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Effects of chronic copper exposure on the macrophage chemiluminescent response and gill histology in goldfish (*Carassius auratus* L.)

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The effects of chronic copper exposure on the macrophage chemiluminescent response and gill histology in goldfish (*Carassius auratus* L.) were determined. Fish were exposed to sublethal concentrations (30, 65, 100, and 175 ppb) of copper sulphate for 96 h. Pronephric (head kidney) cells were collected and were comprised on lymphocytes, thrombocytes, monocytes/macrophages neutrophils and eosinophils/basophils (cell types grouped due to difficulty in identification). There were no significant differences in population profiles of cells used for chemiluminescence studies after exposure to 100 ppb copper. Density gradient-separated cells were stimulated with zymosan, the luminol-enhanced chemiluminescence (CL) response was measured and the results were compared to non-copper exposed controls. There was a significant correlation between copper concentration and reduction in the time at which the peak CL response [time to peak (TTP) CL] occurred. In addition, there was a significant parabolic dose response curve for peak CL response, which suggests increased production of reactive oxygen intermediates by phagocytes from fish exposed to concentrations up to 100 ppb, followed by inhibition at 175 ppb. These results indicate that copper affects phagocyte function in a complex manner, stimulating or inhibiting production of reactive oxygen intermediates depending on copper concentration. Exposure to concentrations of copper also resulted in varying degrees of gill hyperplasia. The relative median severity score (a score assigned to each gill based upon the amount of hyperplasia seen in a histological section) correlated significantly with the copper concentration. In addition, there was a very high correlation between the degree of hyperplasia and the TTP CL response. Levels of copper in the head kidney of goldfish exposed to 100 ppb were similar to controls as measured by atomic absorption spectroscopy. Thus, changes in the CL response of head kidney phagocytes of fish exposed to copper may be caused indirectly, via effects on other immunoregulatory cells or other physiological mechanisms. We conclude that addition of heavy metals such as copper to water can modify the CL responses of phagocytes and alter the histopathology of the gills in *C. auratus.*

Key words: *Carassius auratus*, goldfish, non-specific immunity, copper, chemiluminescence, gill histology.

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I. Introduction

Toxicants can be lethal or can produce sub-lethal damage according to critical parameters such as dose and time of exposure. Heavy metals in the water, such as copper, can affect the immunological status of fish (Zelikoff, 1993). Exposure of aquatic organisms to sub-lethal copper concentrations has been shown to increase susceptibility to infectious disease (Stevens, 1977; Hetrick *et al.*, 1979), whereas others have reported conflicting results (Snarski, 1982; MacFarlane *et al.*, 1986). Copper exposure of fish also results in histopathological changes such as damage to gill lamellae, lateral line canals, chemoreceptors, liver, kidney and haematopoietic organs (Baker, 1969; Sorensen, 1991). The mechanism and specific effects on phagocyte function and gill histology are not well characterized.

The chemiluminescent response (CL) may be used to monitor immune system alterations after exposure to metals (Elsasser *et al.*, 1986). CL measures the respiratory burst activity of phagocytic cells in which oxygen is converted into reactive oxygen intermediates (ROIs). The ROIs can activate probes such as luminol, triggering the emission of photons which can then be measured photometrically. The production of ROIs as measured by CL is important in host defence since killing of micro-organisms is at least partially dependent on the production of ROIs such as H_2O_2 and O_2^- (Babior, 1984) in fish (Chung & Secombes, 1988) and $HOCl^-$ in mammals (Klebanoff, 1968). These reactions have been demonstrated in isolated fish macrophages (Zelikoff *et al.*, 1991), in neutrophils (Waterstrat *et al.*, 1991), and in cell free systems (Karczewski *et al.*, 1991).

