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SOCIAL INTERACTIONS AFFECT PHYSIOLOGICAL CONSEQUENCES OF SUBLETHAL COPPER EXPOSURE IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

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Abstract—The interactions of sublethal waterborne copper exposure and social dominance behavior were examined in juvenile rainbow trout. Dominance hierarchies were determined between pairs of fish by behavioral observations and among groups of 10 fish by the use of passive integrated transponder (PIT) tagging equipment. The present study is one of the first to utilize this novel PIT tag method for behavioral assessment. Feeding behavior was quantified by placing a PIT tag recorder at the entrance to the feeding area. Linear dominance hierarchies were documented based on these observations of feeding behavior. Dominance hierarchies established in control water were not altered by exposure to 30 μg/L of copper; however, physiological responses of fish to sublethal concentrations of copper were related to social rank. Subordinate fish exhibited a higher accumulation of copper in both gill and liver tissue. Subordinates of paired fish were also shown to have a higher uptake of sodium than dominant fish, and the uptake of sodium was correlated with uptake of copper from the water. Therefore, within a population of fish, it cannot be assumed that individuals of different social status will exhibit the same physiological responses to the presence of copper.

Keywords—Copper Passive integrated transponder tagging Dominance Behavior

INTRODUCTION

Copper is an essential element to many functions in the vertebrate body. However, in excess, copper can be toxic. Therefore, homeostatic mechanisms have evolved to regulate internal copper to optimum levels [1]. In fish, physiological effects of excess copper may include changes in hematology [2,3], ionoregulation [4,5], olfaction [6], and swimming performance [7]. Although much is now known about the physiological effects of copper, less is known about the effects of copper on behavior. The majority of behavioral studies involving copper have focused on avoidance behavior and cough and ventilatory responses as sensitive endpoints to toxicological trials [8–11]. However, there are many other sensitive behaviors exhibited by fish exposed to copper that also are ecologically relevant to natural populations of fish. Impairment of swimming performance and olfaction may affect the competitive ability of a fish and influence the ability of fish to form social hierarchies. Stability of population structure among groups of salmonid fish in the natural environment could therefore be threatened by the presence of copper.

Linear dominance hierarchies, consisting of one dominant fish at the top of the hierarchy, are frequent characteristics of stream-dwelling salmonid fish. These hierarchies have been successfully studied in both natural [12] and laboratory conditions [13–17] among groups of fish ranging in number from two to a hundred. Dominance hierarchies among juvenile fish form as a result of competition for food and space within a stream environment [18,19], with dominant fish acquiring the most profitable positions. Confinement of fish in pairs within artificial laboratory conditions has demonstrated physiological consequences, including elevated plasma cortisol concentrations [17,20,21] and metabolic rate [22], in subordinates of paired fish. Some physiological disadvantages have also been seen in subordinate fish held in stream tanks, including increases in interrenal cell activity [14] and decreases in growth [16]. The ability to replicate this social structure in the lab both between pairs of fish (an approach designed to maximize social interaction) and in stream tanks (a situation designed to replicate the natural environment of the fish as much as possible) makes this characteristic social behavior an ideal subject for further toxicological studies.

The aim of the present study was to examine the effects of sublethal concentrations of copper on dominance hierarchies under different levels of social complexity. Previous research has shown that dominance hierarchies remain stable under controlled conditions but may be disrupted by environmental perturbations such as lowered water levels [23]. Disruption of dominance hierarchies under these conditions may be attributable to the fact that the change in environment (in particular topography and mean water depth) may have favored individual fish, resulting in a change in dominance relationships. Exposure of social hierarchies to copper may affect olfactory receptors [6] and swimming performance [7] and thus disrupt social interactions. Physiological differences that arise as a result of social interactions (e.g., differences in specific growth rates [13,15,16,23]) may also be affected by the presence of copper. In this study, we studied the effects of copper on both hierarchy structure and the physiology of different social ranks among groups of rainbow trout, *Oncorhynchus mykiss*. Hierarchy structure was defined by measurement of feeding behavior that was indicative of dominance. Physiological measurements of different social ranks included growth and sodium and copper uptake.

METHODS

Rainbow trout, *Oncorhynchus mykiss*, (young of the year), were obtained from Rainbow Springs Hatchery (Thamesford, ON, Canada) and held in flow-through tanks (500 L) supplied with Hamilton City tap water (hardness ~ 120 mg/L as $CaCO_3$,

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$Na^+ \sim 14$ mg/L; $Cl^- \sim 25$ mg/L; $Ca^{2+} \sim 40$ mg/L; 13°C; pH ~ 8.0; natural background copper concentration of 2.46 ± 0.16 μg/L [$n = 8$]; dissolved organic carbon ~ 3 mg/L) for at least two weeks before experiments were performed. All experiments were carried out in this water. Fish were fed a 1% ration (food wt/wet body wt) daily of commercial trout pellets (Martins Feed Mill, Elmira, ON, Canada; copper concentration ~ 3 μg/g). A 1% ration was used to ensure that rapid growth of the fish did not occur during acclimation to the aquarium conditions.

