INTERACTIONS OF TEMPERATURE AND SUBLETHAL ENVIRONMENTAL COPPER EXPOSURE ON THE ENERGY METABOLISM OF BLUEGILL (*LEPOMIS MACROCHIRUS*)

by

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Thesis submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Zoology

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May, 1983

Blacksburg, Virginia

ACKNOWLEDGEMENTS:

I would like to gratefully acknowledge the help of the members of my graduate committee, Walter Schwab, Anne McNabb, and especially Alan Heath without whose assistance this research would not have been possible. In addition, I would like to thank those friends, fellow graduate students, and family members who provided so much encouragement and support throughout my graduate career.

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INTRODUCTION

Depending on its concentration in the water, copper may serve as an essential trace element, a chronic toxicant, or an acute toxicant bringing on sudden death. Copper can be found as a trace element in nearly all waters, however it is also encountered as a toxicant chiefly from two sources, industrial pollution produced by mining and plating, and in compounds applied as algicides.

Several effects of exposure to sublethal concentrations of copper have been reported in fish. These effects include decreased survival, growth, and reproduction (Mount and Stephan, 1969; McKim and Benoit, 1971), lowering of the preferred temperature in fathead minnows (Opuszynski, 1971), elevated plasma corticosteroid levels in salmon (Donaldson and Dye, 1975; Schreck and Lorz, 1978), tissue damage in the gills of the winter flounder (Baker, 1969), and decreased activity of gill Na^+, K^+-ATPase from coho salmon exposed to copper in fresh water (Lorz and McPherson, 1976). In addition, it has been shown that fish exposed to sublethal copper concentrations accumulate copper in certain tissues, particularly gills and liver (Brungs et al., 1973; Benoit, 1975).

Two studies have examined the effect of copper exposure on whole body energy metabolism. Waiwood and Beamish (1978a) reported a decrease in the maximum oxygen consumption of

rainbow trout exposed to sublethal levels of copper. O'Hara (1971) found a dose dependent increase in oxygen consumption in bluegill exposed to copper levels between 0.5 and 5mg/L (96 hour LC_{50}=2.4mg/L). The increase was transient and lasted between 2 and 18 hours. The increase was followed by a dose dependent depression in oxygen consumption which was maintained for the seven days of exposure (copper levels 0.3-3.0mg/L) after which time the copper contaminated water was replaced with freshwater.

In light of the findings of Sellers et al. (1975) that exposure of rainbow trout to their 48-hour LC_{50} for 24 hours did not result in a reduction in dorsal aortic PO_2, it is probable that the decrease in oxygen consumption found by O'Hara is not merely a result of the failure of the oxygen delivery mechanism, but rather a lowered uptake and/or utilization of oxygen from the blood by the tissues of the body. Since copper does accumulate in certain tissues during a sublethal exposure, the metal may be acting internally to suppress oxygen consumption. However whether the copper is acting on systems which are responsible for integration of whole body oxygen consumption such as nervous and endocrine tissues, or directly on the individual cells is not known. One of the primary purposes of this study was to establish the effect of chronic exposure to copper on oxygen consumption and to identify the level of action of copper in bluegills.

This was determined by exposing bluegill to sublethal copper concentrations for up to 32 days and measuring oxygen consumption in the whole body and certain tissues.

Any long term exposure of bluegill in the natural environment will almost inevitably be accompanied by changes in temperature, whether natural and/or anthropogenic in origin. And, changes in temperature in ectothermic animals result in changes in the oxygen consumption of the animal, making this variable important for study in conjunction with copper exposure, particularly the phenomenon of temperature acclimation. Through the process of temperature acclimation, it has been found that many ectothermic animals have the ability to achieve a partial metabolic homeostasis (Hochachka and Somero, 1973). Temperature acclimation has been shown to involve several physiological changes. These include changes in endocrine functions (Precht, 1964; Rao, 1967), increased fatty acid saturation at higher temperatures (Caldwell and Vernberg, 1970), and most importantly, changes in enzyme activity (Shaklee et al., 1977). Since temperature acclimation involves enzyme modulation, and given the affinity of copper for proteins, copper may have an effect on the dynamic process of temperature acclimation, perhaps delaying or preventing it. The effect of sublethal exposure to copper and a temperature change was studied by exposing

bluegill to both and measuring their whole body oxygen consumption and the oxygen consumption of certain tissues.

LITERATURE REVIEW

This study is aimed at gaining an understanding of the implications of chronic copper exposure on fish, specifically concerning its effects on energy metabolism, the process of acclimation to a temperature change, and the accumulation of copper in fish tissue. The literature on these three aspects of copper will be reviewed.

As is true for a number of metals, copper is a trace element required for several physiological functions. It is transported in the blood of mammals bound to a protein called ceruloplasmin. Recent evidence suggests the presence of this protein in fish blood (Fletcher and Fletcher, 1980). Ceruloplasmin is a ferrous oxidase and the recent discovery of the copper dependency of another ferrous oxidase, Ferroxidase II, (Garnier et al., 1981) illustrates the dependency of iron utilization (and ultimately hematopoiesis) on copper proteins. Copper is also a cofactor of the enzyme lysyl oxidase which is necessary for the synthesis of mature elastin and collagen (Harris et al., 1980). The metalloenzyme superoxidase dismutase (copper-zinc type), which removes the toxic superoxide radical, is also a copper dependent enzyme (Hassan, 1980).

The lethal concentration of copper for different species of fish under various conditions is variable. Acutely lethal concentrations to freshwater fish ranging over 1½ orders of magnitude have been reported (EIFAC, 1978). Preliminary

experiments in this lab have shown that a concentration of 2.4mg Cu/L is lethal to 50% of the bluegill (Lepomis macrochirus) exposed to this level for 96 hours. The acute lethality of copper has historically been attributed to gill damage and mucous precipitation with subsequent asphyxiation (Ellis, 1937). And indeed, a more recent study (Baker, 1969) has found changes in gill morphology following exposure to acutely lethal copper levels and, mucous has been shown to be a barrier to oxygen diffusion (Ultsch and Gros, 1979). However, Sellers et al. (1975) found no reduction in dorsal aortic PO_2 in rainbow trout (Salmo gairdneri) exposed to their LC_{50} for 24 hours, an indication that asphyxiation may not be the principle cause of copper induced lethality.

Further evidence for this is the finding of Smith and Heath (1979) that over rather wide temperature ranges, the toxicity of copper is only slightly increased at higher temperatures in goldfish (Carassius auratus), golden shiner (Notemigonus crysoleucus), and rainbow trout, and is actually slightly decreased at higher temperatures in bluegill. Rehwoldt et al., (1972) found no significant increase in the toxicity of copper with increasing temperature. Yet, if asphyxiation were the principal cause of mortality, one would expect that the lower solubility of oxygen, coupled with the increase

in respiration and hence oxygen demand at higher temperatures, would lead to increased toxicity.

Several other effects of acute copper poisoning have also been reported. Baker (1969) found hepatic fatty metamorphosis and kidney necrosis in winter flounder exposed to a high concentration of copper. Renal lesions were also found in the killifish (Fundulus heteroclitus) by Gardner and LaRoche (1973), as well as lesions in the chemoreceptive sites of the olfactory organs and cellular changes in the mechanoreceptors of the lateral line of the killifish and Atlantic silversides (Menidia menidia). Jackim et al. (1970) determined the enzyme activity of five liver enzymes from killifish which survived a 96 hour exposure to their 96 hour LC_{50} for copper. They found no change in alkaline phosphatase, catalase, and ribonuclease and an increase in activity of xanthine oxidase as compared to control values. They also found that in vitro exposure of the enzymes to copper caused inhibition in acid and alkaline phosphatase, xanthine oxidase, and catalase, a finding also reported by Christensen (1971) for plasma glutamic oxalacetic transaminase and lactic dehydrogenase. However in both these studies levels of copper were probably higher than would be encountered in vivo. Bilinski and Jonas (1973) also found that the oxidative ability of rainbow trout gills were reduced following 48 hours of exposure to a lethal copper concentration.

