fish, salt depleted 12 hour copper exposed values were based on 3 fish, and the values for chloride in salt depleted fish with 2 day copper exposure were based on 12 samples. The vertical bars represent ± SEM.


Salt Depleted, Control Fish
Salt Depleted, 12 hour Copper Exposed Fish
Non Salt Depleted, Control Fish
Non Salt Depleted, 12 hour Copper Exposed Fish
Salt Depleted, 2 day Copper Exposed Fish
Urine Excretion Rate
(mEq/Kg/day)
1.50
1.25
1.00
0.75
0.50
0.25
Sodium
Chloride

DISCUSSION

An LC_{50} value for copper in goldfish has not been reported by other authors. However, the LC_{50} value of 60 µg/l total copper, found in this study, is comparable to LC_{50} values reported by researchers using salmonids (Appendix I). One toxic unit has been defined as the LC_{50} and sublethal concentrations are reported as fractions of 1 toxic unit (Sprague 1964). The copper concentrations used in this study, 0.67 toxic units, should be large enough to allow an effect of copper to be seen if some action of copper on sodium and chloride transport is the mechanism responsible for copper toxicity.

In each of the groups studied, copper exposure starting 12 hours prior to flux measurements, had no effect on sodium or chloride flux values. The lack of an observed effect on sodium transport agrees with conclusions drawn from studies of frog skin *in vitro* (Ussing and Zerahn, 1951; Koefoed-Johnsen and Ussing, 1958; Zadunaisky *et al.*, 1963). Alvarado *et al.* (1975b) have reported that copper has no effect on chloride flux across *in vitro* frog skin, however, most authors have reported that copper inhibits chloride influx across the *in vitro* frog skin (Ussing and Zerahn, 1951; Koefoed-Johnsen and Ussing, 1958; Zadunaisky *et al.*, 1963; Koefoed-Johnsen *et al.*, 1973).

The mechanism of ion flux differs between *in vivo* and *in vitro* membranes. The influx of chloride across *in vitro* frog skin is a passive process while chloride influx across *in vivo* frog skin and fish gill are similar and active processes (Motais and Garcia-Romeu, 1972). Since the transport processes are similar, the reported difference in the effect of copper on *in vitro* frog skin and the results presented here for *in vivo* fish gills may be due to a difference in the mechanism of chloride

transport. Copper seems to inhibit passive chloride influx, however, there is little passive influx across *in vivo* membranes (Alvarado *et al.*, 1975a). Therefore, copper would have no detectable effect on influx in fish gills *in vivo*.

The inhibitory effect of copper on chloride permeability, seen in frog skin *in vitro*, is not supported on fish gills *in vivo* in this study. Passive efflux is partially dependent on plasma concentrations and membrane permeability and since 12 hour copper exposure did not affect chloride efflux or plasma chloride concentrations it would seem that chloride permeability was not affected. This was also observed for sodium permeability following 12 hour copper exposure, however, 2 day copper exposure seems to have inhibited sodium permeability. This was concluded from the observed decrease in sodium efflux and plasma sodium concentrations following 2 day copper exposure. The ratio of sodium efflux to plasma sodium concentrations would give some measure of the effective permeability of the gill to sodium. This ratio (1.47) for control salt depleted fish was not affected by 12 hour copper exposure but decreased to 0.82 with 2 day copper exposure. This supports the conclusion that 12 hour copper exposure did not affect sodium permeability but 2 day copper exposure did reduce sodium permeability in the gill.

Since experiments were not performed for Group D controls a comparison of Group D copper exposed fish to Group A controls will not be as valid as desired, however, the lack of any seasonal or weight specific changes in influx indicates that such a comparison would be of some value. Copper exposure starting 2 days prior to flux measurements, had no effect on sodium or chloride netflux, however, it did reduce sodium influx and efflux. Studies with *in vitro* frog skin have reported

no effect of copper on sodium transport (Ussing and Zerahn, 1951; Koefoed-Johnsen and Ussing, 1958; Zadunaisky et al., 1963).

