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Susceptibility of chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), and rainbow trout, *Salmo gairdneri* Richardson, to infection with *Vibrio anguillarum* following sublethal copper exposure*

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Abstract. Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), and rainbow trout, *Salmo gairdneri* Richardson, were used to determine if sublethal copper exposure would increase their susceptibility to *Vibrio anguillarum* infection. Fish were pretreated with copper at fractional levels of the 96 h copper LC_{50} before exposure to the pathogen. Mortality by vibriosis was greater among fish exposed to 9% of the copper LC_{50} for 96 h than unexposed fish. Peak susceptibility to vibriosis depended in part on the interaction of exposure time and copper concentration. The higher copper concentrations produced peak susceptibility to infection in shorter time periods. After the peak of susceptibility, sensitivity to infection declined to near control levels in those fish where exposure was continued. Rainbow trout stressed by copper required about 50% fewer pathogens to induce a fatal infection than non-copper exposed fish.

Introduction

Pollutants have been implicated in outbreaks of disease in fish populations either by reactivation of carrier states or by predisposition to infection by common waterborne pathogens (Snieszko 1974; Wedemeyer, Meyer & Smith 1976; Rodsaether, Olafsen, Rea, Myhre & Steen 1977). Much of this evidence is circumstantial due to the time lag between initial contact with the stressor and the onset of the first mortalities due to the disease organism. Recently, controlled laboratory experiments have been used to more precisely demonstrate this interaction. Hetrick, Knittel & Fryer (1979) exposed rainbow trout, *Salmo gairdneri* Richardson, to infectious haematopoietic necrosis virus following sublethal copper exposure and demonstrated a significant increase in susceptibility to infection. Coho salmon, *Oncorhynchus kisutch* (Walbaum), exposed to sublethal chromium concentrations became more susceptible to subcutaneously injected *Vibrio anguillarum* (Sugatt 1980). Knittel (1981) has also demonstrated a decreased disease resistance to a low virulence pathogen, *Yersinia ruckeri*, when steelhead trout, *Salmo*

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gairdneri, were pre-exposed to copper. Our research demonstrates that stressful levels of copper can also promote infection by a virulent strain of *V. anguillarum* in rainbow trout and chinook salmon, *Oncorhynchus tshawytscha* (Walbaum).

Materials and methods

Fish

Chinook salmon and rainbow trout were obtained as 'eyed' eggs from the Willamette River Hatchery of the Oregon Department of Fish and Wildlife. Upon arrival at Western Fish Toxicology Station, Corvallis, Oregon, the eggs were sanitized and reared in a manner similar to that described by Knittel (1981).

Water chemistry

Water hardness varied in our well water supply during the rainy season (Samuelson 1976) which was found to affect the toxicity of copper (Chapman & McCrady 1977). For this reason, copper concentrations were tested as percentages of the 96 h LC_{50} for each fish species. Copper concentrations were expressed as toxic units, one toxic unit (TU) being equal to the 96 h LC_{50} concentration of the fish species studied. The concept of TU is described in detail by Sprague & Ramsey (1965). Total hardness, alkalinity and pH were determined daily during each experiment.

Toxicant exposure system

A proportional flow-through toxicant diluter (Garton 1980) was used to distribute the desired concentrations of copper to the aquaria at 1·0 to 1·5 l/min/aquarium. Copper stock solutions were prepared and were analysed as in Chapman (1978). The copper concentrations chosen were sublethal over a 96 h exposure. Test fish were acclimatized to 16°C water for a minimum of 96 h prior to the start of copper exposure. During this time, fish were fed Oregon moist pellets *ad libitum* once daily and were fasted 24–48 h before start of copper exposure. Photoperiod simulated the local day–night conditions.

The proportional diluter was later modified to supply a single concentration of copper to the aquaria to allow testing of the effect of the duration of copper exposure on susceptibility to infection and the effect of bacterial concentration.