Evidence for the failure of osmoregulatory mechanisms in channel catfish (Ictalurus punctatus) and golden shiner following exposure to copper was provided by Lewis and Lewis (1971). They found that the osmolality of blood serum decreased in fish exposed to lethal levels of copper. Schreck and Lorz (1978) however found no decrease in serum osmolarity in coho salmon (Oncorhynchus kisutch) exposed to lethal copper concentrations but they did find decreased serum chlorinity. Further evidence for osmoregulatory effects was provided by the finding of Birdsong and Avault (1971) that the lethality of copper for juvenile pompano (Trachinotus carolinus) decrease with increasing salinity.

Finally, Donaldson and Dye (1975) working with sockeye salmon (Oncorhynchus nerka) and Schreck and Lorz (1978) working with coho salmon found an increase in corticosteroids in fish exposed to lethal concentrations of copper. This is a classical form of stress response and may not be particularly specific to any pollutant.

Unfortunately, much of the data generated concerning the toxicity of copper is of little use for comparative purposes. Studies within the last decade have shown that the total concentration of copper in a solution is not well correlated with toxicity, but rather, it appears that only certain of the many forms of copper in solution participate in the toxic

mechanism. Several authors have reported that toxicity is related to the cupric ion (Cu^{++}) concentration (Howarth and Sprague, 1978; Waiwood and Beamish, 1978a; Waiwood and Beamish, 1978b; Pagenkopf et al., 1974; Shaw and Brown, 1974).

In addition to the lethal actions of copper, several effects of exposure to sublethal concentrations have been investigated. Mount (1968) found that no spawning occured in fathead minnow (Pimephales promelas) exposed to copper levels greater than 32µg/L in water with a hardness of 200mg/L as $CaCO_3$. In water with a hardness of 30mg/L as $CaCO_3$, Mount and Stephan (1969) found that survival, growth, and reproduction in brook trout (Salvelinus fontinalis) was reduced, however concentrations between 17.4 and 3.4 µg/L had no effect. McKim and Benoit (1974) also showed that progeny of brook trout exposed to copper concentrations as high as 9.5µg/L were not more susceptible to the effects of copper than those from unexposed parents. Thus decreased resistance does not appear to be passed on by affected parent fish. Benoit (1975) exposed bluegill for 22 months to copper concentrations of 162, 77, 40, 21, and 12µg/L and found that survival, growth, and reproduction were decreased at the highest concentration but not affected by lower concentrations. In general these studies have also shown that larval forms of freshwater fish are more susceptible to copper effects on growth and survival than are adults (Benoit, 1975;

McKim and Benoit, 1971). Finally, in a related study, Murai et al. (1981) fed diets containing known amounts of copper to channel catfish. They found that at the two highest concentrations, 16 and 32mg copper supplement/Kg diet, growth was reduced after 16 and 4 weeks respectively.

Several studies have investigated the avoidance of copper-containing water by fish and the effect of sublethal concentrations of copper on preferred temperature. Sprague (1964) found that Atlantic salmon (Salmo salar) parr avoided water containing copper down to a threshold of 2.3 µg/L. In a natural avoidance experiment, Saunders and Sprague (1967) monitored the effect of mining activity on the Northwest Miramichi River on spawning migration of the Atlantic salmon. They found significant increases in premature downstream returns following the start of mining activity. Opuszynski (1971) reported a lowering of the preferred temperature in the fathead minnow following exposure to a sublethal level of copper. However, Peterson (1976) found no significant change in the preferred temperature of Atlantic salmon exposed to copper.

A transient decrease in feeding activity following copper exposure has been reported for the brook trout (Lett et al., 1976). This decrease may be related to the effect of copper on the olfactory response as Hara et al. (1976) found that a concentration of copper as low as 8µg/L caused appreciable

depression in the electrical response of the olfactory bulb of rainbow trout to L-serine solution.

Sublethal exposure to copper has recently been implicated in decreasing the resistance of fish to disease. Rodsaether et al. (1977) found that eels (Anguilla anguilla) exposed to copper concentrations between 30 and 60µg/L died of vibriosis (Vibrio angiollarum) within 120 days, however eels kept in noncontaminated freshwater (6µgCu/L) remained healthy for at least 1 year. In a study on the increased pathogenicity of externally applied agents in fish exposed to copper, Hetrick et al. (1979) found that rainbow trout exposed to copper as low as 3.9µg/L were more susceptible to infectious hematopoietic necrosis virus than were fish exposed to the virus alone. Similar results were obtained by Knittel (1981) in steelhead trout (Salmo gairdneri) exposed to redmouth infection (Yersinia ruckeri). Trout exposed to 7 or 10µg Cu/L for 96 hours were more likely to die of infection than were control fish. Also, the infectious dose was lower in 10µg/L exposed fish than in controls.

Waiwood and Beamish (1978a) investigated the effect of low levels of copper on critical swimming performance (highest swimming speed maintainable for a defined period) in rainbow trout at various hardnesses. They found decreases in critical swimming performance of 23, 12, and 6% in trout exposed

to the 240 hour LC_{50} for hardnesses 30, 100, and 360 mg/L (as $CaCO_3$) respectively. Drummond et al. (1973) reported transient increases in locomotor activity in brook trout exposed to levels of copper between 6 and 115 µg/L and increases in cough frequencies in this same range. However, Sellers et al. (1975) found no increase in cough frequency in rainbow trout exposed to levels of copper as high as 90 µg/L.

In general, these studies on the sublethal effects of copper have been descriptive of the external manifestations of copper exposure. Several studies have, however, examined the internal effects of sublethal exposure. These studies include investigations of the effect of copper exposure on blood parameters, enzymes, osmoregulatory competency, the endocrine system, tissue damage, and tissue copper concentration.

McKim et al. (1970) found increases in red blood cell count, hematocrit, total protein, plasma glutamic oxalacetic transaminase (PGOT), and hemoglobin and a decrease in plasma chloride levels and osmolarity in brook trout exposed for 6 and 21 days. However, the only difference from controls observable after 337 days of exposure was a reduction in PGOT, an enzyme which, when found in excess in mammals, is considered indicative of internal tissue damage. Christensen et al. (1972) found a similar decrease in the PGOT activity

of brown bullhead (Ictalurus nebulosus) exposed to 27 and 53 µgCu/L for 600 days. This suggests a direct inhibitory effect of this enzyme by copper. They also found increases in glucose and hematocrit following 6 days of exposure. Following 21 days of exposure, hematocrit, hemoglobin, and glucose were still elevated but plasma chloride and plasma total protein were decreased.

The studies which have examined the effect of sublethal concentration of copper on enzymes fall into two categories. The first includes two investigations which determined the effect of copper on δ-aminolevulinate dehydrase (an enzyme involved in porphobilinogen synthesis) parenthetical to investigations of the effect of lead on this enzyme. Jackim (1973) reported a small decrease in liver δ-aminolevulinate dehydrase activity in copper exposed killifish. However, Hodson et al. (1977) reported no change in erythrocyte δ-aminolevulinate dehydrase activity from copper exposed rainbow trout. The secondary category of investigations consists of two studies which examined the effect of copper on gill enzymes. Bilinski and Jonas (1973) found that exposure to 64µg Cu/L for 48 hours caused a reduction in the oxidation of ^{14}C lactate to $^{14}CO_2$ by rainbow trout gills in vitro. This effect was not seen with 24 hours of exposure, and lower metal concentrations resulted in some mortalities while having no effect on oxidative activity. They also examined gill structure

and found few histopathological changes, leading them to conclude that the effect of copper on oxidative activity was due to its action on enzymic processes. Lorz and McPherson (1976) found decreased activity of Na^+, K^+-ATPase in coho salmon exposed to copper in freshwater, and decreased survivability when exposed animals were transferred to sea water. They speculate that death in sea water was caused by loss of osmoregulatory ability due to decreased ATPase activity.