As with chloride there is a difference in the mechanism of sodium transport in in vivo and in vitro preparations of frog skin. Motais and Garcia-Romeu (1972) reported that there was an obligatory sodium/hydrogen exchange in vivo, however, in vitro there was no obligatory sodium/hydrogen exchange. This change in the transport mechanism from in vitro to in vivo preparations may account for the difference in the effect of copper on sodium influx.

Sodium and chloride influx increases as plasma ion concentrations decrease below normal levels (Richards and Fromm, 1970; Mackay, 1974). Since the 2 day copper exposed fish have the lowest plasma sodium concentrations, they would be expected to have the largest influx values. However, the 2 day copper exposed fish had the lowest sodium influx values. This provides further evidence of an inhibitory effect of copper on sodium influx. Plasma chloride concentrations were not significantly different between controls, 12 hour, and 2 day copper exposed fish indicating that chloride influx probably would not vary between these groups. Since chloride netflux was not significantly different between these groups it seems reasonable to conclude that copper exposure, even starting 2 days prior to flux measurements, had no effect on chloride transport.

Two theories have been proposed to account for the effect of heavy metals on active sodium transport (Schwartz and Flamenbaum, 1976). The first theory is that heavy metals bind to the mucosal membrane, altering specific entry sites which are functionally coupled to or in series with the active transport site in the serosal membrane. Alternatively,

the heavy metals may enter the cell and then bind with specific enzymes or inhibit the active transport site itself. Since this study shows an inhibition of efflux and there was no effect on transport without long term exposure to copper it may be that the inhibition by copper is working in both ways.

A relationship between Na-K-ATPase activity and sodium influx has been noted by several authors (Jampol and Epstein, 1970; Fisher, 1975; Pfeiler, 1976). Since copper inhibits Na-K-ATPase activity (Bowler, 1970; Ting-Beall et al., 1973; Hexum, 1974; Lorz and MacPherson, 1976) it is possible that inhibition of active sodium transport may be the result of copper binding to the Na-K-ATPase enzyme.

McKim et al. (1970) and Christensen et al. (1972) reported an initial decrease and subsequent recovery in chloride concentrations following 6 day copper exposure. Williams (personal communications) reported the same observation for both sodium and chloride ions. If this is happening in the two day copper exposed group they may not have been able to replace lost ions as copper exposure and salt depletion occured at the same time. This may account for the lowered plasma sodium chloride concentrations following 2 day exposure to copper.

Comparison of sodium influx values for non blood sampled, salt depleted fish and salt depleted fish from which blood samples were taken 48 hours prior to flux measurements indicates that blood sampling has an inhibitory effect on influx and efflux. Speiler (1974), working with goldfish (Carassius auratus), observed an increase in cortisol which promotes the retention of sodium by the kidney in salt water eels (Anguilla anguilla) (Chan et al., 1963; Henderson and Chester-Jones, 1967; Mayer et al., 1967; Butler et al., 1969). Since in freshwater

fish the main tissue across which salt enters and on which cortisol could act is the gill, the inhibition of sodium influx in the blood sampled group could be explained as a general stress response producing increased concentrations of cortisol.