Infection of fish

The strain of *V. anguillarum* type I (LS-174), was originally isolated from an epizootic of fall chinook salmon at Lint Slough, Waldport, Oregon. The bacterium was isolated by Dr J. S. Rohovec and obtained from Oregon State University Department of Microbiology, Corvallis, Oregon. Lyophilized cultures were prepared and stored at −10°C until needed. Cultures were grown from the lyophilized cells in sterile trypticase soy

broth (TSB) at 18°C for 24 h, diluted with TSB to an optical density of 0·90 at 525 nm, and a volume added to the aquaria water to give a final concentration of $1{\cdot}5 \times 10^5$ cells/ml. Static conditions were maintained for 1 h (with aeration) and water flow (at one l/min) resumed. This method of infection was similar to that reported by Gould, O' Leary, Garrison, Rohovec & Fryer (1978). Mortalities were collected and recorded daily until the seventh day after exposure to the bacterium.

Necropsy

Dead fish were swabbed with 10% (v/v) iodophor and kidney tissue aseptically removed and streaked on to trypticase soy agar (TSA) plates. The presumptive evidence of *V. anguillarum* infection was whether growth was inhibited by novobiocin and vibriostatic agent 0/129 (2,4-diamino-6,7-di-isopropyl pteridine) and characteristic colony morphology. Twenty per cent of presumptive isolates were also tested for cytochrome oxidase,

Table 1. Replicate experiments determining change in susceptibility of chinook salmon to *Vibrio anguillarum* infection following a 96 h exposure to a range of sublethal copper concentrations

Copper concentration (toxic units*)†	Number of deaths‡ due to vibriosis / Number of fish surviving copper exposure	Percentage mortality due to vibrio
Experiment I		
0·56	8/25	32·0
0·34	10/24§	41·7
0·20	15/25	60·0
0·09	10/25	40·0
0·00 *Vibrio* only	7/25	28·0
Experiment II		
0·52	7/25	28·0
0·33	8/24§	33·3
0·20	8/25	32·0
0·15	12/24§	50·0
0·08	10/25	40·0
0·00 *Vibrio* only	8/25	32·0

* Toxic unit (TU) defined as 1·0 TU is equal to the 96 h LC_{50} of copper ($CuCl_2.2H_2O$) to chinook salmon. Experiment I, 1·0 TU is equal to 38 μg/l; experiment II, 1·0 TU is equal to 40 μg/l.

† Water chemistry: experiment I, mean hardness 23·5 ± 0·71 mg/l; mean alkalinity 25·0 ± 1·41 mg/l; pH range 7·01 to 7·09. Experiment II, mean hardness 26·3 ± 1·15 mg/l; mean alkalinity 26·7 ± 1·15 mg/l; pH range 7·00 to 7·25.

‡ Mean weight of test fish: experiment I, 9·9 ± 2·91 g; experiment II, 20·2 ± 4·86 g.

§ All aquaria contained 25 fish at the beginning of the experiment. Reduced numbers reflect fish unaccounted for at the end of the test.

motility, Gram stain and rapid slide agglutination with specific *V. anguillarum* antiserum. Only those fish from which *V. anguillarum* was isolated were considered to have died of vibriosis.

Results

Copper concentration was not linearly related to the incidence of mortality by *V. anguillarum* (Table 1). Maximum susceptibility to vibriosis in chinook salmon occurred after 96 h exposure to concentrations of copper ranging from 8–20% of the 96 h LC_{50} value (0·08–0·20 TU). The difference in mean weight of chinook in replicate experiments had no apparent effect on survival of fish to copper or *Vibrio* exposure. Rainbow trout showed a similar peak of susceptibility (Table 2), but at copper concentrations of

Table 2. Replicate experiments determining change in susceptibility of rainbow trout to *Vibrio anguillarum* infection following a 96 h exposure to a range of sub-lethal copper concentrations