The effect of copper on corticosteroid levels was investigated in two studies previously mentioned in the acute effects section. Donaldson and Dye (1975) found that total corticosteroids as well as cortisol and cortisone were elevated in sockeye salmon exposed to 63.5µg Cu/L for 1 hour. And, fish exposed to 6.35µg Cu/L showed increased total corticosteroids after 2 hours of exposure and increased cortisone after 4 hours of exposure. Schreck and Lorz (1978) found an immediate dose dependent increase in the plasma cortisol levels in coho salmon exposed to sublethal levels of copper. This was followed by a return to near control levels after 24 hours of exposure. However, those fish exposed to higher sublethal levels (60 and 90µg Cu/L) then exhibited an increase in plasma cortisol over controls between 36 and 78 hours, which was maintained for the remainder of the 170 hours of exposure.

Several studies have also found that sublethal exposures

to copper can cause tissue damage. Baker (1969) observed that in the gills of the winter flounder exposed to copper the epithelial layer was vacuolated and basal lamina reduced in thickness compared to controls. In addition, chloride cells appeared to increase in number, however the number of mucous cells decreased. Gardner and LaRoche (1973) found alterations in the epithelium and mechanoreceptors of the lateral line canals of killifish and Atlantic silversides as well as lesions in the olfactory organs in fish exposed to sublethal (and lethal) levels of copper. In a related study which may shed some light on the mechanism of tissue damage, Young et al. (1981) found that copper injured the gills of the polychaete Eudistylia vancouveri and that the copper was localized subcellularly in membrane bound vesicles similar to lysosomes. They theorize that cell damage may be a result of the labilization of these vesicles.

Related to this is the finding of many researchers that sublethal exposure to copper can cause increases in the concentration of copper in certain tissues. Brungs et al. (1973) found that brown bullheads exposed to sublethal levels of copper as low as 27μg/L accumulated copper in their liver and gill, reaching equilibrium levels within 30 days. However copper levels did not increase in red blood cells or blood plasma following 6 days, 30 days, and 20 months of exposure. McKim and Benoit (1974) exposed brook trout to

sublethal copper levels up to 9.4 µg/L for 24 months and found no increase in the copper concentration in the gill, liver, kidney, muscles, or eggs. However, Benoit (1975) found significant increases in the gill and liver copper concentrations in bluegill exposed to copper levels between 40 and 162µg/L for 22 months. In addition, at the highest exposure level (162µg/L) there was an increase in kidney copper levels. And, Solbe and Cooper (1975) found that stone loach (Noemacheilus barbatulus) which survived a 64 day exposure to 120µg Cu/L (63 day LC_{50} was approximately 250ug Cµ/L) lost significant amounts of copper from their gill, muscles, eye, and vertebrae upon being placed in clean water for 7 days.

Two studies have examined the effect of exposure to sublethal concentrations of copper on oxygen consumption, a major focus of the present study. Waiwood and Beamish (1978a) reported a decrease in the maximum oxygen consumption of rainbow trout exposed to sublethal levels of copper. O'Hara (1971) reported a dose dependent increase in oxygen consumption in bluegill exposed to copper levels between 0.5 and 5mg/L (96 hour LC_{50}= 2.4mg/L). The increase was transient and lasted between two and eighteen hours. The increase was followed by a dose dependent decrease in oxygen consumption. This decrease was maintained for the seven days of exposure (concentrations of 3, 2, 1, 0.5, and 0.3 mg Cu/L) after which time

the copper contaminated water was replaced with freshwater. Following this, a gradual return toward normal levels was noted. Unfortunately, two problems with this study make the assessment of the effect of low sublethal copper concentrations on oxygen consumption difficult. The first is the short exposure period. Brungs et al. (1973) reported that accumulation of copper in tissues of sublethally exposed brown bullheads reached equilibrium by 30 days however levels at 6 days of exposure were well below equilibrium values. And, secondly, while O'Hara does provide statistics to differentiate the responses between various copper concentrations from one another, he does not provide them in relation to control values. Since the decrease in oxygen consumption at his two lowest concentrations, 0.5 and 0.3mg Cu/L are statistically different from each other, it is probable that the oxygen consumption rate at 0.5 mg/l is significantly less than the control value. However, it is not possible to determine if the decrease in oxygen consumption at 0.3 mg/L is significantly less than control values. It is interesting to note that in the introduction to his report, O'Hara mentions 0.3mg Cu/L as a frequently used application level of copper for algae control.

No reports of the effects of copper on respiration of fish tissues exists, however some related work has been done on the mussel Mytilus edulis. Brown and Newell (1972) exposed

mussels to a high but not immediately lethal concentration of copper and observed a 50% reduction in oxygen consumption. They then exposed excised gill and digestive gland tissue to similar copper levels *in vitro* and found that the gill tissue respiration was inhibited but that of the digestive gland was not. They attributed the reduction in the gill tissue oxygen consumption to a cessation of ciliary action.

METHODS AND MATERIALS

Fish Maintenance and Water Chemistry:

Bluegill (Lepomis macrochirus) with an average weight of 10.11g $\pm$ 0.61g (S.E.) were obtained from Perry Minnow Farm, Windsor, Virginia and Kurtz's Fish Hatchery, Elverson, Pennsylvania. Fish were maintained for a minimum of three weeks prior to experimentation in charcoal filtered Blacksburg tap water at 20° $\pm$ 1° C (hardness = 59 mg $CaCO_3$/L, alkalinity = 46 mg $CaCO_3$/L, pH = 7.55). The photoperiod was 15 L (beginning at 6:00 a.m.), 9 D, and most of the measurements were made during spring and summer. Fish were fed daily on an ad libitum diet of partially ground Purina Trout Chow.

Copper stock solution was prepared by adding cupric nitrate ($Cu(NO_3)_2:3H_2O$) to distilled water, and this mixture was then acidified with 1ml of concentrated sulfuric acid per 15L stock solution. Stock solution was diluted and delivered to experimental tanks using part of a proportional diluter (Mount and Brungs, 1967) modified to deliver a single toxicant concentration (Fig. 1). As water descended from the clean water tank, it passed a venturi which initiated siphoning of stock solution from a delivery bottle. The amount of stock solution in the delivery bottle was maintained at a constant level by an overflow. During experiments, the flow of solution from the stock bottle was adjusted so that filling