This study consisted of three separate experiments. Experiments 1 and 2 were short-term studies utilizing pairs of fish held for 48 h under conditions designed to maximize social interaction between the fish. The objective of experiment 1 was to consider both the behavioral and physiological effects of exposure of pairs of fish to 30 μg/L copper. Experiment 2 aimed to clarify that the higher gill copper concentration exhibited by subordinate fish in experiment 1 was indicative of a higher uptake rate of copper from the water and to investigate whether this increased copper uptake was related to increased sodium uptake. Experiment 3 was a two-week study using stream tanks to observe groups of 10 fish in conditions designed to simulate those of the natural environment. The objective of experiment 3 was to consider the effects of 30 μg/L copper on the behavior and physiology of larger social groups of fish.

Experiment 1—Effect of exposure to 30 μg/L of copper on behavior and tissue copper concentrations of pairs of fish

Fish (weight = 1.45 ± 0.09 g; length = 5.19 ± 0.08 cm; $n = 18$) were anesthetized in MS222 (tricaine methane sulfonate; 0.08 g/L) and individually marked with alcian blue dye injected into their fins [24]. Initial fork lengths and weights were recorded. Fish were then placed in size-matched pairs in flow-through 4.5-L glass aquaria but were separated from each other by an opaque plastic partition. After a 48-h acclimation (fasting conditions) to the aquaria and recovery from handling stress, the partition was removed to allow the fish to swim in the same area, and behavioral observations were made three times daily for the remainder of the experiment (complete details on behavioral observations are given later). Refuges (polyvinyl chloride tubes ~2 cm in diameter, 5 cm in length) were added to the tanks at the time that the partitions were removed to provide shelter for the subordinate fish from the continual aggression of the dominant fish. Fish were confined for 48 h in pairs under control conditions for social acclimation. At $T = 48$ h, copper was introduced into the mixing tanks by using a copper sulfate stock (0.1886 g/L $CuSO4 \cdot 5H_2O$; Fisher Scientific, Toronto, ON, Canada) at a rate of 0.5 ml/min. Mixer tanks were supplied with control water at a rate of 800 ml/min to dilute the stock to the final exposure concentration of 29.10 ± 0.23 μg/L. The copper solution was introduced to replicate experimental tanks at a rate of 100 ml/min. A 50% molecular exchange occurred within the experimental tanks every 31.5 min. The copper concentration in the tanks was maintained for the next 48 h and behavioral observations were continued. The final copper exposure concentration was approximately 30% of the acute 96-h LC50 (the concentration at which 50% mortality is seen at 96 h). This exposure concentration is reflective of copper concentrations in highly contaminated waters.

At 48-h postexposure to copper, the fish were killed by a blow to the head. Gill (branchial basket), liver, kidney, and remaining carcass were removed, weighed, and stored for later analysis. Tissues were digested in a known volume of 1 N HNO_3 (Fisher Scientific; trace metal analysis grade) for 48 h at 55°C. Water samples and the supernatants from the gill, liver, kidney, and carcass tissues were analyzed for copper by graphite furnace atomic absorption spectrophotometry (Varian AA-220, GTA 110, Varian, Walnut Creek, CA, USA) using Fisher Scientific-certified copper standards and operating conditions as documented by the manufacturer.

Experiment 2—Uptake of copper and sodium in pairs of fish

Fish (weight = 3.69 ± 0.5 g; length = 6.13 ± 0.4 cm; $n = 18$) were anesthetized, individually marked with alcian blue dye, and placed in size-matched pairs. Behavioral observations were made exactly as in experiment 1, allowing 48 h of acclimation to the aquaria and recovery from handling stress before initiation of behavioral measurements. Complete details on behavioral observations are given below. After 48 h of confinement in pairs, water inflow to the tanks was stopped and a combination of ^{64}Cu (McMaster Nuclear Reactor, Hamilton, ON, Canada; 0.05 μCi/L) and ^{22}Na (NEN–Dupont, Boston, MA, USA; 1 μCi/L) was added to the tanks. A water sample was taken 15 min after the addition of radioisotope and analyzed to determine initial specific activity. A spike of copper stock was also added to the tank at the same time to yield a measured concentration of 19.89 ± 0.55 μg/L copper. Two hours after the addition of radioisotopes, another reference water sample was taken for calculation of final specific activity and the live fish were transferred for a 2-min period to a cold displacement rinse to remove any surface-bound isotope. The rinse consisted of 600 mM NaCl and 30 mg/L Cu. Fish were then killed by a blow to the head and placed in preweighed scintillation vials. Copper concentrations of the water samples were determined by graphite furnace atomic absorption spectrophotometry as for experiment 1. Sodium concentrations were measured by flame atomic absorption spectrophotometry (Varian AA-220, Varian, Walnut Creek, CA, USA) using Fisher Scientific-certified sodium standards. The ^{22}Na and ^{64}Cu activity was determined in the reference water samples and the whole fish using an 8-cm well NaI crystal gamma counter (Canberra Packard MINAXI Auto Gamma 5000, Mississauga, ON, Canada). Due to the short half-life of ^{64}Cu (12.7 h), samples were counted once immediately after the experiment and again one week (~13 half-lives) later. The difference in counts was equal to the activity of ^{64}Cu for each sample, the remainder of the original count being equal to the activity of ^{22}Na for each sample. Uptake of both copper and sodium was calculated using the equation

$$\text{uptake} = ([a/b]/c)/d$$

where a = counts per minute, b = mean specific activity (activity per unit concentration), c = weight of fish (g), and d = duration of exposure (hours) [25]. The experiment was repeated using ^{22}Na alone to ensure that sodium uptake rates were not significantly affected by the copper spike.

