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Oxygen Tolerance of Fathead Minnows Previously Exposed to Copper

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Copper is a classic limiting factor of fishes, as it is both essential and toxic. In small amounts the element is a vital micronutrient needed for hemoglobin synthesis and a major component of cytochrome oxidase (Birge and Black 1979). Indeed, most fishes will selectively accumulate copper from surrounding water independent of actual concentrations (Stokes 1979; Luoma 1983). As concentrations exceed metabolic requirements, however, copper becomes injurious to fishes, and ultimately reaches lethal levels if concentrations continue to increase.

Because copper rarely reaches lethal levels in natural waters, fishes are more likely to encounter sublethal concentrations (Kleerekopper 1975). These concentrations can change behavior, growth, reproduction and physiology of fishes (see review of Birge and Black 1979). In all cases, copper-exposed fishes display a reduction in performance capacity. Because many of these processes indirectly affect species survival, sublethal effects of copper exposure may have the same ecological relevance as direct lethality.

Disruption of normal gill physiology of fishes exposed to copper prompted O'Hara (1971) to suggest that impaired oxygen uptake may be a major cause of copper toxicity in fishes. Copper has been shown to adversely affect gill mucus (Westfall 1945; Stokes 1979), lamellae (Gardner and LaRoche 1973), and ATPase (Birge and Black 1979) of fishes exposed to sublethal concentrations. These changes imply that fishes exposed to the element may be less tolerant of low oxygen concentrations than unexposed fishes. Although oxygen minimum techniques are often used as a bio-indicator of fish stress (see review by Beitinger and McCauley 1990), few data are available on responses of copper-exposed fishes to progressive hypoxia. We quantified the effects of copper concentration and exposure time on low oxygen tolerance of fathead minnows, *Pimephales promelas*, and evaluated the usefulness of oxygen minimum techniques as a potential bioassay tool.

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MATERIALS AND METHODS

Fathead minnows (60- to 90-d-old) obtained from a culture maintained at the University of North Texas (mean standard length = 3.3 cm, SD = 0.44 and mean weight = 0.7 g, SD = 0.30) were held under a LD 12:12 photoperiod, 23 ± 0.5°C and fed frozen adult brine shrimp (*Artemia* sp.) daily. Fish were not fed 24 hr prior to, or during, the experiments.

Copper concentrations and exposure times for our oxygen tolerance experiments were derived from a static 96-hr lethality test. Fish used for the copper toxicity studies were randomly allocated to six treatment groups of 30 fish each. Treatment groups were divided into three replicates of ten fish and placed into 40-L glass aquaria. Moderate aeration was provided to each aquarium. Treatment groups were exposed to copper sulfate concentrations equivalent to total copper concentrations of 0 (control), 93, 319, 505, 607 or 897 µg Cu/L for 96 hr.

Temperature (± 0.1°C), dissolved oxygen (± 0.1 mg/L), pH (± 0.01 pH unit), hardness (± 0.5 mg/L CaCO3), alkalinity (± 0.5 mg/L $CaCO_3$), conductivity (± 5.0 µmho/cm), and total copper (± 10 µg/L) were measured daily in each aquarium (data available upon request). Copper concentrations were measured with a Perkin-Elmer Model 2380 atomic absorption spectrophotometer. Prior to each set of measures, a standard curve was generated from five copper standards ranging from 0 to 1000 µg Cu/L. Water samples from each aquarium and a blank (triple distilled water) were acidified with trace-metal grade nitric acid and measured within 1 hr of collection. Total copper content of each sample was determined from the standard curve. Copper concentrations in all aquaria remained relatively stable over the 96-hr period.

Mortality was checked hourly during the first 24 hr and at least every 6 hr for 96 hr during lethality trials. Dead fish were removed, measured to the nearest 0.1 cm, and weighed to the nearest 0.1 g at each observation period. Fish surviving trials were measured and weighed at the experiment's termination. The 96-hr LC50 for copper-exposed fathead minnows was estimated from the mortality-concentration data using probit analysis. These data, in turn, were used to estimate copper concentrations equivalent to LC10, LC30, LC50 and LC90 which became the exposure concentrations in our minimum oxygen tolerance experiments.