Copper concentration (toxic units*)†	Number of deaths‡§ due to vibriosis / Number of fish surviving copper exposure	Percentage mortality due to vibrio
Experiment I		
0·80	9/18	50·0
0·62	15/21	71·4
0·43	14/21	66·7
0·33	18/24	75·0
0·18	20/24	83·3
0·00 *Vibrio* only	14/25	56·0
Experiment II		
0·98	4/21	19·0
0·69	8/24	33·3
0·50	5/22	22·7
0·31	19/24	79·2
0·15	4/25	16·0
0·00 *Vibrio* only	5/24	20·8

* Toxic unit (TU) defined as 1·0 TU is equal to the 96 h LC_{50} of copper ($CuCl_2.2H_2O$) to rainbow trout. Experiment I, 1·0 TU is equal to 30 μg/l; experiment II, 1·0 TU is equal to 26 μg/l.

† Water chemistry: experiment I, mean hardness 49·0 ± 3·16 mg/l; mean alkalinity 43·0 ± 2·00 mg/l; pH range 6·83 to 7·01; experiment II, mean hardness 36·6 ± 5·50 mg/l; mean alkalinity 32·2 ± 4·44 mg/l; pH range 6·80 to 7·10.

‡ Mean weight of test fish: experiment I, 15·1 ± 5·8 g; experiment II, 17·9 ± 3·95 g.

§ All aquaria contained 25 fish prior to copper exposure and deviations from this reflect deaths during copper exposure.

18–31% of their 96 h LC_{50} value. Logistic regression analysis* of data from Tables 1 and 2 revealed that both species of fish reach peak susceptibility to *Vibrio* infection at 9% of their 96 h copper LC_{50} (0·09 TU), even though the acutely lethal concentration of copper was different for each species of fish. Fish which were first exposed to copper, then *V. anguillarum*, were observed to die more rapidly than those only exposed to the bacterium; however, these values were not statistically analysed.

Table 3. Influence of time of exposure to 0·8 TU of copper on susceptibility of rainbow trout to infection by *Vibrio anguillarum*

Hours of copper exposure	Copper concentration (toxic units*)	Number of deaths†‡§ due to vibriosis / Number of fish surviving copper exposure	Percentage mortality due to vibrio
192	0·76	8/10	80·0¶
96	0·77	11/25	44·0
48	0·80	16/24	66·7‖
24	0·83	18/24	75·0¶
0 *Vibrio* only	0·0	10/25	40·0

* Toxic unit defined as 1·0 TU is equal to the 96 h LC_{50} of copper ($CuCl_2 \cdot 2H_2O$) to rainbow trout. 1·0 TU is equal to 25 μg/l copper.

† All aquaria contained 25 fish prior to copper exposure and deviations from this reflect deaths during the copper exposure.

‡ Water chemistry: mean hardness, 36·1 ± 1·46 mg/l; mean alkalinity, 28·2 ± 2·22 mg/l; pH range 6·91 to 7·05.

§ Mean weight of test fish: 20·2 ± 4·30 g.

¶ Significantly higher than control $P = 0{\cdot}05$.

‖ Significantly higher than control $P = 0{\cdot}10$.

The effect of exposure time on susceptibility to vibriosis was studied. Rainbow trout treated with about 0·8 TU of copper for up to 192 h became significantly more susceptible to vibriosis after 24 and 48 h of copper exposure than their controls (Table 3). Fish treated with copper for 48 h had slightly less mortality than those treated for 24 h. Fish exposed for 96 h to about 0·8 TU showed no more susceptibility to vibriosis than non-copper control groups. Fish exposed to copper for longer than 96 h were more susceptible to infection; however, this may have been an artefact because the sample size (10) of fish which survived the copper exposure was small.

* The computed equation which best describes the data is:

$$\text{Logit } Y = B_0 + 1{\cdot}883\ (\log Cu \text{ conc.}) - 0{\cdot}9581\ (\log Cu \text{ conc.}^2).$$

where: $\text{Logit } Y = \ln \dfrac{\%\text{ mortality}}{100 - \%\text{ mortality}}$.