Behavioral observations—Experiments 1 and 2

Fish were observed three times daily (9:00 AM, 12 noon, and 4:00 PM) after removal of the partition and were scored according to their position in the tank, their food intake, and their coloration. Position and food scores were based on methods used by Sloman et al. [17]. Fish patrolling in the water

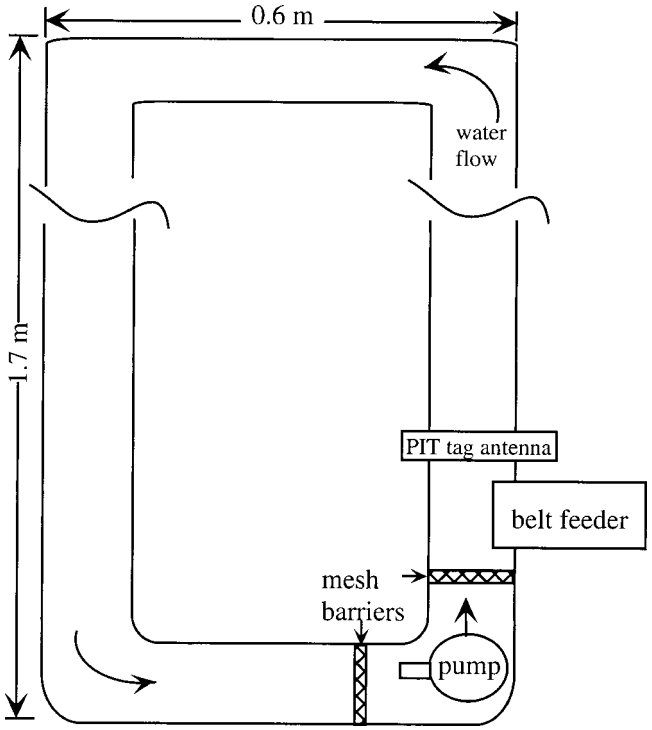


Fig. 1. Plan of experimental stream tanks. The direction of water flow is indicated. Water was driven by a pump with an outflow also in the pump area. Fish could not cross the pump area. Food was delivered by a belt feeder to the upstream end of the tank. Depth of the stream tank was 5 cm. PIT = passive integrated transponder.

column scored three points; fish hovering above the bottom of the tank scored two points; those fish resting on the bottom of the tank or hiding in refuges scored one point; and those fish swimming at the water surface scored zero points. Avoiding aggression from the dominant fish by swimming at the water surface is a behavior frequently recorded in subordinate fish [17,21,23]. At the time of each set of behavioral observations, a food pellet was introduced to the tank and the fish that consumed the food particle scored one point; the other fish scored zero points. It has been demonstrated previously [26] that subordinate fish will darken in coloration as a signal of submissiveness. Therefore, fish were also scored according to their coloration: those that were light scoring one point and those that were dark scored zero points. Behavioral scores from each fish were then combined and analyzed using a principal-components analysis to generate a single behavior score for each fish (see *Statistical analysis* for further details).

Experiment 3—Exposure of groups of fish in stream tanks to 30 μg/L copper

One week prior to the start of the experiment, fish (weight = 2.74 ± 0.11 g; length = 0.6 ± 0.1 cm; n = 100) were anesthetized in MS222 (0.08 g/L) and each individually tagged by the implantation of a passive integrated transponder (PIT) tag (12-mm identity tags TX1400L, 125 kHz, Destron Fearing, South St. Paul, MN, USA) in the peritoneal cavity. After a one-week recovery period, fish were size matched and placed in groups of 10 in a 10-L stream tank (Fig. 1). Water was pumped around the stream tank, producing a unidirectional flow rate of 5 cm/s, and food was introduced at the upstream end of the tank; fish could only access food by swimming upstream to the food source. A PIT tag transceiver (portable transceiver system FS2001, Destron Fearing) was placed slightly downstream of the food entry point to record any fish attempting to enter the feeding area. The PIT tag data were recorded continuously from the transceiver system through a pocket reader (HS 6100L, Destron Fearing) to a computer and read using Software Wedge for Windows 1.2v (TAL Technologies, Philadelphia, PA, USA). The software program Software Wedge then collected the PIT tag data in an Excel® (Microsoft, Redmond, WA, USA) spreadsheet that could be used for later analysis. A 3% food ration was supplied daily at a continuous rate over a 12-h period using a belt feeder (Aquatic Ecosystems, Apopka, FL, USA). To avoid complications with diurnal behavioral changes, fish were kept in continuous dim light conditions throughout the experiment.

Over the first week in the stream tank, fish were left undisturbed to allow a dominance hierarchy to be established. After week one, fish were either left for an additional week in normal water (control) or were exposed to a copper concentration of 30.69 ± 0.75 μg/L for a further week (copper treatment). In the same way as in experiment 1, copper sulfate stock was introduced into a mixing tank, which supplied the stream tank with water at a rate of 250 ml/min. Fifty percent molecular exchange occurred within the stream tank every 28 min. Five replicates of both control and experimental treatments were carried out and the results combined for statistical analysis (see below).