Several studies have demonstrated dose-dependent modification in ROI formation as measured by the CL response of fish phagocytes, after *in vitro* and *in vivo* exposure to environmental pollutants (Anderson, 1993). Most studies have shown suppression at high doses, while others have shown slight activation at lower doses. *In vitro* studies have been used to evaluate the effects of tributyltin (TBT), a marine anti-fouling agent, on the CL response of fish macrophages. Pronephric phagocytes from several species of estuarine fish were exposed to TBT, and the CL response was found to be suppressed at high concentrations (40–400 ppb) (Wishkovsky *et al.*, 1989). Oyster toadfish (*Opsanus tau*) peritoneal macrophages exposed to lower concentrations of TBT (5 and 50 ppb) resulted in increased peak CL responses, while 500 ppb TBT caused the CL responses to be suppressed to baseline values (Rice & Weeks, 1989). *In vivo* exposure studies have revealed that oyster haemocytes (Larson *et al.*, 1989) and fish macrophages (Weeks & Warinner, 1984; Warinner *et al.*, 1988) exposed to environmental pollutants such as polynuclear aromatic hydrocarbons (PAH), pesticides and other organic chemicals, exhibit decreased phagocytic and CL responses. However, in one *in vitro* exposure study, macrophages from minnows, (*Fundulus heteroclitis*) exposed to PAH showed increased CL responses (Kelly-Reay & Weeks-Perkins, 1994).

Copper exposure has also been shown to decrease the CL response in aquatic organisms, depending on exposure time and dose. *In vitro* exposure of rainbow trout (*Oncorhynchus mykiss*) to copper (10 ppb) resulted in

significantly decreased CL activity (Elsasser *et al.*, 1986). *In vivo* exposure of oysters (*Crassostrea virginica*) to copper resulted in varying reductions in the haematocyte CL response (Larson *et al.*, 1989).

Copper exposure can also induce a stress response as shown by increased blood cortisol levels (Donaldson & Dye, 1975; Schreck & Lorz, 1978). Chronic stress has been theorized to increase susceptibility to infectious disease (Griffin, 1989). However, Carballo *et al.* (1992) showed that copper exposure and associated increased cortisol levels did not increase susceptibility to infectious disease. They suggested that copper may act directly on the immune system instead of via a stress-mediated mechanism. *In vitro* exposure of striped bass (*Morone saxatilis*) phagocytes to hydrocortisone (Stave & Roberson, 1985) and phagocytes from stressed fish (Angelidis *et al.*, 1987) demonstrated suppression of the CL response. CL responses in phagocytes from fish exposed to copper *in vivo* have not been measured prior to this study.

Therefore, these experiments were designed to test the immunological and histological effects of sub-lethal copper exposures on *C. auratus* after 96 h. Copper challenges were performed at varying levels below the LC_{50} (determined to be 175 ppb in our laboratory). Differential staining and enumeration of cells in pronephros homogenates was used to quantify possible changes in the sub-populations of leucocytes. The non-specific immune response of pronephrocytes was assessed using the CL assay. Copper levels were measured by atomic absorption spectroscopy in pronephros samples taken after 96 h of exposure. The degree of gill hyperplasia from fish exposed to varying levels of copper was quantified by assigning a severity score to each gill based upon the amount of hyperplasia seen in a histological section.

II. Materials and Methods

ANIMALS

C. auratus (7–25 cm, 10–60 g), obtained from Hunting Creek Fisheries, Thurmont, MD, were laboratory acclimated for a minimum of 3 weeks. The fish received trout grower diet (Ziegler Bros, Gardners, PA) daily and were maintained in recirculating 190 l fibreglass tanks (12 fish per tank), an average ambient temperature of 21°C and a 16 h light:8 h dark photoperiod. The water was passed through coarse fibre filters (Hayward Industrial Products, Elizabeth, NJ) and activated charcoal (Atochem, Pryor, OK). Test animals were not fed for 1 day prior to or during testing.

CHEMICAL EXPOSURE

Culpric sulphate (J. T. Baker Chemical Co., Phillipsburg, NJ) was dissolved in distilled water for stock concentrations before dilution to final concentrations of 30, 65, 100 and 175 ppb in freshly dechlorinated tap water. The concentrations used were 17, 37, 57 and 100% of the LC_{50} determined for *C. auratus* in our laboratory. The control tanks containing one to five fish per tank contained dechlorinated tap water only. Static exposure of one to five

fish per tank to 0–175 ppb copper with aeration was carried out for 96 h. Water copper concentrations were measured from each tank. These water samples were nitric acid-fixed, filtered and then analysed by atomic absorption spectroscopy.