During the minimum oxygen tolerance experiments, fish in each treatment group were randomly allocated to three 40-L aquaria containing 15 fish each. Replicates within treatment groups were separated by 24 hr so that fish could be tested on consecutive days. In addition, more fish were exposed to copper (n = 45), than were used in the subsequent low oxygen experiments (n = 20) to compensate for possible mortality during exposure. Following copper exposure, fish were moved to the oxygen test chamber and minimum oxygen tolerance estimated.

Minimal oxygen tolerance estimates were made using the apparatus and approach of Bennett and Beitinger (1995). This apparatus was a 20-L glass aquarium fitted with

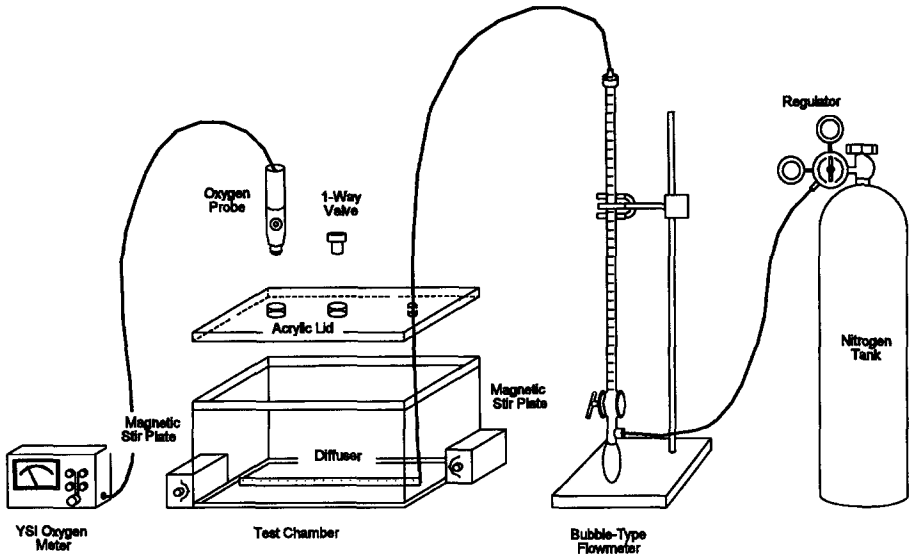

Figure 1. Apparatus used to determine low oxygen tolerance of fathead minnows.

a 1-cm thick plexiglass lid. Plastic screening (6.3 mm mesh size) separated the apparatus into 10 chambers. Stopcock grease sealed the lid to the top of the apparatus to prevent oxygen influx (Fig. 1).

Oxygen was removed by diffusing 0.005 L of nitrogen per second through a porous polyethylene tube located at the bottom of the apparatus. Excess gas escaped through a one-way valve in the lid. Under these conditions, chamber oxygen content was reduced by approximately ½ every 20 min. A typical trial lasted about 1.5 hr. Oxygen concentrations were continually monitored (± 0.05 mg/L) during each oxygen tolerance experiment by a Yellow Springs Instrument Company (YSI), Model 54 oxygen analyzer. The oxygen probe was calibrated following manufacturer's instructions and standardized against Winkler-titrated water samples before each experiment.

Homogeneous oxygen levels were maintained throughout the chambers by vigorous mixing of water, both vertically, by the action of nitrogen bubbles, and horizontally, by magnetic stir bars. Oxygen concentrations measured by the YSI meter were corroborated at the start and end of each trial by Winkler-titration of samples collected from random locations within the chamber. Oxygen concentration differences between Winkler-titration and YSI probe values never varied more than ± 0.15 mg/L for any measurement (mean = 0.00, SD = 0.073, n = 36).

For each trial, ten randomly selected fish from the appropriate copper exposure group were placed into copper-free fresh water (mean dissolved oxygen = 7.5 ± 0.32 mg/L) within the oxygen apparatus, and exposed to progressive hypoxia. Loss of equilibrium (LOE) was the selected experimental endpoint because it represents ecological death, i.e., oxygen concentrations below which fishes in nature can no longer escape conditions that ultimately lead to physiological death. Oxygen concentration (mg/L) was recorded for each fish as it lost equilibrium. Tests were stopped when the last fish of a trial group lost equilibrium; fish were then removed from the test chamber, weighed to the nearest 0.1 g and standard length measured to the nearest 0.1 cm. Nonparametric statistics were used because oxygen minimum data were not normally distributed (Shapiro-Wilks normality test, $P < 0.05$).