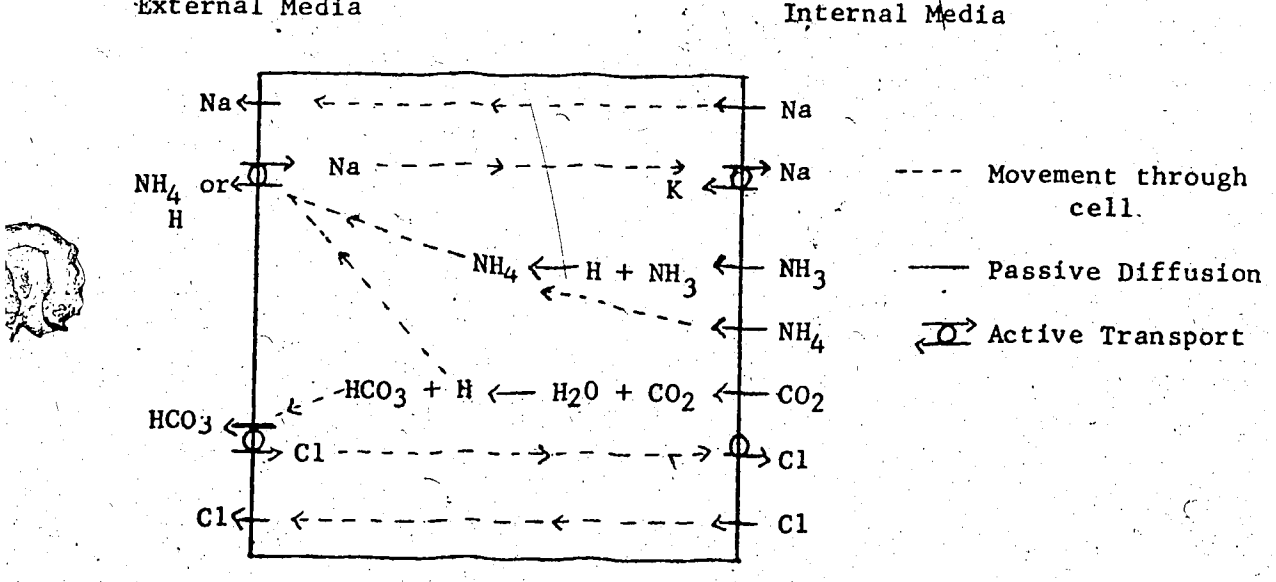
Copper exposure also produces stress leading to increased blood cortisol levels in fish (Donaldson and Dye, 1975; Schreck and Lorz, 1978). Copper levels in this study would produce an elevation of cortisol levels. Since an effect of cortisol would not be seen immediately a stress induced cortisol increase may have caused at least partial inhibition of sodium transport with 2 day copper exposure (Appendix VI).

Flux values for non salt depleted fish show a larger variation than values for salt depleted fish, indicating that influx is not as uniform. The netflux of sodium and chloride in the non salt depleted fish was not significantly different from zero. Salt depletion stimulated net sodium uptake but had no significant effect on chloride netflux. Plasma sodium concentrations showed a significant decrease after salt depletion whereas plasma chloride concentrations did not decrease proportionally. Therefore, it would be expected that net sodium uptake would be much larger in order to replace lost sodium.

As stated earlier, no studies have been performed on the effect of copper on sodium and chloride transport across fish gills. However, a comparison of control values to other author's work indicates that the experimental protocol used for this study does yeild reproducible values indicating a possible validity of the results. The flux values of sodium for salt depleted fish compare closely with values reported by Eddy (1975). Cuthbert and Maetz (1972) and Maetz (1972) reported influx values similar to those reported here, however, efflux values

were lower and netflux values higher than in this study. Chloride influx and efflux values are within the limits of values reported by De Renzis and Maetz (1973) although chloride netflux values were higher then in this study. Plasma sodium concentrations for non salt depleted fish are comparable to those reported for goldfish by Lahlou et al. (1969) and De Renzis and Maetz (1973). Plasma concentrations for sodium and chloride in salt depleted fish, are also comparable to values reported by De Renzis and Maetz (1973). The urinary sodium excretion rates are comparable to those of Maetz (1972) and Mackay (1974) while chloride excretion rates comparable to values calculated from data reported by Hunn and Willford (1970).

The following diagram indicates a basic model of the accepted theory for sodium and chloride transport in fish gill. This model was originally proposed by Maetz (1971) and supported by further studies (De Renzis and Maetz, 1973; Maetz, 1973; Eddy, 1975).



The production of HCO_3 and NH_4 are thought to be catalyzed by carbonic anhydrase and amino acid deaminases, respectively. Na-K-ATPase is thought to be involved in the active transport of sodium (Maetz 1971).

Since sodium influx values showed a direct relationship to plasma concentrations, even with copper exposure, and copper affected sodium efflux it must be assumed that long term exposure affected the sodium permeability of the gill membrane. The inhibiting effect of copper on active sodium influx and the length of exposure to copper required may be due to a summation of factors. Copper must enter the cell, bind to the enzyme and affect the binding capacity of the enzyme. Assuming the the enzyme levels are higher than necessary to maintain influx values it may also take time to reduce enzyme activity to where influx is also affected.

Although copper does have an inhibitory effect on sodium transport it would seem unlikely that this is the mechanism of copper toxicity in freshwater fish.