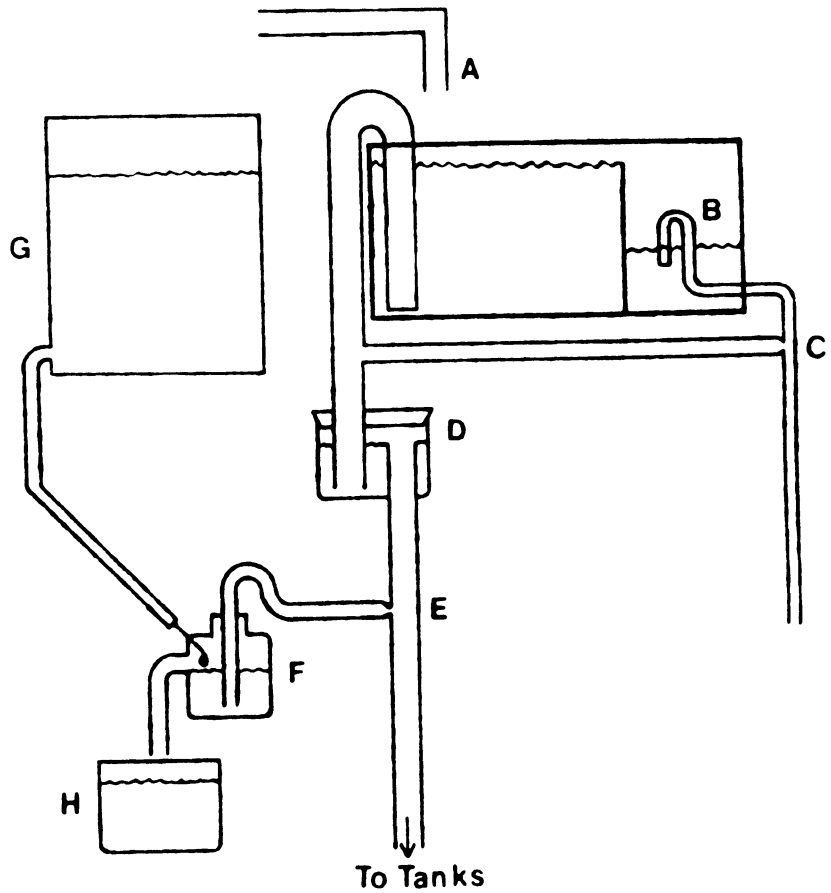

Figure 1. Diagram of diluter used to deliver toxicant. Tube A delivers dechlorinated tap water to the left side of the upper tank (capacity 1200 ml) which, after filling, overflows into the right chamber (capacity 400 ml), eventually reaching a level which allows the automatic siphon (B) to begin. Water siphoning out of the upper right tank passes a venturi (C) which starts a siphon in the large U-shaped tube in the left side of the upper tank. Air is prevented from entering the U tube by use of a manifold (D). Water siphoning out of the upper left tank goes through the manifold and passes a second venturi (E). The suction provided by this venturi causes the emptying of a bottle containing 15 ml of stock toxicant solution (F). Stock solution is delivered from a large reservoir (G) to the bottle through a small guage needle. The level of stock solution in the bottle is maintained at a constant level by an overflow tube which leads to an overflow tank (H). The concentration delivered by the diluter can be rapidly adjusted by simply raising or lowering the tube in the constant volume bottle. Cycle time was approximately 4 minutes.

of the delivery bottle occured only slightly more rapidly than the cycling time of the upper tank and manifold system. This system was not strictly a proportional diluter since the flow of clean water and stock solution into the upper tank and stock delivery bottle (respectively) was maintained during the emptying phase of the cycle. Thus any variations in these flow rates would have some effect on the final concentration of the toxicant. However, the emptying cycle was so short compared with the filling phase (approximately one-tenth as long) that minor fluctuations in flow rate would produce very small changes in the final toxicant concentration. This system had the advantage of allowing minor adjustments in concentration to be made by simply lowering (thus increasing concentration) or raising (thus decreasing concentration) the siphon tube in the delivery bottle.

The concentration of copper in the experimental tanks was determined at approximately one week intervals by the cuprethol method (APHA, 1975) utilizing a Bausch and Lomb model 88 spectrophotometer. Water samples for copper determination were obtained in acid rinsed glassware and were tested immediately for both total copper and, following filtration through a 0.45μm membrane filter, dissolved copper.

The sublethal concentration of copper to be used for the experiments was determined by a preliminary toxicity study. A value of 0.62 mg/L dissolved copper was obtained

as a 96 hour LC_{50} for this species under the aforementioned conditions using probit analysis. By linear extrapolation of the probit line, a value of 0.28 mg/L was obtained for 98% survivability. This value served as a target concentration for all the experiments. The measured dissolved copper concentration in the tanks during the course of the experiments was 0.21 mg/L $\pm$ .035 mg/L (mean $\pm$ 2 S.E.).

Oxygen Consumption Measurements (Whole Animal):

Animals utilized in the 20°C experiments were transferred three at a time to 34 liter tanks receiving a flow of either clean water or copper contaminated water at 20°C. The following day they (3 control animals, 3 experimental animals) were placed individually in 910ml glass chambers which, after they were sealed, received a flow of approximately 200 ml/min from either the control or copper contaminated tanks. The chambers were then placed in a constant temperature bath at 20°C ($\pm$0.1°C) which contained dividers providing visual isolation from one another and the experimenter. They were then allowed 24 hours to acclimate to the chambers. At this point, which coincides with day 3 of exposure to copper, oxygen consumption measurements began. Samples of the outflow from each chamber were removed with 25 ml syringes and analysed for dissolved oxygen with a Yellow Springs model 54A oxygen analyser. Electric solenoid

valves were used to allow simultaneous collection from all six chambers. Flow was then terminated on all six chambers. Thirty minutes later flow was again initiated and the outflow coming from the middle of the chambers was again obtained with syringes and analysed for dissolved oxygen. Each day of experimentation consisted of 4 trials per fish occuring between the hours of 10:00 a.m. and 4:00 p.m. From the difference in dissolved oxygen, the weight of the fish, and volume of the jar, the weight specific oxygen consumption ($\dot{V}O_2$) was calculated.

Fish remained in the chambers and were retested on day 4 of exposure. They were then returned to their respective control or experimental tanks. On days 8 and 31 of exposure they were again placed in the chambers and, 24 hours later, the $\dot{V}O_2$ was measured as before (days 9 and 32 respectively).

In order to determine the effect of copper exposure on temperature acclimation, a similar procedure was used, however between day 3 and 4, the temperature in the water bath and test chambers was gradually increased from 20°C to 30°C over a period of 6 hours. After $\dot{V}O_2$ measurements at 30°C on day 4 the fish were placed in control or experimental tanks at 30°C. They were returned to the chambers on days 8 and 31 and tested on days 9 and 32.

Tissue Oxygen Consumption Measurements:

Fish utilized for tissue oxygen consumption determinations

(QO_2) underwent the same procedure of copper exposure, transfer, and, in the case of those which would be exposed to an increase in temperature, gradual temperature increase on day 4 of exposure. Just prior to QO_2 measurement, fish were stunned by a blow to the head and killed by spinal transection. They were then placed on ice and the liver and brain removed and placed in cold Hickman's saline (Hale, 1965). In addition, bile was removed from the gall bladder with a 1 ml syringe. The gills were then repeatedly washed with cold Hickman's saline and the arches excised and placed in cold saline. The left front gill arch was fixed in 2% gluteraldehyde for later histological examination.

Tissues were prepared for in vitro oxygen consumption measurements as follows: The liver was blotted dry and pressed through a stainless steel screen with mesh of approximately 0.5 mm. To this, 1 ml. of Hickman's saline with 1% glucose was added. The brain was blotted dry and placed in 1 ml of Hickman's saline with 1% glucose and finely minced with scissors. The gill arches were blotted dry, and the filaments cut from the arches with scissors and placed in 1 ml of saline with 1% glucose.

