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Effect of Cupric Ion Activity on Calcium Accumulation in Juvenile Flounder (*Paralichthys* spp.)

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ABSTRACT

*To test the effects of cupric ion activity on calcium accumulation, juvenile flounder (*Paralichthys *spp.) were exposed to five different activities of ionic copper for 14 days. The negative log of the cupric ion activity (pCu) of the seawater-based exposure media were controlled with a nitrilotriacetic acid (NTA) trace metal buffer system. The pCu ranged from 13·0 (analogous to background cupric ion activities measured in Beaufort seawater) to 9·0 (which could exist in grossly contaminated environments). The rates of ^{45}Ca accumulation by flounder were significantly reduced with increasing cupric ion activities. The rates at pCu 10·0, 9·5, and 9·0 were lower than those at pCu 13·0 and 11·0. Additionally, it was shown that after the fish were exposed to the different activities for 14 days and then placed in seawater without added copper or NTA, there was increased ^{45}Ca accumulation at the two highest pCu of 9·5 and 9·0. This increased accumulation suggests that the fishes were trying to overcome a calcium deficit that was induced by the copper exposure. The demonstrated non-lethal effects of copper/cupric ion activity on flounder occurred at cupric ion concentrations that were two to three orders of magnitude lower than the lethal concentrations.*

INTRODUCTION

Estuarine-dependent fish, such as juvenile flounder, spend the majority of their first year in habitats where the food supply is abundant, but where they

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may be exposed to a variety of physical and chemical stressors. Among the chemical stressors, transition metals such as Cd, Cu, Hg and Zn are important. These metals can enter the estuary naturally through the weathering process or from the discharge of domestic, industrial or agricultural effluents. Copper is a ubiquitous metal and is nutritionally required for both growth and development and can be toxic at elevated concentrations (Gardner & LaRoche, 1973; Brown *et al.*, 1974; Harrison & Rice, 1981).

Chemical speciation has a major influence on the toxicity and bioavailability of copper in seawater. Numerous investigations have shown that cupric ion activity rather than total dissolved copper concentration controls bioavailability (Sunda & Guillard, 1976; Engel & Sunda, 1979; Guida *et al.*, 1980; Zamuda & Sunda, 1982). Free ion activity is therefore important in laboratory toxicity experiments and in assessing the potential toxicity of copper in the environment.

Life stage has been shown also to affect the sensitivity of fish to copper toxicity. Eggs, larvae and early juveniles are somewhat more sensitive to elevated concentrations of copper in seawater than older fish (Blaxter, 1977; Engel & Sunda, 1979; Simon, 1979; Engel *et al.*, 1981). In earlier experiments on the toxic effects of copper on juvenile flounders, we demonstrated that after a 28-day exposure to different dissolved concentrations, the removal of copper caused an increased accumulation of ^{45}Ca that was three to four times greater than that accumulated by controls. These data suggested that copper dissolved in seawater interfered with calcium accumulation and/or metabolism, that copper poisoning was reversible and that the resultant increase in ^{45}Ca accumulation was the result of a calcium deficit produced by copper exposure.

To investigate these observations further, experiments were designed to determine if cupric ion activity influenced the accumulation of calcium by juvenile flounder. Two types of experiment were conducted: determination of the effect of copper on the rate of calcium accumulation, and determination of whether copper inhibition of calcium accumulation was reversible.

MATERIALS AND METHODS

Juvenile flounder (*Paralichthys* spp.) used in these experiments were collected from the Newport River estuary with a bridge net near our laboratory during January and February. After collection the fish were held in the laboratory in 30 ppt seawater at 20°C in large glass finger bowls and fed newly hatched brine shrimp daily. The fish were maintained under these conditions for a minimum of 1 week to allow capture-induced mortalities to occur. Also, in all of the copper exposure experiments, the flounder were fed newly hatched brine shrimp nauplii that were filtered and blotted to remove excess seawater. This technique prevented the dilution of the exposure media with variable amounts of seawater.