After two weeks confinement in groups of 10, either as control (no added copper) or copper treatments (copper ~ 30 μg/L), fish were killed by a blow to the head and final fork lengths and weights recorded. Gill (branchial basket), liver, and kidney tissues were removed from the fish, weighed, and stored in separate containers. The rest of the carcass was also stored for later analysis of copper content as in experiment 1.

Behavioral analyses were carried out by ranking the fish according to their number of PIT tag entries (i.e., number of times a tag was detected by the reader). Dominant fish within each group (i.e., the fish with the highest number of PIT tag readings) were ranked as one, the second highest number of readings as two, and so forth. Fish ranked five or above were not easy to differentiate due to the infrequency of entry to the feeding area and so were grouped together for statistical analyses. A few random mortalities of fish in ranks 5 to 10 account for the smaller sample sizes in these groups.

Statistical analysis

Specific growth rates were calculated on individual fish as percent change in weight per day $([\ln y_2 - \ln y_1]/[t_2 - t_1]\cdot 100$, where y_2 = final weight, y_1 = initial weight, $[t_2 - t_1]$ = duration of experiment [days]). Data are given as means ± standard error of the mean. Physiological and behavioral values were compared between pairs of fish in experiments 1 and 2 using independent samples t test analyses. A paired t test was used in experiment 2 to compare sodium uptake rates of subordinate fish with those of its dominant pair. Comparisons of physiological and behavioral values among ranks of fish in experiment 3 were made using a two-way analysis of variance followed by Scheffé's tests for multiple comparisons. Linear regression analyses were also carried out on both behavioral and physiological data. SPSS® software (SPSS, Chicago, IL, USA) was used for statistical analyses, and the limit of significance in all analyses was $p < 0.05$. In each of experiments 1 and 2,

Table 1. Tissue copper concentrations (μg/g tissue) for paired dominant and subordinate fish exposed to 30 μg/L for 48 h; data are expressed as mean ± standard error of the mean (n = 9)

	Tissue copper concentrations (μg/g wet tissue)			
	Gill	Carcass	Liver	Kidney
Dominant	1.14 ± 0.09	1.93 ± 0.23	51.99 ± 9.61	5.70 ± 1.17
Subordinate	1.95* ± 0.39	2.76 ± 0.64	49.17 ± 10.88	5.98 ± 0.73

* Significant difference ($p < 0.05$) between dominant and subordinate groups.

a principal-components analysis was used to combine the behavioral scores obtained for each fish during the observation period. This statistical test is designed to weight all the available variables and provide the maximum discrimination between the individuals. The different behavioral measurements (food intake, position in the tank, number of attacks) are ranked according to the extent to which they correlate with the derived principal axis (the line of best fit of the data), and an overall behavior score is generated for each fish. This method has been used previously to successfully analyze fish behavioral data (see reference 17 for further details).

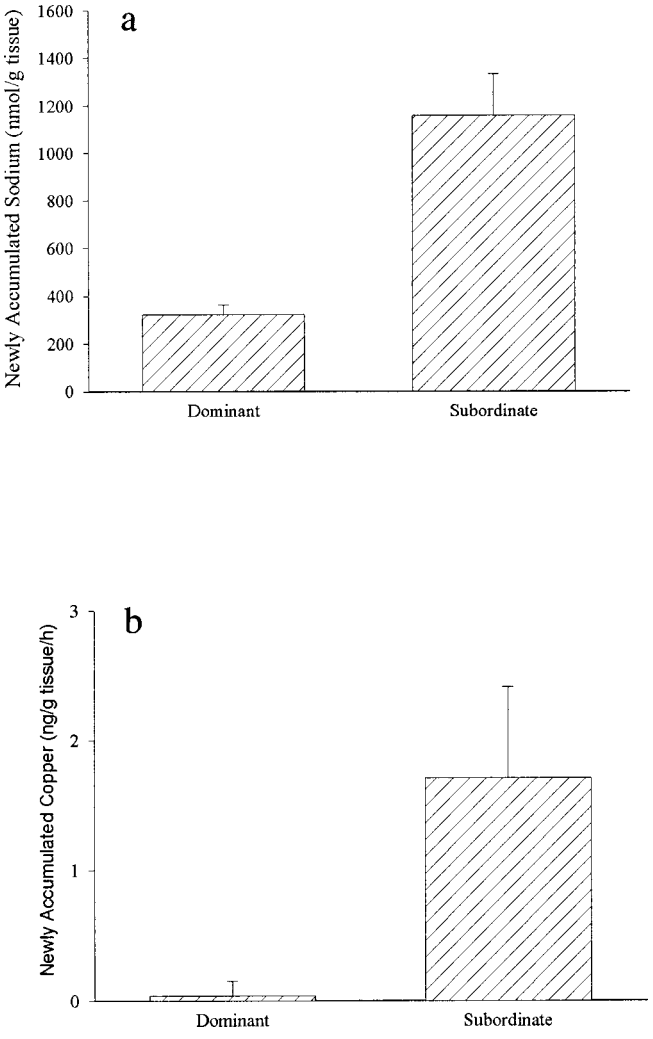


Fig. 2. Uptake of (**a**) waterborne sodium (measured using ^{22}Na) and (**b**) waterborne copper (measured using ^{64}Cu) from a dual labeling experiment. Data are presented as mean ± standard error of the mean (independent samples t test: for sodium, $t = -4.624$, $p < 0.001$; for copper, $t = -2.333$, $p = 0.035$; $n = 8$).