SUMMARY

1) Short term exposure (12 hours) to a sublethal copper concentration, 0.67 of the LC_{50}, had no effect on sodium transport across the gill of goldfish.

2) Longer exposure (2 days) to 0.67 toxic units of copper reduced both passive and active fluxes of sodium.

3) Exposure to sublethal copper concentrations had no effect on chloride transport with up to 2 day exposure to copper.

4) General stress effects, such as blood sampling, had an effect on sodium transport in goldfish gills.

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APPENDIX I Determination of a 96 hour LC_{50} Value for Goldfish

To determine a copper concentration which would stress the fish, without producing mortality, a 96 hour LC_{50} was determined on 24.5 ± 7.9 gram (mean ± standadrd deviation) goldfish. The tests were performed in the recirculating system used for flux measurements using a temperature of 23 ± 0.5°C, a pH of 7.3 ± 0.3 and a total hardness of 12 mg/l $CaCO_3$. Infusion of 4500 µg/l copper at 0.05 ml/hr kept dissolved copper concentrations stable.

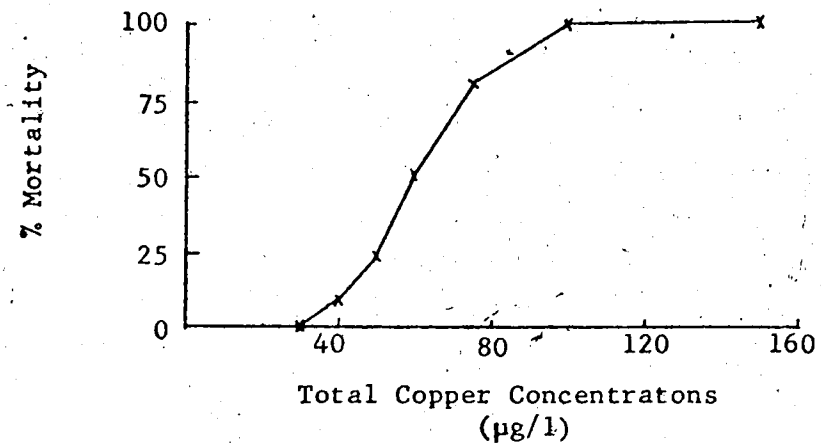
Toxicity Test # 1:

	Total Copper Concentration			
	150 µg/l	100 µg/l	75 µg/l	50 µg/l
# of fish	10	10	10	10
# of deaths After 96 hr	10	10	8	2
% Mortality	100%	100%	80%	20%

Toxicity Test # 2:

	Total Copper Concentration			
	60 µg/l	50 µg/l	40 µg/l	30 µg/l
# of fish	12	12	12	13
# of deaths After 96 hr	6	3	1	0
% Mortality	50%	25%	8%	0%

Mortality in Varying Copper Concentrations:



The 96 hour LC_{50} value was taken to be 60 µg/l.

There have been several studies on goldfish using copper as the toxicant but a toxic value has not been determined. However, the toxic value of copper for several salmonids has been determined. The 96 hour LC_{50} was 100 µg/l of copper for 14 month old trout (Salvelinus fontinalis) in water with a temperature of 12 ± 1°C, a pH of 7.5 and a hardness of 42 mg/l $CaCO_3$ (McKim and Benoit, 1971). For juvenile coho salmon, (Oncorhynchus kisutch), the 96 hour LC_{50} ranged from 60-74 µg/l copper depending on the time of year (Lorz and MacPherson, 1976). The incipient lethal level (ILL) for 14.3 cm Salmo salar was 48 µg/l of copper at 15° C, 20 mg/l $CaCO_3$ and a pH of 7.1-7.5 (Sprague, 1964). At a temperature of 17°C, pH of 7.0-7.4 and 20 mg/l $CaCO_3$ the ILL for 9.2 cm Salmo salar was found to be 32 µg/l of copper (Sprague, 1965). The LC_{50} of 60 µg/l, for goldfish (Carassius auratus), found in this study is well within the range of LC_{50} values for other freshwater fish.