In the copper exposure experiments, the free cupric ion activity was controlled using a 30 ppt seawater-based trace metal buffer system that contained 0·1 mM nitrilotriacetic acid (NTA) and added trace metals (0·1 μM Fe, 0·5 μM Mn, 0·6 μM Zn, 0·3 μM Co and 0·012–1200 μM Cu. The seawater used was collected from the Newport River estuary at our laboratory dock and filtered through 0·4-μm Nucleopore filters. The negative log of the cupric ion activity (pCu) of the media used in these experiments were computed from equilibrium calculations (Zamuda & Sunda, 1982) and equalled 13, 11, 10, 9·5, and 9·0. A pCu of 13 falls within the measured range in natural seawater collected from our dock (Sunda & Huntsman, unpublished data) and therefore can be considered a control treatment. The benefit of metal buffers is that they reduce the experimental uncertainties caused by incidental contamination and uncontrolled metal speciation that can occur during both the preparation of exposure media and the experiments.

In the first set of experiments, flounder were exposed to five different pCu in the presence of ^{45}Ca for 3, 7 and 14 days to determine the effect of copper on calcium accumulation. Five juvenile fish, 10–15 mm standard length, were placed into each of twenty 500-ml polystyrene cups containing 200 ml of the medium together with ^{45}Ca 18 μCi to give a final concentration of 0·9 μCi/ml. The availability of calcium was not affected significantly by the presence of NTA because the concentration of calcium in the seawater was about 11 mM while that of NTA concentration was 0·1 mM. After specified periods of time, flounder were removed from the media and measured for ^{45}Ca accumulation and dry weight.

In the second set of experiments, flounder were exposed to five different pCu for 14 days, after which the copper-containing media were removed and the flounder placed in 200 ml of filtered dock seawater containing ^{45}Ca 0·09 μCi/ml and no added copper or NTA buffer. After 7-days incubation with ^{45}Ca, the concentrations of ^{45}Ca in the fish were measured.

The concentrations of ^{45}Ca in individual flounder were determined using liquid scintillation counting techniques. The flounder were removed from the containers, washed with seawater, blotted, placed in preweighed scintillation vials and dried for 24 h at 109°C to determine dry weight. After drying, the fish were digested at 90°C in 1·0 ml of concentrated HNO_3 and the digests were evaporated to dryness in the scintillation vials. ICN Ecolume scintillation fluid (20 ml) was then added to each vial and the radioactivity measured in a Beckman LS-9000 scintillation counter. The ^{45}Ca concentration was expressed as CPM/mg dry weight of fish after correction for radioactive decay and quench.

Statistical significance of the effects of copper (pCu) and time of exposure on calcium accumulation was tested using two-way analysis of variance (ANOVA) and regression analysis.

RESULTS

The accumulation of calcium by juvenile flounder was affected by cupric ion activity and time exposure (Fig. 1). Cupric ion activity had only a slight effect on calcium accumulation during the first 3 days but by day 7 a dose-dependent trend was apparent. This trend persisted and was most obvious by day 14. Regression analysis of the data set showed that both pCu and time of exposure exerted a significant effect ($p < 0{\cdot}05$) on the accumulation of ^{45}Ca by flounder.

Instantaneous rates of calcium influx on the three sampling days were

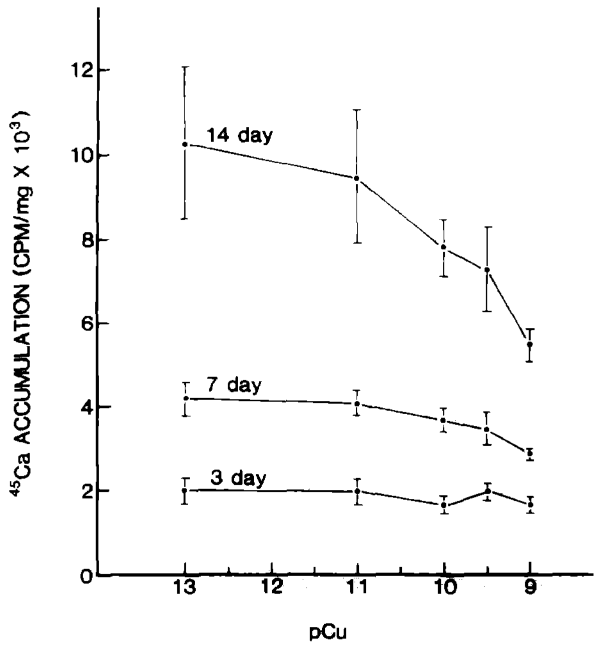

Fig. 1. The concentration of ^{45}Ca in juvenile flounder on each of the four sampling days as a function of the pCu of the medium. All radioactivity measurements are expressed as counts per minute (CPM) per mg dry weight. Each point represents a mean of five fish and the vertical lines represent ± 1 SE. pCu is the negative log of the cupric ion activity, and is analogous to pH. Therefore, a higher pCu has a lower cupric ion activity.