RESULTS

Experiment 1—Effect of exposure to 30 μg/L of copper on behavior and tissue copper concentrations of pairs of fish

Dominant and subordinate fish were distinguished from one another using the behavior scores generated by the principal-components analysis for the whole of the experimental period. Within each pair of fish, the fish with the highest behavior score was the dominant fish, and overall, dominant fish had significantly higher behavior scores than subordinate fish (dominant = 0.896 ± 0.1423; subordinate = −0.896 ± 0.123; independent samples t test: $t = 9.531$, $df = 16$, $p < 0.001$). Positive scores were indicative of dominant fish, whereas more negative scores were indicative of the more submissive fish. There was no effect of the introduction of 30 μg/L copper on previously established dominance. The behavior scores of each fish during the first 48 h (control conditions) of the experiment and the second 48 h (~30 μg/L) of the experiment were positively correlated, indicating that fish that were dominant in control conditions remained dominant after the addition of 30 μg/L copper (linear regression: $F = 1{,}919$, $r^2 = 0.991$; $p < 0.001$). However, there was a significant difference in the gill copper concentration, with subordinate fish displaying higher gill copper concentrations than dominant fish (independent samples t test: $t = 2.314$, $df = 16$, $p = 0.034$; Table 1). The significant difference in copper concentration measured at the level of the gill was not reflected in any of the other tissues measured (independent samples t test: for liver, $t = -0.237$, $df = 16$, $p = 0.816$; for kidney, $t = 0.034$, $df = 16$, $p = 0.973$; for carcass, $t = 0.999$, $df = 16$, $p = 0.333$; Table 1).

Experiment 2—Uptake of copper and sodium in pairs of fish

Subordinate fish had a higher whole-body sodium uptake (independent samples t test: $t = -4.624$, $df = 14$, $p < 0.001$;

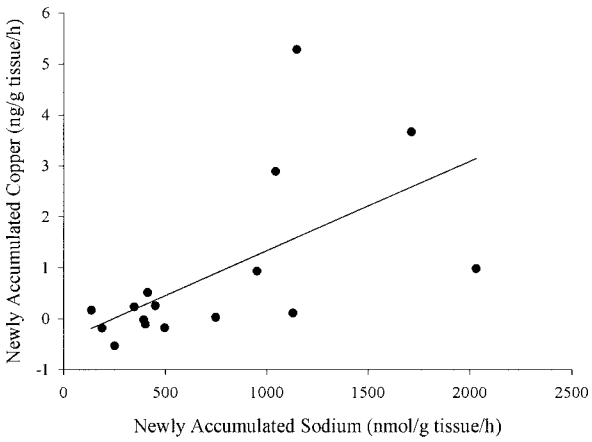


Fig. 3. Relationship between newly accumulated sodium (measured using ^{22}Na) and newly accumulated copper (measured using ^{64}Cu) (linear regression: $F = 7.776$, $r^2 = 0.357$, $p = 0.015$; $n = 16$).