APPENDIX II Copper Concentrations in the Artificial Media

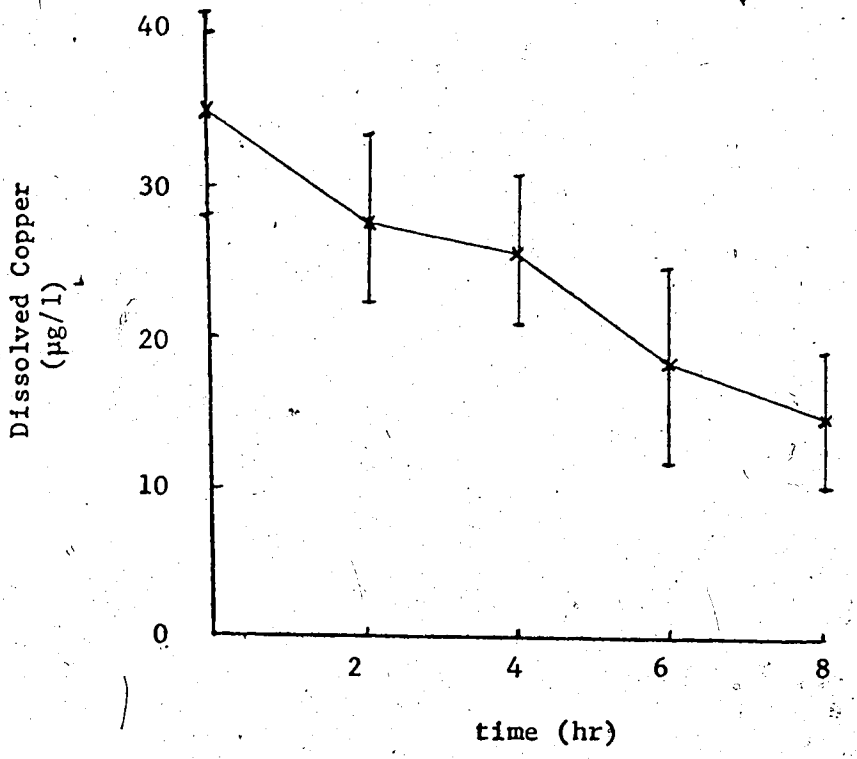
Initial experiments, performed with 60 µg/l total copper, started with actual concentrations of 57 ± 7.97 µg/l total copper, 34.6 ± 6.79 µg/l dissolved copper, and 26.0 ± 5.08 µg/l ionic copper. (Concentrations expressed as mean ± standard deviation).

During the flux measurements, mucus secretions by the fish prevented measurements for total copper. During analysis the mucus was suspended in the methyl isobutyl ketone along with the copper. This suspended mucus increased the viscosity of the sample compared to the standard solution, so that the aspiration rates into the Spectrophotometer differed. For this reason accurate estimates of total copper concentrations could not be made.

Ionic copper concentrations rapidly diminished until they reached undetectable levels after one half hour. Recent papers (Pagenkogf, 1974; Andrews et al., 1976) indicate that the copper ion is possibly the active toxicant, rather than the dissolved copper salts, but the decrease in free copper ions observed in this study indicates that this may not be the case since toxicity occured even when ionic copper was undectable.

Measurements for dissolved copper concentrations indicate an approximate 2.5 ug/hr decrease (See graph below). A concentrated copper sulfate solution, 4500 µg/l, was infused in at 0.05 ml/hr to maintain stable dissolved copper concentrations. Sprague (1964) assumed that this dissappearance of copper was due to the binding of the copper to the walls of the apparatus, taken up by the fish, and reacting with organic matter and colloids in the water. He assumed that this copper was undissociated and therefore had no direct lethal affect on the fish.

Decrease in Dissolved Copper Concentrations Over Time



APPENDIX III Analyses Procedure

Sodium Analysis:

Water samples were diluted 1:1 with 30 mM $LiNO_3$ dilutent (Instrumentation Lab Inc.) and plasma and urine samples were diluted 1:200 with 15 mM $LiNO_3$. Standards were produced to yeild desired sodium concentrations in 15 mM $LiNO_3$ and then run on the IL 143 Flame Photometer. The machine readings were plotted versus sodium concentration of known standards and the sodium concentration of duplicate samples were interpolated from the graph. Interpolated concentrations were then corrected for the dilution factor.