TABLE 1

Effect of pCu (−Log Cupric Ion Activity) on the Rate of Accumulation of Calcium by Juvenile Flounder as a Function of Time of Exposure. Accumulation Rates Expressed as μM Ca/g Dry Weight/h (Mean ± SE)[a]

pCu	*Exposure time (days)* 3	7	14
13·0	1·22 ± 0·19 ($n = 5$)	1·0 ± 0·13 ($n = 5$)	1·40 ± 0·3 ($n = 3$)
11·0	1·27 ± 0·22 ($n = 5$)	1·09 ± 0·09 ($n = 5$)	1·27 ± 0·3 ($n = 4$)
10·0	1·11 ± 0·12 ($n = 5$)	1·03 ± 0·10 ($n = 5$)	1·02 ± 0·10 ($n = 5$)
9·5	1·09 ± 0·20 ($n = 5$)	0·93 ± 0·12 ($n = 5$)	0·97 ± 0·16 ($n = 4$)
9·0	1·02 ± 0·10 ($n = 5$)	0·78 ± 0·03 ($n = 5$)	0·74 ± 0·06 ($n = 2$)

[a] $-Jin = Cwb(SAw \times W \times t) - 1$, [$Jin = Ca^{2+}$ influx; Cwb = whole body radioactivity in μCi; SAw = specific activity of the water; W = dry weight in grams; t = time in hours of exposure] (Reid & McDonald, 1988).

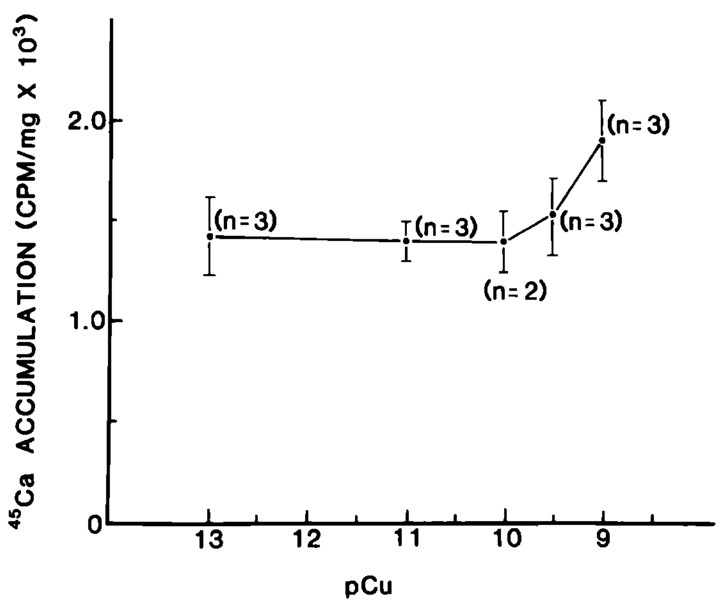

Fig. 2. Concentrations of ^{45}Ca in juvenile flounder exposed to different pCu for 14 days and then removed from the trace metal buffers and exposed to radioactive calcium in filtered seawater for 7 days. *n* represents the number of surviving flounder that were used in these determinations.

calculated for the juvenile flounder exposed to different cupric ion activities (Table 1) using the relation of Reid & McDonald (1988). There was an inverse correlation between the rates of calcium influx and cupric ion activity for each of the sampling days. A two-way ANOVA showed that only pCu was significant ($p < 0.05$) and that there was no signficant day × pCu interaction in this data set. While the instantaneous rates were calculated on specific days, they represent the integrated accumulation of calcium up to that sampling day.