Oxygen consumption measurements of the tissue suspensions were carried out in a constant temperature chamber fabricated from a 10 ml graduated cylinder with a surrounding water

jacket which was maintained at the fish experimental temperature. The sample was aerated by bubbling compressed air through it for approximately one minute. The aerator was then withdrawn, a miniature magnetic stirring bar added, and a Beckman model 325814 oxygen macroelectrode was introduced into the chamber in such a way that all bubbles in the solution were excluded. The electrode was connected to a Radiometer model PHA927b gas monitor and the decline in PO_2 was recorded on a chart recorder while the suspension was stirred with a magnetic stirrer. Following each measurement the mixture was removed, placed in a tared plastic weighing boat and dried to constant weight. Oxygen consumption was calculated by first measuring the length of time necessary for the oxygen in the sample to be reduced from 80 mmHg to 40 mmHg PO_2. The amount of oxygen represented by this reduction was calculated from the oxygen solubility. Finally, the small amount of oxygen consumed by the electrode, which was measured following each day's experiment, was subtracted from this value. The oxygen consumption was then divided by the corrected dry weight of the tissue (corrected by subtracting the added weight of the salts and glucose in the saline) to provide a weight specific rate of oxygen consumption. All whole body and tissue oxygen consumption measurements were adjusted to standard temperature and pressure.

Following the dry weight determination, each tissue sample was analysed for copper using the method of Leonard (1971). Samples were digested in 30 ml of concentrated nitric acid and 5 ml of concentrated perchloric acid. This mixture was heated to 100°C and allowed to evaporate to dryness. Additional nitric acid was added to samples in which the fats came out of solution and they were allowed to evaporate to dryness. The residues were then dissolved in 3.0 ml of deionized distilled water. Analysis was performed with a Perkin-Elmer model atomic absorption spectrophotometer at the settings recommended by the manufacturer.

Bile samples were weighed and 0.15 ml of concentrated nitric acid and 0.5 ml of deionized distilled water were added. The copper content was measured with the atomic absorption spectrophotometer.

The fixed gill lamellae from each group were infiltrated with paraffin and sectioned. The paraffin was then dissolved with xylene and the tissues were hydrated with an alcohol series, stained with hematoxylin and eosin, dehydrated with an alcohol series and mounted.

All means were compared utilizing the Student's T-test. Statistical significance was assigned at the 0.05 probability level. In addition, probabilities falling between 0.1 and 0.05 were noted as trends.

RESULTS

20°C Experiments

Whole animal oxygen consumption of copper exposed fish was not significantly different from controls through day 9 of exposure (Fig. 2). However, exposed animals were found to have significantly lower oxygen consumption than controls on day 32 of exposure.

Tissue oxygen consumption on day 3 of exposure (Fig.3a) showed no significant differences between exposed and control animals, however the oxygen consumption of the liver from exposed animals showed a trend toward decreased oxygen consumption. By day 32 of exposure (Fig.3b) this trend had reversed itself and the oxygen consumption of the liver tissue from exposed fish was significantly higher than the oxygen consumption of controls. In addition, the brain tissue of bluegill exposed to copper for 32 days showed a trend toward lowered oxygen consumption as compared to day 32 controls. There was no difference in the oxygen consumption rate of gill tissue from exposed and control animals at either day 3 or day 32 of exposure.

Tissue copper analyses on day 3 of exposure (Fig.4a) revealed no increase in the concentration of copper in the liver or brain of exposed animals, however, the gills of exposed animals did show significantly higher concentrations of copper than controls. On day 32 of exposure (Fig.4b), the

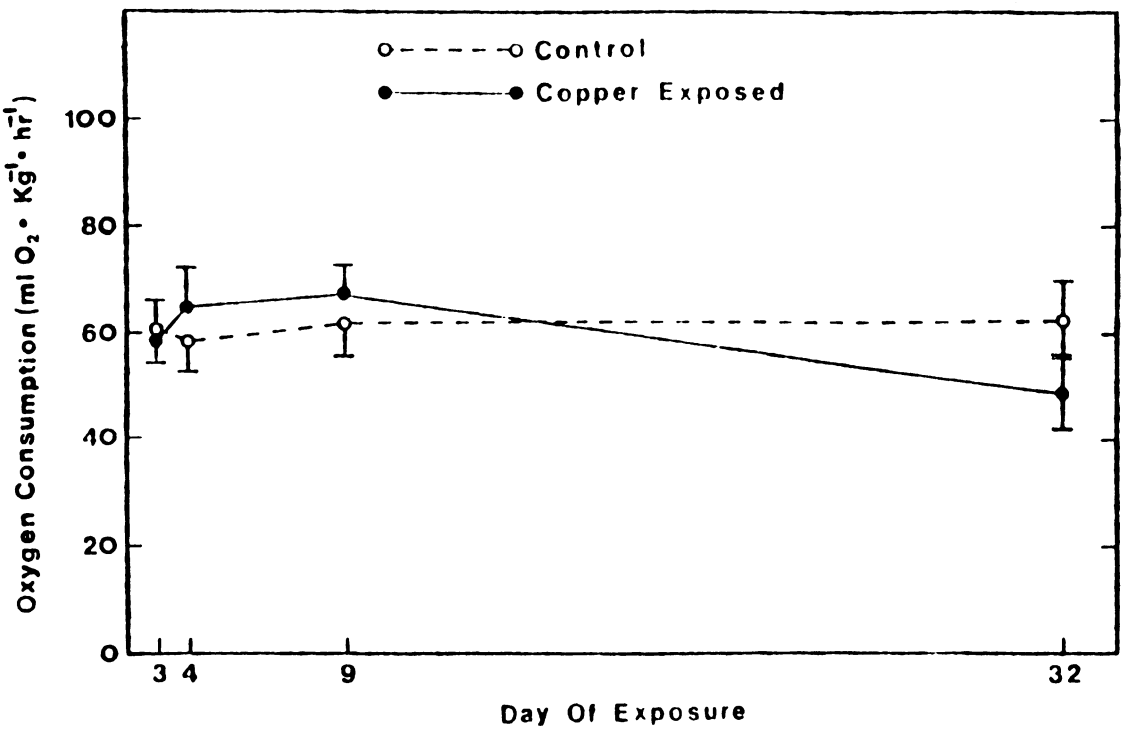

Figure 2. Whole body oxygen consumption of bluegill exposed to sublethal copper concentrations for various periods of time at 20°C. Bars represent ± 2 standard errors of the mean, n numbers ranged from 24 to 48.