Chloride Analysis:

Water samples were diluted 1:1 with a nitric acid-acetic acid solution (200 ml acetic acid and 12.8 ml nitric acid per liter) while plasma and urine samples were diluted 1:40 with the acid solution. After dilution, 4 drops of gelatin reagent (Buchler Instruments Division, Nuclear-Chicago Corp.) were added. Standards were produced from NaCl solutions diluted with the acid solution as the samples were.

Chloride samples were titrated using a Buchler-Cotlove Chloridometer, which determines the length of time needed for precipitation of the chloride ions by silver ions. Chloride concentrations were determined, in duplicate, using the formula:

$$\text{Sample Concentration} = \text{Sample Time} - \text{Blank Time} \times \frac{\text{Standard Concentration}}{\text{Standard Time} - \text{Blank Time}}$$

Copper Analysis:

Total copper concentrations were determined on 100 ml samples while

dissolved copper concentrations were determined on 100 ml samples which had been passed through a 0.45 micron filter. Five ml of a 4.25 pH sodium acetate buffer were added followed by 5 ml of ammonium pyrrolidine dithiocarbamate, which extracts the copper in the sample. The extracted copper was then suspended in 10 ml of methyl isobutyl ketone. The ketone mixture was then aspirated in the Jarrel-Ash Flame Emission-Atomic Absorption Spectrophotometer and absorption measured at a wavelength of 324.7. Standards were produced using the same procedure and then the percent absorption was plotted versus copper concentrations of known standards. Sample copper concentration were interpolated from the graph.

^{22}Na and ^{36}Cl Activity Analysis:

These isotopes are beta emitters, ^{22}Na emits with an energy of 2.44 MeV while ^{36}Cl produces a 0.71 MeV energy, for each atom decay. This energy produces light flashes in the Aquasol fluor used. Each light flash, representing 1 decay, is registered on the Nuclear Chicago Mark 1 Liquid Scintillation System. Each sample was measured over a 10 minute period to give an activity in decays per minute. Each activity was corrected for the background activity.

Sodium flux Values for Control Fish:

	Group A Salt Depleted Fish Non Blood Sampled	Group B Salt Depleted Fish Blood Sampled	Group C Non Salt Depleted Fish Blood Sampled
influx	225.6 ± 13.9*(33)**	180.6 ± 13.4 (17)	284.9 ± 26.0 (19)
efflux	181.9 ± 18.1 (33)	111.7 ± 19.1 (17)	277.2 ± 31.4 (19)
netflux	43.6 + 14.6 (33)	68.9 + 20.6 (17)	7.7 + 23.0 (19)

Sodium Flux Values for Copper Exposed Fish

	Group A Salt Depleted Fish Non Blood Sampled 12 hour Exposure	Group B Salt Depleted Fish Blood Sampled 12 hour Exposure	Group C Non Salt Depleted Fish Blood Sampled 12 hour Exposure	Group D Salt Depleted Fish Non Blood Sampled 2 day Exposure
influx	235.3 ± 14.3 (35)	195.8 ± 12.5 (16)	268.9 ± 26.4 (20)	140.7 ± 8.8 (23)
efflux	182.8 ± 15.9 (35)	149.1 ± 18.5 (16)	220.6 ± 25.7 (20)	66.7 ± 9.3 (23)
netflux	52.5 + 13.5 (35)	46.8 + 13.5 (16)	48.3 + 23.5 (20)	74.4 + 8.0 (23)

* Values Expressed as mean ± 1 S.E.M. ($\mu Eq/hr/kg^{0.67}$)
** Values are followed by the number of fish sampled.