Flounders exposed to cupric ion buffered media for 14 days reacted to the removal of copper from the water with increased accumulation of ^{45}Ca at the two highest cupric ion activities (Fig. 2). These experiments showed considerable variability but the results indicate that copper inhibition of calcium accumulation was alleviated by the removal of copper from the medium.

DISCUSSION

Investigations of trace metal effects on marine and estuarine organisms are for the most part concerned with mortality, but subtle, non-lethal, metal-induced changes in the physiology of fish can have an impact at the population level. Pathological conditions, such as the 'broken-back' syndrome and scoliosis, have been correlated with degraded environmental quality. Such conditions could result from interferences in normal calcium uptake, metabolism and bone formation. Disrupted calcium uptake and metabolism could be used, therefore, as an indicator of pollution stress, particularly in larval and juvenile fish.

During the early life stages of fish, bone formation and calcification are critical for production of a stable skeletal system to anchor the developing musculature. Since the skeleton acts as the base of muscle attachment, it is necessary that the bones be adequately calcified or they will break or deform under the pressure of muscular activity. In the literature on fish diseases there are numerous references to conditions known as the 'broken-back' syndrome and scoliosis (Sindermann, 1979). The etiology of many of these deformities are not known, but their occurrence, in some cases, are correlated positively with degraded water quality and industrial or sewage inputs (Hansson *et al.*, 1984; Bengtsson *et al.*, 1988). Laboratory investigations have also shown that pathological conditions characteristic of scoliosis or the broken-back syndrome are produced by toxicants such as cadmium (Muramoto, 1981), Kepone (Couch *et al.*, 1977; Stehlik & Merriner, 1983), and trifluralin (Couch *et al.*, 1979). These data suggest that some toxic substances interfere with normal calcium metabolism in fish and cause anomalies of the vertebral column, such as stunting of growth and scoliosis.

Our data on juvenile flounder indicates that copper suppresses the accumulation of calcium by these rapidly growing fish. Since the experiments reported here were of relatively short duration, the fish did not have adequate time to develop observable pathological changes. For example, Muramoto (1981) exposed fish to elevated cadmium concentrations for more than 80 days to produce vertebral damage, while our longest exposure was only 14 days. Longer exposures might also allow for examination of skeletal development and determination of whether correlations exist between calcium accumulation and skeletal integrity. Such information would give added insights into the etiology of scoliosis in fish.

An important finding in our experiments was that inhibition of calcium accumulation could be reversed by removing excess copper from the water (Fig. 2). Also, the increased ^{45}Ca uptake was shown only at the two highest cupric ion activities. Similar results also were shown in preliminary experiments where juvenile flounder were exposed to dissolved copper in unbuffered seawater for 28 days. Since calcium accumulation and metabolism is highly regulated, the increased accumulation of calcium following copper exposure may represent an attempt by the flounder to compensate for a calcium deficit incurred during the exposure to copper.

The lack of change in calcium influx rates with time (Table 1) and the reversible nature of copper inhibition (Fig. 2), suggest that copper acts on calcium accumulation at the animal/water interface. Flounder of this age do not have well formed scales, so both the gills and body epithelium could be affected by copper. The mechanism of copper poisoning was not measured directly, but the data suggest that copper blocks calcium transport. Sauer & Watabe (1984) demonstrated reductions in calcium uptake after exposing *Fundulus heteroclitus* to cadmium and zinc. They suggested that the chloride cells in the gills might be affected by the metals which in turn affected calcium accumulation. Similar effects have also been shown in freshwater fish (Payan *et al.*, 1981).

Our experiments have demonstrated responses of flounder at copper exposure levels far below those that are lethal. In our laboratory we have determined the LC_{50} for total dissolved copper at 14 days to be 7×10^{-6} M in unbuffered dock seawater for flounder of the same age as used in the present experiments (Engel, unpublished data). This dissolved copper concentration is at least 10 times the measured chelation capacity in water from the Newport River estuary (Sunda *et al.*, 1984) and this translates to a pCu value of approximately 7·2 based on inorganic speciation of copper (Byrne & Miller, 1985). In our present experiments we demonstrated reduced calcium accumulation at cupric ion activities of $\leq 10^{-10}$ M which is approximately two to three orders of magnitude lower than the concentrations required to produce mortality.

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