B_0 = The Y intercept of the group receiving only *Vibrio*, no copper.
Constants = description of the curvature of four parallel lines, one for each replicate.
Equation was statistically significant at $P = 0{\cdot}005$.

Fish exposed to about 0·2 TU copper showed increased infection at 24 to 96 h (Table 4); however, fish exposed longer (192 h), were no more susceptible than the control fish. Statistical analysis of these data revealed no significant difference between treatment groups. This was probably due to the high level of vibriosis in non-copper exposed fish (72·7%). These data are presented because they show a trend consistent with the other experiments presented in this paper.

Table 4. Influence of time of exposure to 0·2 TU of copper on susceptibility of rainbow trout to infection by *Vibrio anguillarum*

Hours of copper exposure	Copper concentration (toxic units*)	Number of deaths†‡§ due to vibriosis / Number of fish surviving copper exposure	Percentage mortality due to vibrio
192	0·20	30/45¶	66·7
96	0·21	19/22	86·4
48	0·21	19/21	90·5
24	0·20	19/22	86·4
0 *Vibrio* only	0·0	16/22	72·7

* Toxic unit defined as 1·0 TU is equal to the 96 h LC_{50} of copper ($CuCl_2 \cdot 2H_2O$) to rainbow trout. 1·0 TU is equal to 21 μg/l copper.

† All aquaria contained 25 fish prior to copper exposure and deviations from this reflect deaths during the copper exposure.

‡ Water chemistry: mean hardness, 29·1 ± 0·93 mg/l; mean alkalinity, 27·4 ± 1·51 mg/l; pH range 6·99 to 7·34.

§ Mean weight of test fish: 25·6 ± 5·22 g.

¶ Combined total of two aquaria receiving identical treatments.

Copper was found to have an effect on the number of pathogens needed to kill 50% of rainbow trout (Fig. 1). Trout exposed to 0·28 TU of copper and infected with *Vibrio* had an LD_{50} of $1{\cdot}4 \times 10^6$ cells/ml of aquarium water, whereas fish not exposed to the copper had an LD_{50} of $2{\cdot}7 \times 10^6$ cells/ml. The difference between the two groups is significant at $P = 0{\cdot}10$, almost a 50% reduction in the number of *V. anguillarum* cells required to cause a fatal infection.

Discussion

The authors chose *V. anguillarum*, an invasive and virulent pathogen, to study the effect of copper on the susceptibility of salmon and trout to infection. Previous work from this laboratory showed that copper could cause an increase in susceptibility to infection of steelhead trout to the opportunistic pathogen, *Y. ruckeri* (Knittel 1981). This experi-