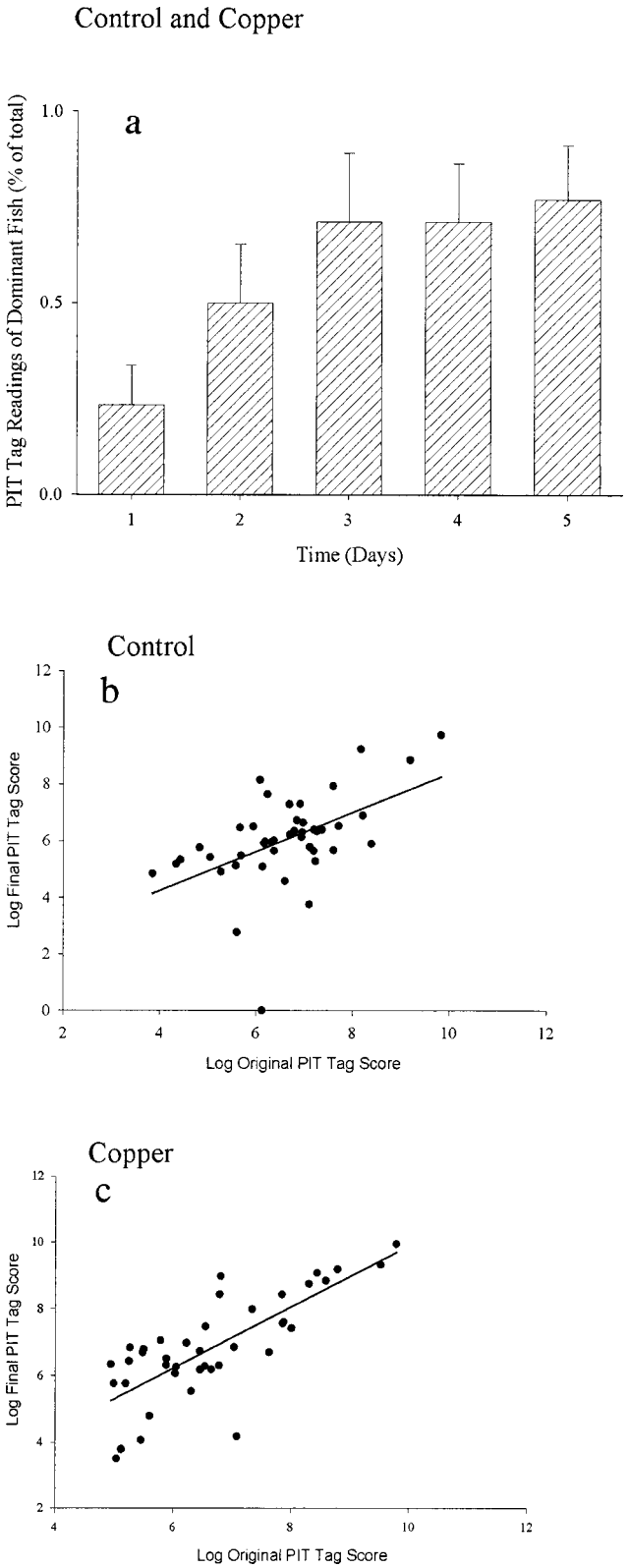


Fig. 4. (**a**) Percentage per day of passive integrated transponder (PIT) tag readings acquired by the fish that became the dominant fish in each replicate. The first 5 d of the experiment are presented, during which time the hierarchies were established. Control and experimental treatments are presented together and data are presented as mean ± standard error of the mean after arcsine transformation. The PIT tag scores (number of PIT tag readings recorded) during the first (original) and second (final) week of the experiment were positively correlated in both control (**b**) (linear regression: $F = 15.228$, $r^2 = 0.266$, $p < 0.001$; $n = 45$) and copper (**c**) treatments (linear regression: F, = 198.5, $r^2 = 0.587$, $p < 0.001$; $n = 45$).

Fig. 2a) than dominant fish and also a higher whole-body copper uptake (independent samples t test: $t = -2.333$, $df = 14$, $p = 0.035$; Fig. 2b). Copper uptake by each fish was also significantly correlated to sodium uptake (linear regression: $F = 7.776$, $r^2 = 0.357$, $p = 0.015$; Fig. 3). A higher uptake of sodium by subordinate fish was also seen in the absence of copper (i.e., at background copper concentrations ~3 μg/L; paired samples t test: $t = -2.324$, $df = 14$, $p = 0.053$; dominants = 833.45 ± 133.25; subordinates = 1,505.35 ± 358.5 nmol/g tissue/h). Although these values were higher than the respective values in the presence of copper (see Fig. 2a), the differences were not significant.

Experiment 3—Exposure of groups of fish in stream tanks to 30 μg/L copper

During the first week of the experiment, when all fish were held in control water, dominance was established around day 3. In the first few days, all ranks of fish had varying numbers of records caused by the fish passing through the PIT tag reader, but by day 3, the dominant fish within each replicate was acquiring above 50% of the total number of PIT tag records per day (Fig. 4a). Within control treatments, there was a significant positive correlation between PIT tag scores (the number of PIT tag records acquired by each fish) during the first and second weeks of the experiment (linear regression: $F = 15.228$, $r^2 = 0.266$; $p < 0.001$; Fig. 4b), indicating that there was little change in hierarchy during the course of the experiment. The same result was also seen in the copper treatments, where copper was added (~30 μg/L) during the second week (linear regression: $F = 198.5$, $r^2 = 0.587$; $p < 0.001$; Fig. 4c). For the remainder of the physiological parameters, only a single terminal measurement was obtained at the end of the experiment, so data are presented for the whole two-week period. The dominant fish, rank 1, had significantly higher specific growth rates than the lowest ranks of subordinate fish, and there were no significant differences in growth rates between copper and control treatments (two-way ANOVA: for rank, $F = 5.696$, $p < 0.001$; for treatment, $F = 0.321$, $p = 0.573$; for rank × treatment interaction, $F = 1.155$, $p = 0.338$; Fig. 5). Specific growth rate was also significantly correlated with PIT tag score in both control (linear regression: $F = 16.706$, $r^2 = 0.279$; $p < 0.001$) and copper (linear regression: $F = 12.924$, $r^2 = 0.255$; $p = 0.001$) treatments, with dominant fish obtaining the highest PIT tag score showing the highest specific growth rates.

There were no significant differences among the ranks of fish in gill copper concentration; fish from copper treatment groups had significantly higher gill copper concentrations than control fish (two-way ANOVA: for rank, $F = 0.239$, $p = 0.915$; for treatment, $F = 69.795$, $p < 0.001$; for rank × treatment interaction, $F = 0.218$, $p = 0.928$; Table 2). Fish from copper treatment groups had significantly higher liver copper concentrations than fish in the control treatments. Also, in contrast with the pairs of fish in experiment 1, fish in the copper treatments demonstrated a trend toward increasing liver copper concentration in subordinate fish (Fig. 6a; two-way ANOVA: for rank, $F = 21.106$, $p = 0.089$; for treatment, $F = 21.174$, $p < 0.001$; for rank × treatment interaction, $F = 1.637$, $p = 0.174$). When the liver copper concentration of the dominant rank of fish (rank 1) was compared with the copper concentrations of fish in ranks 2 to 10, the subordinate ranks of fish had significantly higher liver copper concentrations (independent samples t test: $t = -2.471$, $df = 38$, $p = 0.018$). This