Copper effects on low oxygen tolerance of fathead minnows were determined from two replicate groups of 10 fish exposed to copper concentrations approximating the static 96-hr LC10 (120 µg Cu/L), LC30 (177 µg Cu/L), LC50 (250 µg Cu/L) or LC90 (545 µg Cu/L) for 48 hr. Minimum oxygen tolerance data for treatment groups were tested for significant differences ($\alpha = 0.05$) using the Kruskal-Wallis Nonparametric multisample analysis. Treatment groups were arranged into statistically similar subsets by a Student-Newman-Keuls multiple range test (SNK MRT) on ranked data ($\alpha = 0.05$).

The relationship between copper exposure time and minimal oxygen tolerance was determined from low oxygen tolerance estimates from two replicate groups of 10 fathead minnows exposed to copper concentrations equivalent to the LC90 for 0 (control), 24, 48 and 72 hr. A Kruskal-Wallis multisample analysis and SNK MRT ($\alpha = 0.05$) determined the statistical relationships among these groups.

RESULTS AND DISCUSSION

Our static 96-hr lethality test related mortality of fathead minnows to copper concentration by the following statistically significant model (probit analysis; $R^2 = 0.96$; $P < 0.0001$):

Mortality (probit units) = $7.375 + 3.966 \cdot \log_{10}$ copper concentration (mg/L).

Respective LC10, LC30, LC50 and LC90 values (± 95% fiducial limits) estimated by our model were 120 (74 - 160), 186 (149 - 247), 252 (198 - 302) and 530 (455 - 715) µg Cu/L. These results are similar to previously reported values when differences in water hardness are accounted for. For example, our static 96-hr LC50 for fathead minnows of 252 µg Cu/L (mean hardness = 101 mg/L $CaCO_3$), is similar to Benson and Birge's (1985) estimate of 210 µg Cu/L (mean hardness = 100 mg/L $CaCO_3$). Additionally, Hodson et al. (1979) regression model of static LC50 on hardness predicts a static LC50 of 262 µg Cu/L at hardness of 101 mg/L $CaCO_3$; a value well within the 95% fiducial limits of 198 - 302 µg Cu/L estimated by our experiments.

Our tests of low oxygen tolerance of fathead minnows showed a sigmoid decrease from control levels (i.e., median LOE oxygen concentrations increased) with increasing copper concentrations (Fig. 2). Minnows exposed to static concentrations of 0 µg Cu/L (control), and LC10, LC30, LC50 or LC90 copper levels for 48 hr had median oxygen concentrations (min-max) of 0.7 (0.5-1.1), 0.9 (0.6-1.9), 1.6 (0.9-2.5), 1.9 (1.1-3.2), and 2.0 (1.4-3.5) mg/L, respectively. Highly significant differences were evident among fish exposed to the various copper concentrations (Kruskal-Wallis, $P < 0.0001$), with exposure groups separated into the following statistically distinct subsets: control = LC10 < LC30 < LC50 = LC90 (SNK MRT on ranked data, $\alpha = 0.05$).

Although low oxygen tolerance of control fathead minnows exposed to progressive hypoxia has not been previously determined, our value of 0.7 mg/L is consistent with observations by Gee et al. (1962) that fathead minnows become distressed at 1 mg/L, and Klinger et al. (1982), who showed that these fish do not survive oxygen levels below 0.25 mg/L. Furthermore, low oxygen tolerance of our control fish is similar to values between 0.5 and 1.0 mg/L reported for other closely related fishes exposed to progressive hypoxia (e.g., Moore 1942; Lowe et al. 1967).

The observed pattern of low oxygen tolerance loss in fathead minnows previously exposed to various copper concentrations, suggests progressive physiological collapse as copper concentrations exceed a critical threshold. Fish exposed to copper concentrations equivalent to the LC30 and LC50 showed respective decreases in low oxygen tolerance of 130 and 171% after just 48 hr (Fig. 2). The significant deterioration of oxygen tolerance in response to increasing copper concentrations may have important implications for natural fish populations. Many aquatic systems are naturally or anthropogenically high in metals, including copper, with concentrations sometimes approaching lethal levels (Luoma 1983). Therefore, fathead minnows (and presumably other fishes) living in these environments may be killed by exposure to copper concentrations considered sublethal by standard toxicity tests when oxygen concentrations are low. That fathead minnow populations persist in copper-rich environments (Benson and Birge 1985) implies that these fish ameliorate deleterious oxygen tolerance effects associated with copper exposure.