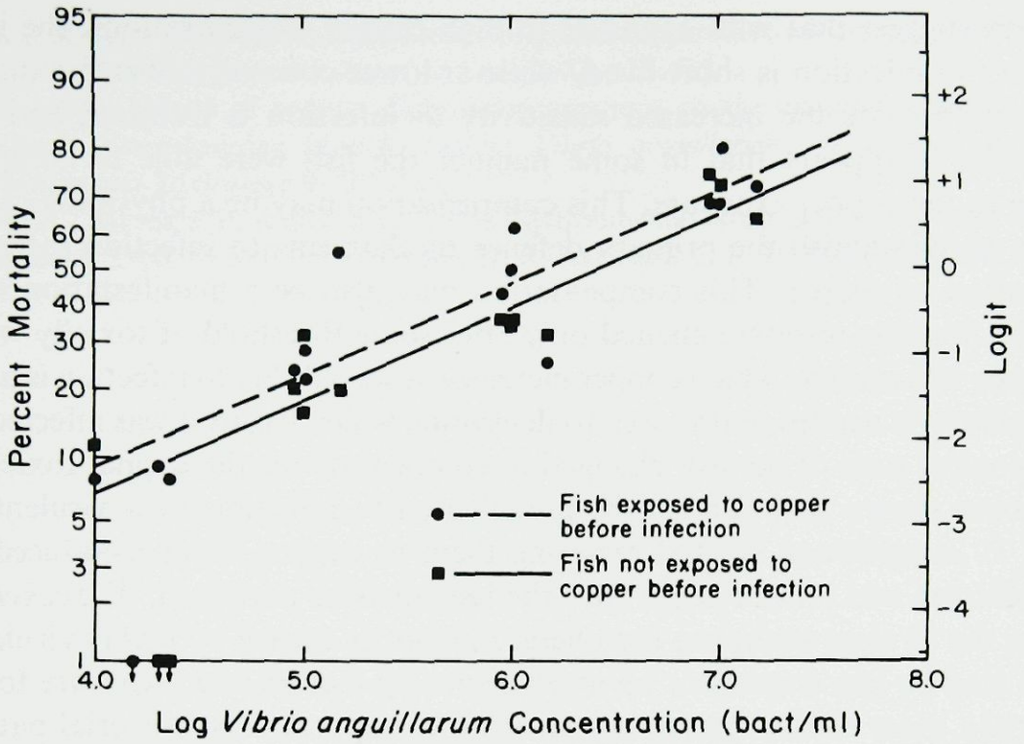

Figure 1. Linear regression comparison of *Vibrio anguillarum* infection in rainbow trout exposed to 0·28 TU copper for 48 h and non-exposed control fish. Significant difference between plots at $P=0{\cdot}10$.

mental work was an extension of observations by fish culturists that epizootics usually followed some stress event (e.g. low dissolved oxygen). *V. anguillarum* answered criteria for both virulence and invasiveness. It could also be transmitted by adding it to the aquarium water, thus eliminating stress caused by injection of the pathogen.

The percentage mortality caused by vibriosis was not linear with increasing copper concentration. In each study, fish showed increased susceptibility to infection at intermediate levels of copper when compared to control groups. At the highest concentrations of copper, the fish were obviously stressed prior to addition of the pathogen, i.e. exhibiting erratic swimming behaviour, 'gasping' at the water surface and general loss of vitality. However, when these fish were infected, no more of them died of the infection than fish not exposed to copper.

Susceptibility to infection was dependent not only upon the concentration of copper but also on the duration of exposure. For example, rainbow trout exposed to about 0·80 TU of copper for 96 h were no more susceptible to vibriosis than the controls (Tables 2 & 3). However, fish exposed to about 0·80 TU and infected after 24 h of exposure were found to have a greater susceptibility to vibriosis (Table 3). With continued exposure at this concentration the susceptibility to infection declined to that of the controls. Lowering the copper concentration to about 0·20 TU (Table 4), the increased susceptibility to vibriosis appeared at 24 h and lasted until 96 h. In both tests (Tables 3 & 4) the fish were infected in an identical manner; however, more controls of the low copper exposure experiment died than in the high copper exposure experiment. The reason for this difference is unknown.

The data suggest that with exposure to high copper concentrations, the increase in susceptibility to infection is short-lived, while at lower concentrations the susceptibility lingers. In both cases the increased sensitivity to infection is transient and returns to control levels. It appears that in some manner the fish were able to compensate or acclimatize to the copper exposure. This compensation may be a physiological response of the fish to re-establish the primary defence mechanisms to infection rather than an acclimatization to copper. This compensation may also be a manifestation of another mode of the immune response elicited only after some threshold of toxicity was passed.

The mode of action by which copper increases susceptibility to infection is not known. Further research is needed in this area to determine which tissue(s) was affected and how the physiological state of the fish changed to compensate for the copper toxicity.

It has been shown that fish can be predisposed to infection by a virulent, invasive pathogen, *V. anguillarum*, by first exposing them to copper. Copper-induced susceptibility to infection was more evident with the less invasive pathogen, *Y. ruckeri* (Knittel 1981), than the virulent pathogen used here. Epizootics caused by highly virulent micro-organisms may be less dependent upon predisposing factors (e.g. exposure to sublethal toxicant) than those caused by the less virulent or opportunistic bacterial pathogens.

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