TISSUE COLLECTION

Fish were killed by decapitation. Head kidneys were removed using sterile forceps and placed in sterile 15 ml glass tissue grinders. Gills from control fish and fish exposed to 100 ppb were removed and fixed in 10% neutral buffered formalin, dehydrated in graded alcohol, embedded in paraffin and sectioned at 6 μm before staining with haematoxylin and eosin.

PRONEPHRIC CELL COLLECTION

Head kidney cells were disrupted in a 15 ml Wheaton tissue grinder (VWR Scientific, Bridgeport, NJ) using approximately 25 strokes in a working solution of Hank's Balanced Salt Solution (wHBSS) without phenol red containing heat inactivated (37°C for 30 min) 10% foetal bovine serum and antibiotics (10 000 U penicillin and 10 000 μg streptomycin ml^{-1}, GIBCO, Gaithersburg, MD). In order to separate leucocytes from red blood cells (RBCs) and other cell debris, cell suspensions were placed on a 3 ml Histopaque gradient (Sigma Chemical Co., St Louis, MO, d=1·077 g cm^{-3}) in 15 ml polypropylene sterile centrifuge tubes, and were centrifuged at 1500 rpm for 20 min at 10°C. The buffy coat was removed from the wHBSS–Histopaque interface and washed twice by centrifugation in wHBSS (5 min at 1000 rpm). Cells were counted using a haemacytometer, and viability was determined to be greater than 95% using the trypan blue exclusion test. Cells were resuspended in wHBSS at a concentration of 5×10^5 cells ml^{-1}.

CHEMILUMINESCENCE ASSAY

The oxidative burst produced by stimulated macrophages was quantified using an ambient temperature scintillation counter (Beckmann LS-7800, Beckmann Products, Palo Alto, CA) in the out-of-coincidence mode using a single photon monitor. Luminol (5-amino-2,3-dihydro-1,4-pathalazinedione, Sigma Chemical Co., St Louis, MO) was used to amplify the CL response of phagocytic cells and was prepared according to the method described by Scott & Klesius (1981). Stock luminol solution containing 0·78 g KOH, 0·618 g boric acid, 0·014 g luminol in 10 ml distilled water (stored for 3 days maximum at 4°C) was diluted 1:1000 in wHBSS for use. Each test vial contained 0·5 ml luminol, 0·5 ml cells in wHBSS and 0·5 ml zymosan (Sigma Chemical Co., St Louis, MO) as the stimulus (1 mg ml^{-1} in wHBSS). Blank vials contained cells and luminol, but no zymosan. Basal CL was monitored by counting the blank vials and remained consistent (between $3–5 \times 10^4$ cpm) throughout the assay. Vials were immediately placed in the counter after addition of cells and reagents, and measurements were made at 4 min intervals for 90 min or until a significant decline in peak CL occurred. All solutions and materials were kept in the dark as much as possible. CL assays were

performed in duplicate and the average of replicate assays was used in subsequent evaluations.

CYTOCENTRIFUGE PREPARATIONS OF THE HEAD KIDNEY

Pronephric cell suspensions were collected from fish exposed to 0 or 100 ppb copper for 96 h. Suspensions were separated on Histopaque gradients as described above. These suspensions were cytocentrifuged (Shandon Elliot, Pittsburg, PA) onto glass microscope slides for 10 min at 2000 rpm. Slides were air-dried, fixed in methanol, stained with Wright-Giemsa (VoluSol, IDE Interstate, Amityville, NY), and various types of cells were counted. Cell types, such as eosinophils and basophils as well as lymphocytes and thrombocytes were grouped together due to the difficulty in differentiating between these cells.