Although low oxygen tolerance of fathead minnows was sensitive to increasing copper concentration, this effect was not persistent. Fish exposed to the static LC90 copper concentration for 0 (control), 24, 48, or 72 hr (Fig. 3) showed highly significant changes in low oxygen tolerance (Kruskal-Wallis, $P < 0.0001$). Significant decreases in oxygen tolerance were seen between controls (median = 0.7 mg/L) and 24-hr exposed fish (median = 1.6 mg/L) and between 24- and 48-hr exposed fish (2.0 mg/L). By 72 hr, however, median LOE oxygen concentration (median = 0.9) decreased to levels not significantly different from the control value (SNK MRT for ranked data, $\alpha = 0.05$). This observation may reflect the induction of some compensatory biochemical/physiological mechanism in these fish. Loss of sensitive individuals during copper exposure could also explain the decreased frequency of extreme values at high oxygen concentrations. However, the increased frequency of fish experiencing LOE at oxygen concentrations lower than either the 24- or 48-hr exposure groups could only result from improved oxygen tolerance.

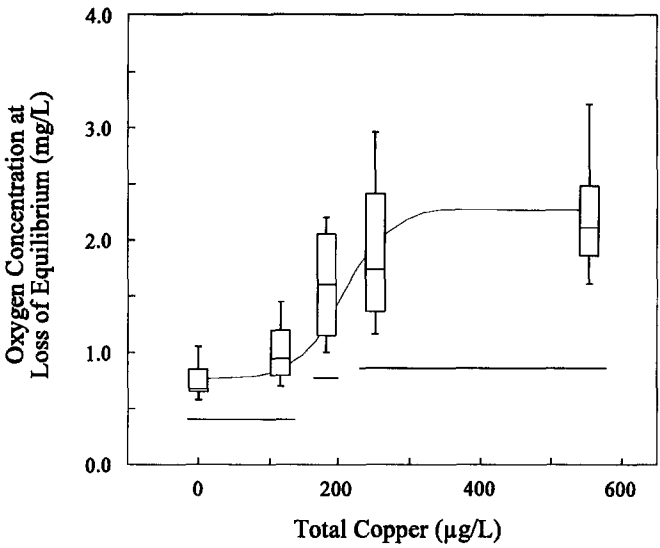

Figure 2. Low oxygen tolerance of fathead minnows (n = 20 for each group) exposed to copper concentrations of 0 to 545 µg/L for 48 hr. Data plotted as median, 10th, 25th, 75th and 90th percentile. Underlined groups do not differ significantly.

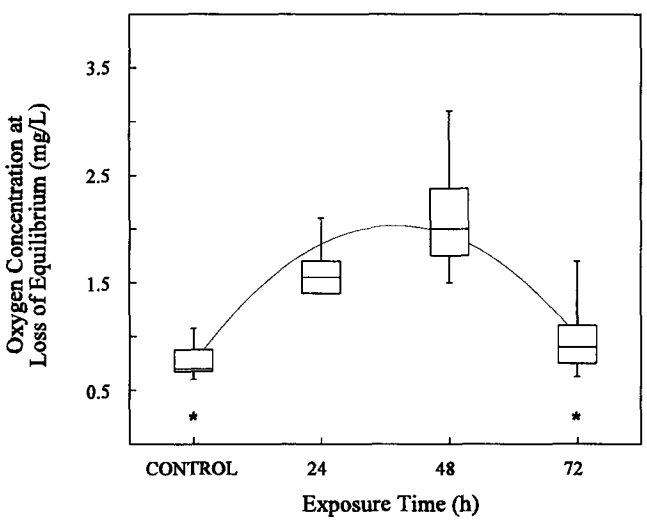

Figure 3. Low oxygen tolerance for groups of fathead minnows (n = 20 for each group) exposed staticly to 96-hr LC90 copper concentrations for 0 (control) 24, 48 or 72 hr. Data plotted as median, 10th, 25th, 75th and 90th percentile. Groups marked with an asterisk do not differ significantly.