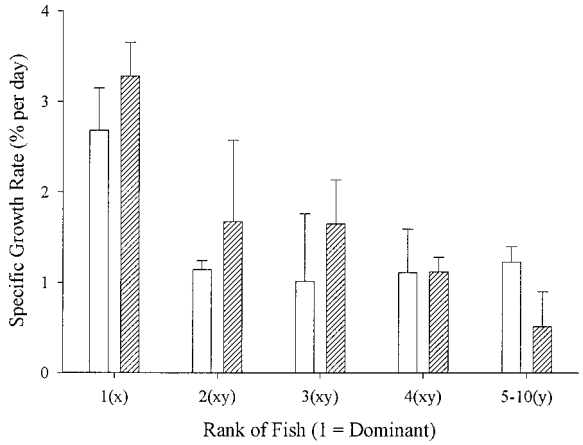
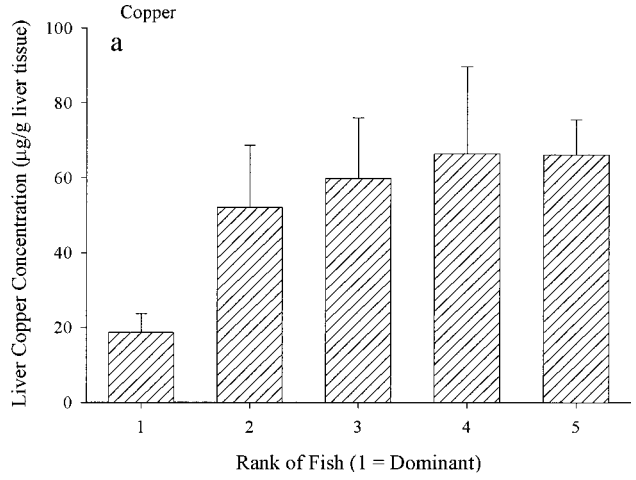


Fig. 5. Specific growth rates (percent increase in weight per day) for the two-week period for ranks of fish in control (open bars) and copper treatments (hatched bars) (for each of ranks 1–4, $n = 5$; for each of ranks 5–10, $n = 20$). Data are presented as mean ± standard error of the mean. Statistical differences are indicated by the letters (two-way analysis of variance: for rank, $F = 5.696$, $p < 0.001$; see text for further analyses); groups sharing the same letters are not significantly different from one another.

did not occur in the control treatment (Table 2). There was also a significant negative correlation between higher specific growth rate and lower liver copper concentration among dominant fish in copper treatments (linear regression: $F = 20.226$, $r^2 = 0.364$; $p = 0.001$; Fig. 6b). This correlation did not occur in the control treatment (linear regression: $F = 1.976$, $r^2 = 0.045$; $p = 0.167$). There were no significant differences among ranks of fish for carcass or kidney copper concentrations, but fish from the copper treatments had significantly higher carcass copper concentrations compared with controls (two-way ANOVA: for carcass and rank, $F = 1.716$, $p = 0.155$; for carcass and treatment, $F = 72.187$, $p < 0.001$; for carcass and rank × treatment interaction, $F = 2.169$, $p = 0.081$; for kidney and rank, $F = 0.823$, $p = 0.514$; for kidney and treatment, $F = 2.237$, $p = 0.131$; for kidney and rank × interaction, $F = 1.006$, $p = 0.410$; Table 2).



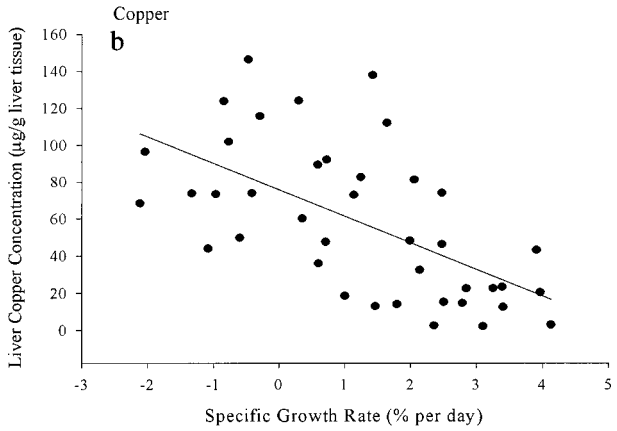


Fig. 6. (**a**) Liver copper concentrations for ranks of fish, where rank 1 is dominant, in the copper treatments (for each of ranks 1–4, $n = 5$; for each of ranks 5–10, $n = 20$). (**b**) Liver copper concentration was negatively correlated with specific growth rate for those fish in the experimental treatments (linear regression: $F = 20.226$, $r^2 = 0.364$, $p < 0.001$; $n = 45$).