TISSUE DETERMINATION OF COPPER CONCENTRATION

Fish were exposed to 0 and 100 ppb copper in 190 l polypropylene tanks. Fish were killed at 96 h; the whole head kidney and a portion of liver were removed using nitric acid-washed instruments. Each tissue was weighed (wet weight) and placed in an acid-washed 50 ml beaker. Accuracy of the measurements was assessed by using reference material, i.e. lyophilized bovine liver no. 1577 from the U.S. National Bureau of Standards (Gaithersburg, MD). Concentrated HNO_3 (10 ml) was added to each sample, and allowed to digest at room temperature for 24 h. The samples were then heated slowly until just boiling, cooled, and filtered through acid washed glass wool. Digested samples were diluted as necessary with nitric acid and analysed for copper using a flame atomic absorption spectrometer (Perkin Elmer 5000, Norwalk, CT). Copper contents in the tissues were calculated by multiplying the concentration of copper measured in digested tissue samples by the total volume of the digested tissue divided by the total weight of the tissue. This value yielded copper tissue concentrations in $\mu g\ g^{-1}$, or ppm.

GILL HISTOPATHOLOGY

Gills were evaluated for hyperplasia by grading on a scale of 1–4, with 1 indicating no hyperplasia, 2=minimal hyperplasia, 3=severe hyperplasia, in which doublets are often apparent (two fused lamellae), and 4=complete fusion of secondary lamellae.

STATISTICS

Where assumptions of normality and homogeneity of variance were valid, Student's *t*-test and analysis of variance linear and quadratic components were used for assessing statistical significance (SAS Institute, Cary, NC). Otherwise, non-parametric tests were performed using SAS or Instat (Graphpad Software, Inc., San Diego, CA) softwares. Regression analyses were performed to determine closeness of fit to a linear model. Correlation

Table 1. Differential cell counts of head kidney cell suspensions separated on density gradients, prepared after 96 h exposure of *Carassius auratus* to 0 and 100 ppb copper sulphate

	Copper sulphate ppb	
Cell type	0	100
Lymphocytes/thrombocytes	27·2 (4·2)*	40·2 (9·0)
Monocytes/macrophages	29·6 (1·4)	25·4 (6·2)
Neutrophils	30·4 (4·0)	23·2 (5·8)
Eosinophils/basophils	2·6 (0·4)	3·3 (1·0)
Blast cells	10·3 (1·4)	7·9 (1·8)

*Mean percentage of 400 cells from six control fish and five treated fish (±S.E.M.). There were no significant differences between these groups using Student's *t*-test or Wilcoxon rank sum test.

analysis was used to determine the strength of the relationship between gill histopathology score and time to peak (TTP).

III. Results

DIFFERENTIAL STAINING OF HEAD KIDNEY CELLS (PRONEPHROCYTES)

Analysis of preparations from whole head kidney homogenates revealed a variety of blood cell types. The terminology used to describe pronephrocytes is based on their morphological resemblance to similar cells found in higher vertebrates. Thus, cells analogous to either lymphocytes or platelets (thrombocytes in lower vertebrates), monocytes or macrophages, neutrophils, eosinophils or basophils, as well as blast cells, were identified. The percentages of the total leucocyte population in the density gradient separated preparations are summarized in Table 1. No significant changes in differential leucocyte counts were observed between control fish and fish exposed to 100 ppb copper sulphate.

ALTERATION OF CL RESPONSE

The maximal (peak) CL response (PCL), and the TTP CL were measured. Significant differences among the TTP means were found ($P<0{\cdot}0001$, least squares means ANOVA). Mean TTP values for 100 and 175 ppb Cu were significantly ($P<0{\cdot}0002$ and $P<0{\cdot}0001$) shorter than the TTP of pronephrocytes from the control fish (Fig. 1). The TTP values for 30 and 65 ppb copper were intermediate between the controls and the highest copper levels tested; however, the differences were not statistically significant. The negative correlation between TTP and copper concentration was demonstrated by linear regression analysis (Fig. 2), $r^2=0{\cdot}706$. The mean PCL showed a tendency to increase with copper concentrations up to 100 ppb, but PCL was apparently inhibited by 175 ppb (LC_{50}). A significant ($P<0{\cdot}05$) dose response curve was calculated for PCL and copper concentration. This curve is parabolic, and is pictured in Fig. 3.