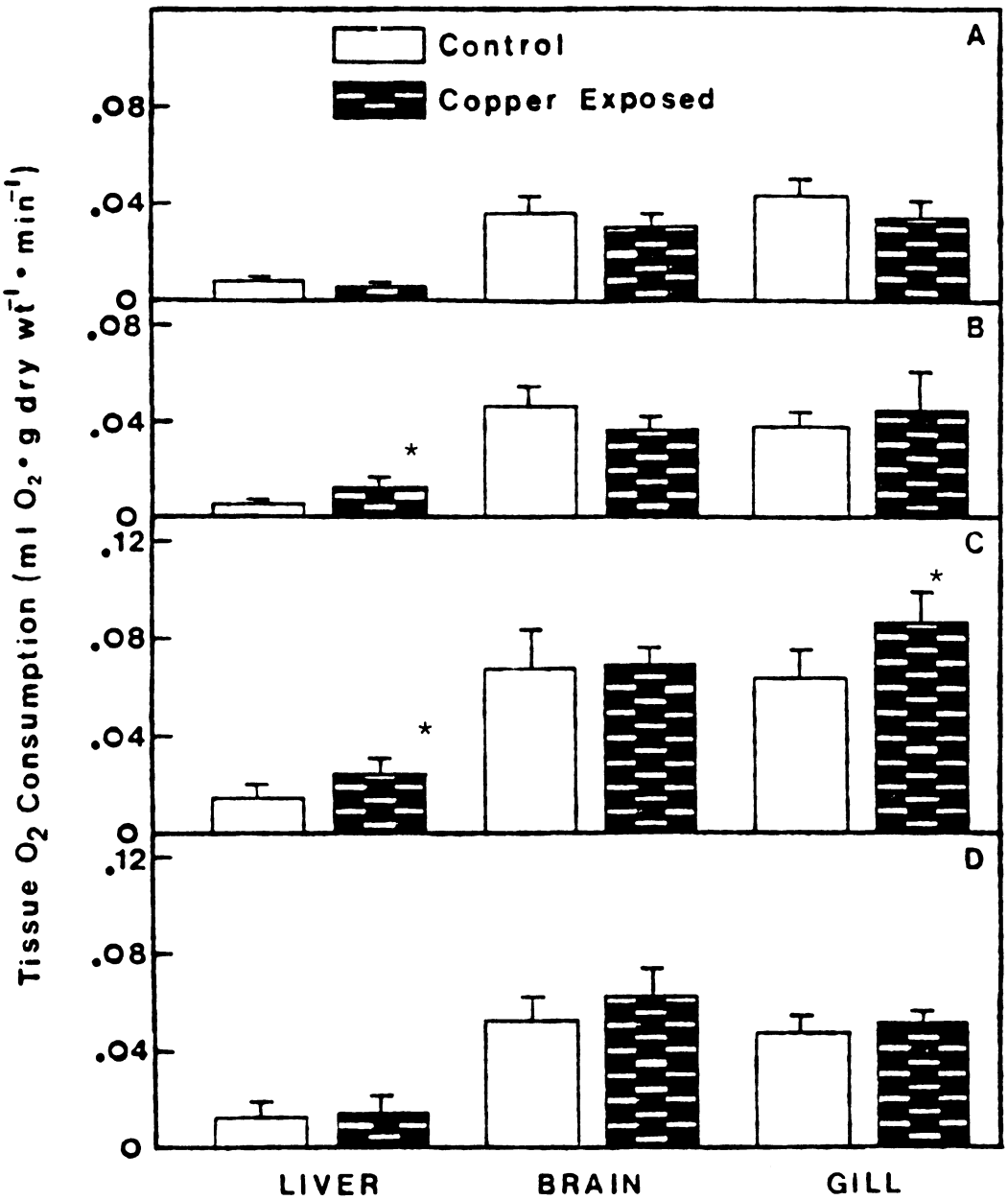

Figure 3. In vitro tissue oxygen consumption (QO_2) of liver, brain and gill from bluegill exposed to sublethal copper. (A) QO_2 for bluegill exposed to sublethal copper for 3 days at 20 C. (B) QO_2 for bluegill exposed to sublethal copper for 32 days at 20 C. (C) QO_2 for bluegill exposed to sublethal copper for 9 days and subjected to an increase from 20 C to 30 C between days 3 and 4 of exposure. (D) QO_2 for bluegill exposed to sublethal copper for 32 days and subjected to an increase from 20 C to 30 C between days 3 and 4 of exposure. Bars represent + 2 standard errors of the mean, n numbers range from 5 to 8. Astericks indicate levels which are significantly different from control values.

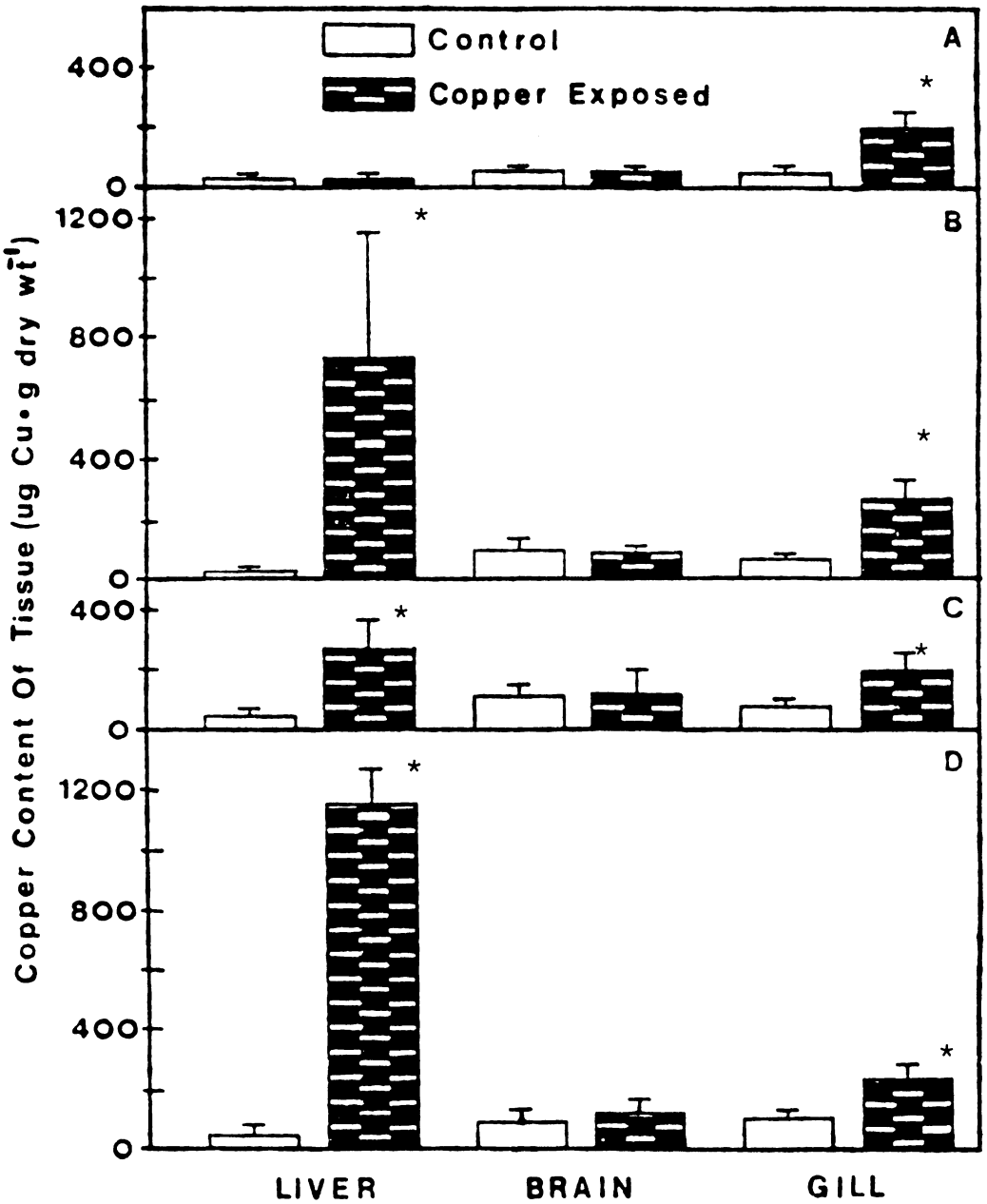

Figure 4. Copper content of liver, brain, and gill from bluegill exposed to sublethal copper concentrations. (A) Bluegill exposed to sublethal copper for 3 days at 20 C. (B) Bluegill exposed to sublethal copper for 32 days at 20 C. (C) Bluegill exposed to sublethal copper for 9 days and subjected to an increase from 20 C to 30 C between days 3 and 4 of exposure. (D) Bluegill exposed to sublethal copper for 32 days and subjected to an increase from 20 C to 30 C between days 3 and 4 of exposure. Bars represent + 2 standard errors of the mean, n numbers range from 5 to 8. Astericks indicate levels which are significantly different from control values.

copper concentration in the gills of exposed animals was again significantly higher than controls, but only slightly higher than day 3. Again, there was no difference in brain copper concentrations. There was however, a large, variable, statistically significant increase in the level of copper in the liver of animals exposed to sublethal copper for 32 days.