APPENDIX y Chloride Flux Values

Chloride Flux Values for Control Fish:

	Group A Salt Depleted Fish Non Blood Sampled	Group B Salt Depleted Fish Blood Sampled	Group C Non Salt Depleted Fish Blood Sampled
influx	160.2 ± 17.7*(29)**		
efflux	158.5 ± 16.6 (29)		
netflux	1.8 + 13.5 (29)	-14.9 + 13.7 (8)	-4.9 + 13.1 (20)

Chloride Flux Values for Copper Exposed Fish:

	Group A Salt Depleted Fish Non Blood Sampled 12 hour Exposure	Group B Salt Depleted Fish Blood Sampled 12 hour Exposure	Group C Non Salt Depleted Fish Blood Sampled 12 hour Exposure	Group D Salt Depleted Fish Non Blood Sampled 2 day Exposure
influx	178.4 ± 14.3 (30)			
efflux	169.1 ± 20.5 (30)			
netflux	9.3 + 15.0 (30)	-38.1 + 13.6 (17)	7.14 + 15.1(22)	-23.9 + 3.6 (23)

* Values Expressed as mean ± S.E.M. ($\mu Eq/hr/kg^{0.67}$)
** Values are followed by the number of fish sampled.

APPENDIX VI Plasma Concentrations and Urine Excretion Rates

Plasma Concentrations:

	Normal Fish	Salt Depleted Fish	After Experiment Controls	After Experiment 12 hour Exposure to Copper	After Experiment 2 day Exposure to Copper
Sodium (mEq/l)	140.7 ± 6.4* (15)**	102.6 ± 5.2 (24)	123.5 ± 5.6 (6)	118.0 ± 10.6 (6)	81.7 ± 3.7 (23)
Chloride (mEq/l)	89.8 ± 3.8 (15)	84.5 ± 3.5 (24)	65.6 ± 6.9 (7)	61.8 ± 5.2 (6)	67.9 ± 2.1 (23)

Urine Analysis:

	Normal Fish Controls	Normal Fish 12 hour Exposure to Copper	Salt Depleted Fish Controls	Salt Depleted Fish 12 hour Copper Exposure	Salt Depleted Fish 2 day Copper Exposure
Sodium Concentration (mEq/l)	17.75 ± 3.61 (14)	19.45 ± 2.9 (10)			11.45 ± 1.94 (15)
Chloride Concentration (mEq/l)	2.12 ± 0.41 (16)	4.83 ± 1.46 (14)	2.15 ± 0.49 (4)	1.85 ± 0.42 (3)	2.9 ± 0.41 (12)
Flow Rate (ml/kg/day)	59.48 ± 3.28 (16)	59.07 ± 2.53 (14)	55.15 ± 2.27 (4)	51.86 ± 8.37 (3)	72.49 ± 4.26 (15)
Sodium Excretion Rate (mEq/kg/day)	1.01 ± 0.19 (14)	1.13 ± 0.18 (10)			0.86 ± 0.19 (15)
Chloride Excretion Rate (mEq/kg/day)	0.12 ± 0.00 (16)	0.28 ± 0.09 (14)	0.12 ± 0.09 (4)	0.10 ± 0.00 (3)	0.19 ± 0.02 (12)

* Values expressed as mean ± SEM.
** Values are followed by the number of fish sampled

APPENDIX VII Relationship of Copper Concentrations to Cortisol Levels

It has been observed that cortisol concentrations in salmonids with exposure to copper (Donaldson and Dye, 1975; Schreck and Lorz, 1978). These authors reported that 60 µg/l copper produced an initial increase in cortisol levels, after 2 hour exposure, followed by a decrease after 8 hours exposure. Cortisol levels had increased to twice the levels found in controls after 24 hour copper exposure.

The 40 µg/l copper concentrations used in this study probably produced a similar effect. Groups A,B, and C, exposed to copper 12 hours prior to flux measurements, should not be affected by elevated cortisol concentrations, as much as 2 day copper exposed fish, since the reported pattern indicates that at the time of flux measurements, cortisol levels would be on the increase and not at the maximum. As well, the copper concentrations used in this study were lower than the 60 µg/l used by Donaldson and Dye (1975) and Schreck and Lorz (1978). These authors also reported that 15 µg/l copper produced no significant increase in cortisol levels, after 8 hour copper exposure. It is expected that the copper concentration of 40 µg/l would not have as strong an effect on cortisol concentrations as higher copper concentrations.

However, the 2 day, 40 µg/l pre exposure to copper used in Group D would produce increases in cortisol levels. These increases in cortisol may not fully account for the large inhibition of influx of sodium in the 2 day copper exposed fish but it must be assumed it had some inhibitory effect.