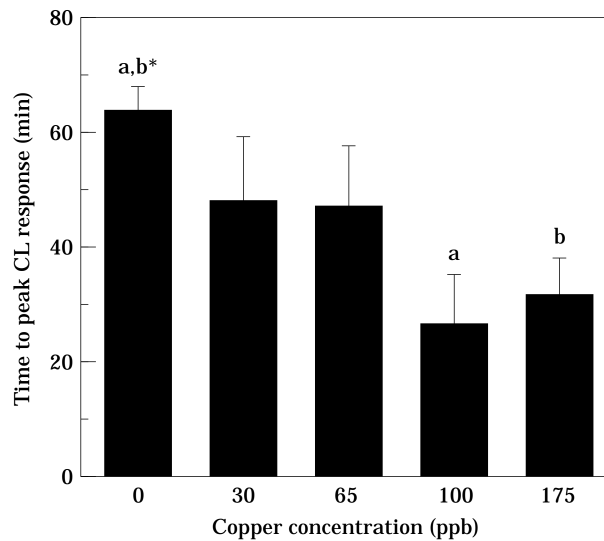

Fig. 1. Time to peak chemiluminescence response (least squares means ± S.E.). *Similar letters denote significant differences between means ($P<0{\cdot}0001$). CL, luminol-enhanced chemiluminescence.

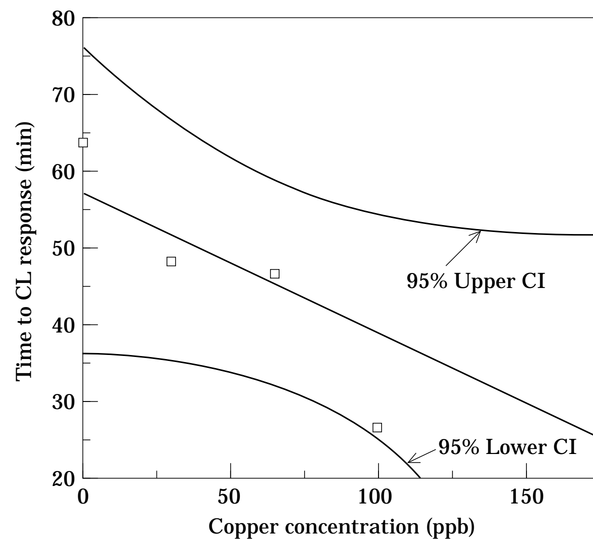

Fig. 2. Time to peak luminol-enhanced chemiluminescence (CL) response and copper dose. Original means and least-squares linear regression analysis (with 95% confidence intervals, CI). $r^2=0{\cdot}706$. (⊡), Actual means; (—), Linear regression.

GILL HISTOPATHOLOGY

Distinct, regular secondary lamellae were seen in control gills (Fig. 4). *In vivo* copper exposure (100 ppb for 96 h) resulted in hyperplasia of the secondary lamellar epithelium (Fig. 5) with a decrease in exposed respiratory epithelium surface.

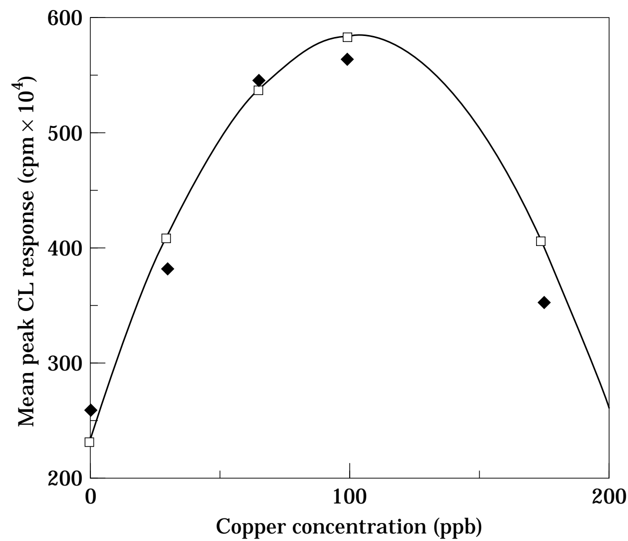

Fig. 3. Mean peak chemiluminescence response (polynomial dose response curve, $P<0.05$). $y=230.20+6.9154\ x-3.378\ \mathrm{Se}-2x^2$. (⊡), Calculated; (◆), actual.