The mechanism used by fathead minnows to mitigate copper-induced oxygen intolerance is unknown, however, many fishes circumvent problems associated with copper exposure by either limiting uptake across gill surfaces (Brungs et al. 1973) or by depuration. Fishes can excrete copper directly via the kidney (Stokes 1979), or complexed with L-6-D liver storage protein (metallothionein), which presumably facilitates copper excretion with bile (Merceau 1979). Benson and Birge (1985) detected relatively high levels of metallothionein in fathead minnows occurring in waters high in copper and induced its production in hatchery fathead minnows by exposure to cadmium. Similar mechanisms may explain the recovery of oxygen tolerance of fish in our experiments, but our experimental design could not discriminate between limited copper uptake and copper excretion. It is clear, however, that fathead minnows invoke some compensatory mechanism and, thus, are susceptible to low oxygen levels only during initial stages of copper exposure.

The narrow temporal window and limited concentration range over which oxygen tolerance is affected in fathead minnows may restrict the usefulness of this technique as a bioassay for copper exposure. To be effective, biological indicators should be persistent, sensitive, and preferably confined to a specific stressor type. Although some investigators have suggested that residual oxygen measurements meet these criteria (e.g., Carter 1962; Gordon and McLeay 1977), our experiments suggest that this may not always be the case for copper. Our technique may be more efficacious, however, if used to bioassay substances producing greater and more persistent oxygen tolerance responses.

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REFERENCES

Beitinger TL, McCauley RW (1990) Whole-animal physiological processes for the assessment of stress in fishes. J Great Lakes Res 16:542-575

Bennett WA, Beitinger TL (1995) Overview of techniques for removing oxygen from water and a description of a new oxygen depletion system. Prog Fish-Cult 57:84-87

Benson WH, Birge WJ (1985) Heavy metal tolerance and metallothionein induction in fathead minnows: Results from field and laboratory investigations. Environ Toxicol Chem 4:209-217

Birge WJ, Black JA (1979) Effects of copper on embryonic and juvenile stages of aquatic animals. In: Nriagu JO (ed) Copper in the Environment, Part 2: Health Effects. John Wiley & Sons Inc., New York, p 373

Brungs WA, Leonard EN, McKim JM (1973) Acute and long-term accumulation of copper by the brown bullhead *Ictalurus nebulosus*. J Fish Res Board Can 30:583-586

Carter L (1962) Bioassay of trade wastes. Nature (Lond) 196:1340

Gardner GR, LaRoche G (1973) Copper induced lesions in estuarine teleosts. J Fish Res Board Can 30:363-368
Gee JH, Tallman RF, Smart HJ (1962) Reactions of some great plains fishes to progressive hypoxia. Can J Zool 56:1962-1966
Gordon MR, McLeay DJ (1977) Sealed-jar bioassays for pulpmill effluent toxicity: Effects of fish species and temperature. J Fish Res Board Can 34:1389-1396
Hodson PV, Borgmann U, Shear H (1979) Toxicity of copper to aquatic biota. In: Nriagu JO (ed) Copper in the Environment, Part 2: Health Effects. John Wiley & Sons Inc., New York. NY. p 307
Kleerekopper H (1975) Effects of sublethal concentrations of pollutants on the behavior of fish. J Fish Res Board Can 33:2036-2039
Klinger SA, Magnuson JJ, Gallepp GW (1982) Survival of the central mudminnow (*Umbra limi*), fathead minnow (*Pimephales promelas*) and brook stickleback (*Culaea inconstans*) for low oxygen in winter. Environ Biol Fish 7:113-120
Lowe CH, Hinds DS, Halpern EA (1967) Experimental catastrophic selection and tolerance to low oxygen concentration in native Arizona freshwater fishes. Ecology 48:1013-1017
Luoma SN (1983) Bio-availability of trace metals to aquatic organisms - a review. Sci Total Environ 28:1-22
Merceau N (1979) The use of radiocopper to trace copper metabolic transfer and utilization. In: Nriagu JO (ed) Copper in the Environment, Part 2: Health Effects. John Wiley & Sons Inc, New York, New York, p 177
Moore WG (1942) Field studies on the oxygen requirements of certain fresh-water fishes. Ecology 23:319-329
O'Hara J (1971) Alterations in oxygen consumption by bluegills exposed to sublethal treatment with copper. Water Res 5:321-327
Stokes PM (1979) Copper accumulations in freshwater biota. In: Nriagu JO (ed) Copper in the Environment, Part 2: Health Effects. John Wiley & Sons Inc, New York, New York, p 357
Westfall BA (1945) Coagulation film anoxia in fishes. Ecology 26:283-287