The concentration of copper in the bile of exposed fish was significantly greater than that in control fish on days 3 and 32 of exposure (Table 1).

30°C Experiments

Control animals which were exposed to a 10°C increase in temperature between days 3 and 4 of the experiment showed a typical increase in whole body oxygen consumption (Q_{10}=1.8) on day 4 followed by a partial compensation by day 9 of the experiment (Fig.5). Copper exposed animals showed a similar increase in oxygen consumption on day 4 of exposure (Q_{10}=1.9) however there was no indication of compensation by day 9 of the experiment. Whole body oxygen consumption on day 9 of the experiment was significantly higher in copper exposed animals than in controls.

This difference between exposed and control fish was reflected in statistically significant higher tissue QO_2 rates in both the liver and gill of animals exposed to sublethal copper for 9 days (Fig.3c). By day 32 of exposure

Table 1. Bile and liver copper concentrations in bluegill following exposure to copper at two temperatures and for various lengths of time.

Temperature (°C)	Length of exposure (days)	Group	n	Bile copper concentration (µg Cu/g bile)	Liver copper concentration (µg Cu/g liver)[a]
20	3	control	5	3.21±2.18[b]	5.37± 3.00
20	3	exposed	6	11.80±5.54	7.05± 2.64
20	32	control	3	1.78±1.08	4.26± 2.07
20	32	exposed	6	21.05±8.04	218.25±127.77
30	9[c]	control	6	3.49±1.03	11.37± 9.21
30	9	exposed	8	10.55±4.02	79.14± 30.09
30	32	control	6	5.36±3.90	14.61± 14.60
30	32	exposed	8	15.98±4.55	345.72± 36.09

a Wet weight of liver based on water weight of 70% for bluegill liver
b Two standard errors of the mean.
c All animals in the 30°C groups began exposure at 20°C and were raised from 20°C to 30°C between days 3 and 4 of exposure.

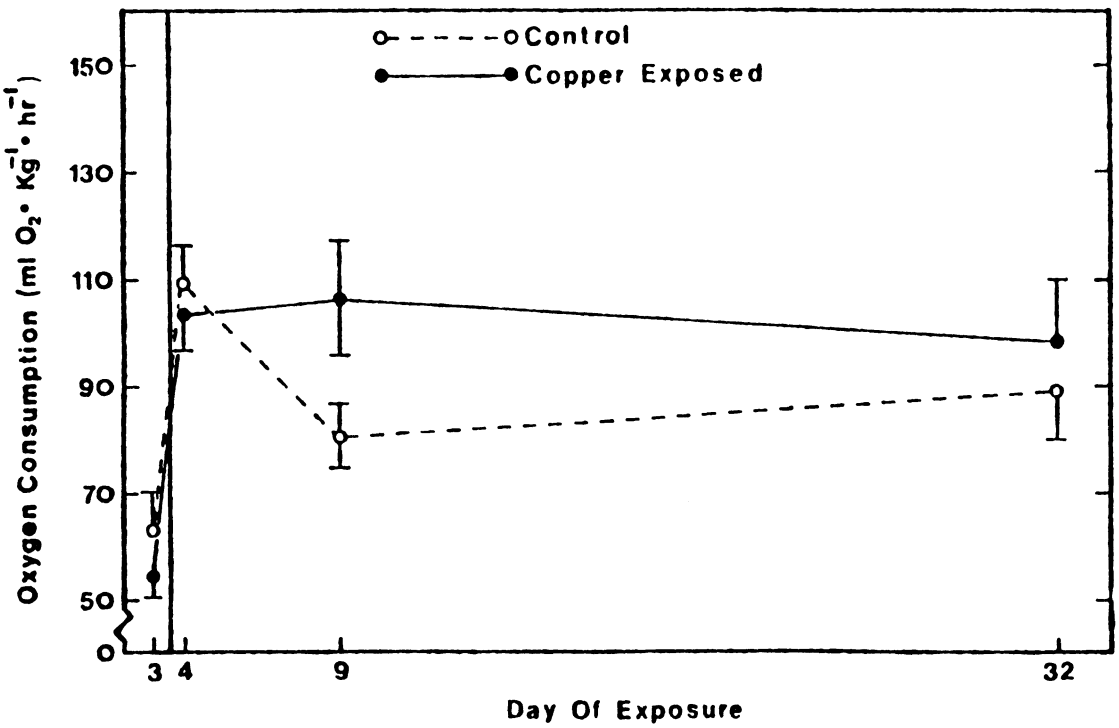

Figure 5. Whole body oxygen consumption of bluegills exposed to sublethal copper. Exposure began at 20°C. Between days 3 and 4 of exposure, the temperature was increased from 20°C to 30°C. This increase is represented by the vertical line between days 3 and 4. Bars represent ± 2 standard errors of the mean, n numbers range from 24 to 48.

there were no significant differences in the tissue QO_2 rates of copper exposed and control animals in any of the tissues tested (Fig.3d).

Tissue copper concentrations in the liver and gill of animals exposed to sublethal copper for 9 days and a 10°C increase in temperature between days 3 and 4 of the experiment were significantly higher than controls, but there was no increase in brain copper levels (Fig.4c). This was also the case on day 32 of exposure (Fig.4d). It should be noted that liver copper concentrations from animals exposed 32 days were approximately four-fold the levels of liver copper concentrations in animals exposed only 9 days.

Bile copper concentrations of animals exposed to sublethal copper for 9 days or 32 days and a temperature increase between days 3 and 4 of the experiment were significantly higher than corresponding controls (Table 1).

Histological Examination

Histological examination of gill tissue from all groups represented in the tissue oxygen consumption experiments (control and exposed animals from 20°day 3 of exposure, 20° day 32 of exposure, 30°day 9 of exposure, and 30°day 32 of exposure) showed no apparent changes in gill morphology in any groups. There was no apparent congestion in the lamellae or separation of the epithelium from the lamellae as was observed by Baker (1969).

DISCUSSION

Whole body oxygen consumption ($\dot{V}O_2$) measurements indicate the energy costs of existence for an animal. Environmental factors which affect that cost can be classified as controlling, limiting, or masking (Fry, 1971). Under this scheme, temperature is a controlling factor, dissolved oxygen a limiting factor. A masking factor is one that modulates the effect of one of the others (e.g. salinity). Regarding copper, O'Hara (1971) found that bluegill exposed to copper (after an initial stimulation of $\dot{V}O_2$) exhibited a dose proportional lowering of $\dot{V}O_2$ which persisted for the 7 days of his experiments. Therefore, using Fry's (1971) terminology, copper was acting as a controlling factor. In the present study, copper was at a lower concentration than used by O'Hara (1971). (It cannot be determined exactly how much lower as he gave only total copper concentrations, not soluble copper as was done here). There was no effect on $\dot{V}O_2$ for the first 9 days of exposure at 20°C, thus at this concentration copper exerted no controlling effect on routine metabolism until the exposure had been prolonged beyond 9 days. When measured at 32 days of exposure, the experimentals did show a lower $\dot{V}O_2$ which is probably due to the time required for copper to accumulate in the tissues (Fig.4).