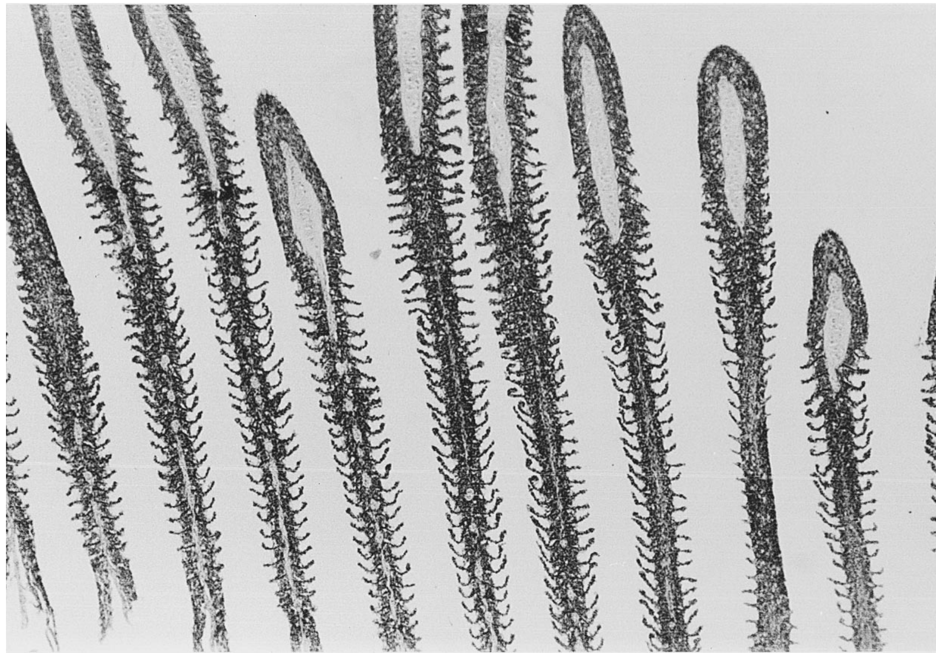

Fig. 4. Histopathology of *Carassius auratus* gills—a typical control. Hyperplasia score=1. (H&E × 16).

The degree of gill hyperplasia in *C. auratus* exposed to varying concentrations of copper was assessed by grading on a scale of 1–4. Significant differences in gill hyperplasia scores were seen in exposures up to 175 ppb ($P<0.0002$). Multiple comparison tests yielded significant differences between

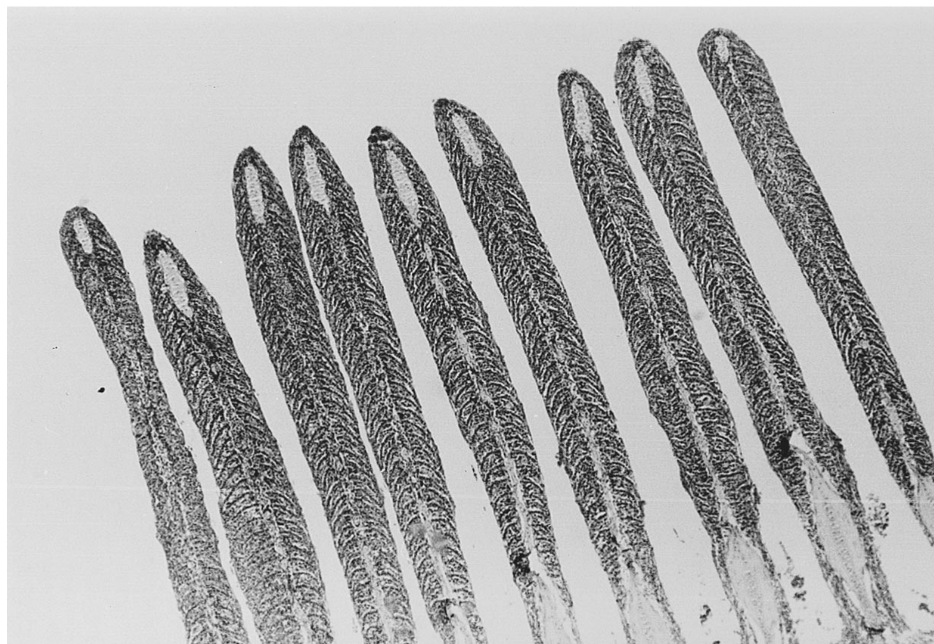

Fig. 5. Histopathology of *Carassius auratus* gills after 96 h exposure to 100 ppb copper sulphate. Hyperplasia score=4. (H&E × 16).

0 and 100 ppb copper ($P<0{\cdot}05$), and 0 and 175 ppb copper ($P<0{\cdot}01$) for gill hyperplasia (Fig. 6). No significant effects were seen at the lower copper concentrations (30 and 65 ppb). There was also a good linear correlation between copper dose and gill hyperplasia ($r^2=0{\cdot}7855$, $P<0{\cdot}05$).

GILL HISTOLOGY AND CL RESPONSE

Variance analysis indicated a significant difference between median gill hyperplasia scores for TTP CL response ($P<0{\cdot}05$, data not shown). When median gill hyperplasia scores were correlated with mean TTP, an $r=0{\cdot}99$ was determined.

COPPER CONCENTRATION IN FISH TISSUE

Digestion of between 0·5–1·0 g samples of head kidney and liver and subsequent analysis by atomic absorption spectroscopy revealed no significant difference between copper concentration in tissues from control and 100 ppb copper-treated fish (Table 2).

IV. Discussion

The phagocyte respiratory burst activity has been studied in aquatic species following xenobiotic exposure (Warinner *et al.*, 1988; Zelikoff *et al.*, 1991; Sharp & Secombes, 1993; Kelly-Reay & Weeks-Perkins, 1994). CL is often used to measure respiratory burst activity (Thomas *et al.*, 1988); changes in CL

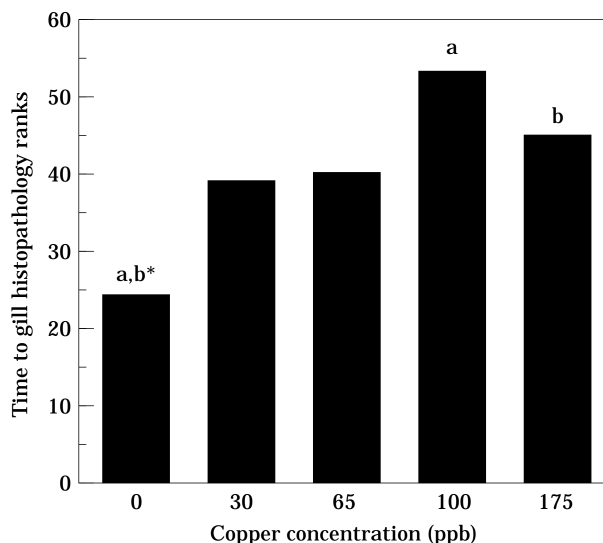

Fig. 6. Gill hyperplasia (mean rank score by Kruskal-Wallis non-parameteric test). *Similar letters denote significant differences ($P<0.05$, Dunn's multiple comparison test).

Table 2. Atomic absorption spectroscopy analysis of pooled tissues from *Carassius auratus* after 96 h *in vivo* exposure to 100 ppb copper sulphate (Cu)

Tissue	Treatment	Fish group	Sample size (g)	Cu concentration (ppm)*	Mean Cu concentration (ppm)†
Pronephros	Control	1	0·520	2·04	
		2	0·497	1·61	1·83
Pronephros	Copper	3	0·654	1·93	
		4	0·601	1·57	1·75
Liver	Control	1	0·679	17·5	
		2	0·806	23·4	20·45
Liver	Copper	3	0·818	21·4	
		4	1·040	24·8	23·1

*Each copper concentration (ppm) is the mean of 12 pooled fish. Average weight of head kidney per fish was 0·054 g for copper exposed and 0·043 g for control fish.