The decrease in oxygen consumption by bluegills exposed

to sublethal copper for 32 days was not reflected in a decrease in *in vitro* oxygen consumption of liver, brain, or gill. Instead, liver *in vitro* oxygen consumption was higher than controls on day 32 and the other two tissues were unchanged, indicating that the effect of copper in depressing oxygen consumption may be at higher levels of integration such as neuromuscular activity. Copper apparently does not accumulate significantly in the central nervous system and therefore is probably not acting directly upon it to decrease oxygen consumption. However, small increases in brain copper concentration which could be masked by the inherent variability may be sufficient to affect brain function. In addition, the brain may be affected secondarily by another effect of copper such as osmotic changes in the plasma. *In vitro* measurements of muscle QO_2 are difficult to interpret because the cell structure is generally destroyed in preparing slices or minces (Umbreit *et al.*, 1967). For that reason, it was not measured in this investigation. However, it seems safe to surmise that a reduction in skeletal muscle metabolism occurred upon prolonged copper exposure, particularly when it is realized that the fishes' body is some 60% muscle (Smith, 1982). Whether this is due to a reduction in nervous tone, spontaneous activity, or a direct effect of copper on muscle metabolism cannot be determined at this time.

The liver, which acts as the major organ of copper homeostasis, showed an elevated energy expenditure which may reflect clearance of the excess copper load as evidenced by the significant increases in bile copper (Table 1). The brain, however, does not accumulate copper and may therefore be protected from its metabolic effects. Gill tissue is the first to accumulate copper but the lack of a change in oxygen consumption may reflect an increase in mucous production by the mucous glands of the gill lamellae (which would increase QO_2) countered by the inhibition of the Na^+, K^+-ATPase system (Lorz and McPherson, 1976).

The whole body oxygen consumption of bluegills in the control portion of the 30°experiments exhibited a classic Type III (Precht, 1958) partial compensation to an increase in temperature. This was not the case with bluegills exposed to sublethal copper (Fig.5). The lack of significantly higher oxygen consumptions in the exposed animals on day 9 of the 20° experiments (Fig.2) indicates that the absence of temperature acclimation by exposed animals between days 4 and 9 of the 30° experiments was not simply an additive effect resulting from the combination of copper increasing oxygen consumption while simultaneously, temperature acclimation tending to decrease oxygen consumption. This indicates, rather, that copper is acting directly on the temperature acclimation process to delay compensation. According to Fry's (1971) scheme, copper must be

acting here as a masking factor. Since the gill and liver show significant increases in *in vitro* oxygen consumption over controls on day 9 of the 30°experiment (Fig.3c) but not on day 32 (Fig.3d), it is probable that copper is acting on the tissues directly to delay acclimation. It is possible that copper is directly influencing enzymes in metabolic pathways since copper is known to bind proteins (chiefly at the site of the imidazole nitrogen of histidine and the thiol group of cysteine (Venugopal and Luckey, 1978)) and temperature acclimation involves both changes in enzyme activities and in isozyme patterns (Shaklee *et al.*, 1977).

The delay in metabolic compensation by fish exposed to sublethal copper and a rise in temperature has ecological implications. Temperature acclimation following exposure to a higher temperature normally provides a decrease in energy consumption to a level below that defined by the Arrhenius equation which would dictate that each 10°rise in temperature results in an approximate doubling of rate (Q_{10}=2). Any interference with the temperature acclimation process would result in an increase in energy demand by the animal and may influence its ability to survive in the environment. Further study is needed to determine the exact mechanism by which copper interferes with temperature acclimation and on the environmental implications this may have on the animal.

The finding of copper accumulating in the liver and gill,

but not in the brain, is in good agreement with the reports of others (Brungs et al., 1973; Benoit, 1975). Copper levels in the gills reached a plateau by day 3 and did not increase further, however copper was being taken up by the animal after day 3 as indicated by the large increase in liver copper by day 32 of both the 20° and 30° experiments. This indicates that copper rapidly reaches equilibrium within the gills after which copper simply passes through the gills into the blood. Recent findings of a copper transport protein in the blood of fish (Fletcher and Fletcher, 1980) similar to the mammalian ceruloplasmin, indicate that copper is carried in the blood of fish bound to a transport protein much as it is in mammals. This protein bound transport may explain the lack of copper accumulation in the brain since the blood-brain barrier is very efficient at preventing the entrance of proteins into the intercellular space of the central nervous system (Bradbury, 1979). In addition, any unbound copper in the blood would probably occur in the ionized state thus it too would be unlikely to cross the blood-brain barrier.

Liver copper was found to be elevated by day 9 of the 30° experiments and highly elevated by day 32 in both the 20° and 30° fish. Bile copper was significantly elevated on day 3 of the 20° experiments indicating that increased copper was reaching the liver by day 3, however, the levels in the liver were not

elevated at this time, thus the liver was able to successfully eliminate the excess copper up to that time. However, this process apparently reaches a plateau such that it does not significantly increase the concentration of copper in the excreted bile after day 3 of exposure while the liver copper concentration continues to rise.

A wet weight liver copper concentration was computed and compared to the bile copper concentration (Table 1). For computational purposes, a water weight of 70% was used for bluegill liver (Heath, unpublished). These data indicate that on day 3, the bile exceeded the liver in copper concentration, but as the exposure period continued, a gradient was established toward the bile. Therefore, an active transport mechanism for copper must be involved, as reported in mammals (Klassen, 1975), but this process became saturated after day 3 so that the copper continued to increase in concentration in the liver but not in the bile.

Lech et al. (1973) found that certain pesticides concentrate in the bile of rainbow trout as does hexavalent chromium (Buhler et al., 1977). The findings that copper also concentrates in fish bile, even prior to the increase in copper in liver tissue, may provide a simple assay tool in investigations of possible copper intoxication. To develop this tool, further research is needed on the time course of copper disappearance from the bile after exposure to the toxicant has ended, and on the

suitability of bile analysis for fish autopsy, where it may be quite useful in fish kill investigations.

In conclusion, sublethal copper appears to suppress whole body oxygen consumption in bluegill exposed at 20°C by acting at higher levels of integration than individual tissues, since tissues from exposed animals showed no decrease in QO_2. A second major effect of sublethal copper was found to be the delay of temperature acclimation in bluegills exposed to an increase in temperature. This effect was reflected in the oxygen consumption of certain tissues from exposed animals indicating that copper acts directly on the tissues to delay temperature acclimation. Increases in the copper concentration of bile from exposed fish were seen by day 3 of exposure and continued throughout the experiment. Copper concentrations in the bile were elevated above control values before higher levels were observed in the liver itself, indicating that in the early stages of copper intoxication, biliary excretion keeps pace with the delivery of copper to the liver. Finally, copper was found to accumulate in the gill as well as the liver of exposed fish, but not in the brain.

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INTERACTIONS OF TEMPERATURE AND SUBLETHAL ENVIRONMENTAL COPPER EXPOSURE ON THE ENERGY METABOLISM OF BLUEGILL (LEPOMIS MACROCHIRUS)

by

Paul Anson Felts

(ABSTRACT)

The effects of sublethal copper on metabolism were investigated in bluegill (Lepomis macrochirus) by measuring whole body oxygen consumption in fish exposed to sublethal copper alone and in conjunction with a temperature increase. In vitro oxygen consumptions of liver, brain, and gill were also measured under these two conditions, as was the accumulation of copper in these tissues. In addition, the concentration of copper in bile was measured.

Copper was found to decrease whole body oxygen consumption in animals exposed to copper alone, although the oxygen consumptions of tissues were not significantly altered. This indicates that copper is acting to decrease $\dot{V}O_2$ at a higher level of integration than the individual tissues.

In animals subjected to an increase in temperature as well as sublethal copper, oxygen consumption was higher than controls five days after the temperature was increased, indicating a delay in temperature acclimation. This increase was reflected in higher in vitro oxygen consumption in the liver and gill indicating that sublethal copper delays temperature acclimation by acting directly on the tissues.

Tissue copper accumulation was seen first in the gills followed by accumulation in the liver. Copper was not found to accumulate in the brain. Increased copper levels were found in the bile at all tested exposure times. A discussion of the ecological implications of these findings is included.