†Mean copper concentration values are expressed as mean of averages obtained from the two groups of fish. Results of mean copper concentrations in pronephros and liver from copper-exposed fish were not significantly different from non-exposed ($P<0.05$, Student's *t*-test).

response induced by xenobiotic exposure (increased or decreased time to peak response, peak CL response, and total CL response) have also been demonstrated (Tam & Hindsdill, 1990). CL was used here to measure altered phagocyte responses of *C. auratus* after *in vivo* copper exposure.

The data presented in this study show that the relationship between peak CL response (PCL) and copper dose can be described by a parabolic curve. An increase in peak CL response was seen at concentrations up to 100 ppb copper;

however, at a higher dose (175 ppb) the CL response was inhibited. Increased ROI production and concomitant increased peak CL response are associated with phagocyte activation in mammals (Johnston, 1981) and in fish (Chung & Secombes, 1987). Thus, phagocytic cells recovered after *in vivo* copper exposure to the lower concentrations may show metabolic changes characteristic of primed or activated macrophages.

In the present study, a dose-dependent increase TTP CL was observed following exposure to increasing copper concentrations. Significant differences in copper concentrations and TTP were found between 0 and 100, and 0 and 175 ppb copper. Differences in time courses for O_2^- production or CL response have been observed in human neutrophils and are linked directly to activation of the respiratory burst enzyme NADPH oxidase (McPhail & Snyderman, 1983). Possibly, copper exposure affected the assembly or activation of NADPH oxidase in the *C. auratus* phagocytes.

The lowest copper concentration found to result in changes in TTP and PCL (100 ppb) was then used to evaluate population profiles of cells and head kidney copper concentrations. No significant differences in copper concentrations in the head kidney were observed between control and 100 ppb treated fish (Table 2). The lack of a significant accumulation of copper in the head kidney of exposed fish leads to the conclusion that copper is probably not directly affecting the pronephric cells. In addition, there was no significant difference in population profiles of cells used for chemiluminescence studies after exposure to 100 ppb copper (Table 1).

In the present experiment, copper exposures of 30, 65, 100 and 175 ppb induced varying degrees of epithelial cell hyperplasia. Along with the hyperplasia, chronic inflammation of the gills of goldfish was sometimes observed. Correlation of the hyperplastic gill response with the copper dose was significant, particularly at doses of 100 and 175 ppb. Since statistical results were obtained for TTP data at the same copper concentrations, correlations between gill hyperplasia and TTP data were investigated. We found a significant correlation between hyperplasia and mean TTP CL response, which indicates that the gill histopathology and the CL response may be interrelated. Physiological effects of gill hyperplasia may be related to hypoxia. Hypoxic changes may result in an indirect impact on the immune response (Kirk, 1974). Gill hyperplasia following copper exposure may also result in an inflammatory response in goldfish gills (Reimscheussel *et al.*, 1991). Infiltrating lymphocytes may release cytokines which in turn can activate mononuclear phagocytes and directly induce synthesis of the enzymes that mediate the respiratory burst (Abbas *et al.*, 1991).

In vivo copper exposures of aquatic organisms have shown apparently contradictory effects on the immune parameters. Although more evidence is shown for immunosuppression as indicated by decreased antibody titres (Stevens, 1977; O'Neill, 1981; Khangarot *et al.*, 1988), no changes in immune parameters such as mitogen response and total immunoglobulin level have also been observed (Viale & Calamari, 1984; Carballo *et al.*, 1992). Enhancement of the non-specific immune system, as evidenced by increased phagocytosis, has also been reported following *in vitro* exposure to copper (Cheng & Sullivan, 1984; Pickwell & Steinert, 1984). These results may vary depending

on dose and higher infectivity rates to *Saprolegnia* have been noted following acute copper exposures (Carballo *et al.*, 1992). Our results indicate that chronic exposure of *C. auratus* at concentrations up to 100 ppb copper results in temporally accelerated and quantitatively greater ROI responses, apparently due to the activation of phagocytic cells, indicating an enhancement of immune status. The impact of xenobiotic-induced immunomodulation such as this on the ultimate health of the fish has yet to be determined. However, the physiological changes described here probably affect the ability of the fish to resist infection.

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