DISCUSSION

Methods of studying dominance

Both paired and grouped methods of studying social behavior utilized in the present study allowed clear identification of dominance in which dominant fish had significantly higher behavior scores than subordinate fish (calculated through both observations and PIT tag readings). Although quantifying dominance between pairs of fish has been done previously [6,22], the use of PIT tags for determining dominance is a

Table 2. Tissue copper concentrations for control (not exposed to copper) and experimental (exposed to ~30 μg/L copper for one week) ranks of fish held in stream tanks (1 = dominant); data are expressed as mean ± standard error of the mean (for each of ranks 1–4, $n = 5$; for each of ranks 5–10, $n = 20$)

	Tissue copper concentrations (μg/g wet tissue)			
Rank	Gill	Carcass	Liver	Kidney
Control				
1	0.48 ± 0.05	0.95 ± 0.18	18.77 ± 4.01	2.39 ± 0.62
2	0.44 ± 0.09	1.08 ± 0.28	21.88 ± 7.32	2.68 ± 0.84
3	0.49 ± 0.10	0.92 ± 0.17	17.72 ± 3.64	2.09 ± 0.44
4	0.46 ± 0.08	0.79 ± 0.10	20.38 ± 3.66	2.09 ± 0.53
5–10	0.48 ± 0.05	0.91 ± 0.09	22.37 ± 2.56	2.98 ± 0.33
Experimental				
1	2.04 ± 0.41	2.17 ± 0.35	18.68 ± 4.99	2.90 ± 0.66
2	1.69 ± 0.34	3.37 ± 0.88	52.10 ± 16.5	3.34 ± 0.87
3	1.70 ± 0.27	3.42 ± 0.211	59.77 ± 16.22	4.73 ± 1.18
4	1.46 ± 0.19	3.92 ± 1.06	66.40 ± 23.30	3.20 ± 0.60
5–10	1.75 ± 0.19	3.84 ± 0.37	66.56 ± 9.42	4.44 ± 1.50

relatively new technique. In the stream tank experiment (experiment 3), PIT tag readings gave a clear indication of dominance. The PIT tag readings identified relative social rank and allowed a correlation between specific growth rate and dominance to be demonstrated. Specific growth rate has been shown in previous studies to be highly correlated with dominance [13,15,16,23,27]. The present study is one of the first to successfully use PIT tagging to monitor social interaction, and it appears that the method of utilizing PIT tags for studying linear dominance hierarchies has an excellent potential as a behavioral tool. Brännäs and Alanärä [28] used a demand feeding system incorporating a PIT tag reader to monitor dominance hierarchies among groups of Arctic charr, *Salvelinus alpinus*. Maclean and Metcalfe [29] also used PIT tagging to monitor feeding behavior of fish of different social status but used a serial removal technique for the determination of dominance. The serial removal technique for determining dominance involves recording the acquisition of food by individuals within a group of fish and identifying which individual is obtaining the most food. This fish is then judged to be the dominant fish and is removed from the tank and the procedure repeated until all fish have been assigned a rank.

Behavioral effects of waterborne copper

There was no apparent effect of 30 μg/L copper on dominance hierarchies either between pairs or among groups of fish. Fish that displayed dominant behaviors when the hierarchy was established in control water maintained their social status after the addition of copper. In contrast, Sloman et al. [23] demonstrated that an environmental perturbation of lowered water levels was sufficient to disrupt linear dominance hierarchies and alter the behavior of the fish. It would seem that the addition of waterborne copper caused a chemical rather than a physical alteration in the stream habitat that may otherwise disrupt the dominance hierarchy; however, effects on other behaviors were not noted. The 96-h LC50 of copper for rainbow trout is approximately 91 μg/L (95% confidence limits, 65.9–107.6 μg/L) at water of a quality similar to that used in the present study (120 mg/L hardness as $CaCO_3$, pH = 8.0, alkalinity = 95 mg/L) [30]. Increasing hardness, alkalinity, and pH generally alter the chemistry of copper and reduce its bioavailability. Waiwood and Beamish [7] clearly demonstrated a significant effect of water hardness on critical swim performance; at a water hardness of 360 mg/L (as $CaCO_3$), 30 μg/L copper did not appreciably alter critical swimming performance, but the same concentration caused a decrease in swimming performance at a water hardness of 30 mg/L (as $CaCO_3$). However, even in water of relatively high water hardness, both physiological and behavioral effects of copper have been observed at levels far below the 96-h LC50. Laurén and McDonald [4] demonstrated disruption of gill ionoregulatory function at concentrations of copper as low as 12.5 μg/L regardless of prior acclimation to hard or soft water. Henry and Atchison [11] noted behavioral changes in bluegills, *Lepomis macrochirus*, at concentrations as low as 34 μg/L copper under hardness condition twofold higher than that used in the present study. The frequency of agonistic and comfort behaviors was seen to increase upon exposure to copper, although relative ranking of individuals within the hierarchy was not affected. At the 30-μg/L concentration used in the present study, no behavioral effects were detected. However, it is possible that behavior of individuals in the present study may have changed but that these changes in behavior were not assessed by the technology used. In any event, if such changes occurred, they were not sufficient to disrupt the social hierarchy.

Uptake of copper by different social ranks

Perhaps the most important conclusion of the present study is that subordinate fish take up more copper from the water than dominant fish. The higher uptake of copper is shown both by higher whole-body uptake of copper (experiment 2) as well as higher gill and liver accumulation of copper (experiments 1 and 3, respectively) in subordinate fish. Water-borne copper is taken up across the gills and then passes into the blood plasma [31], where it is transported to the liver [32]. Higher accumulation of copper was seen in the gills of subordinate fish after 48 h of exposure to copper (experiment 1), but no increase in internal burdens was demonstrated. It is likely that, by 48 h, the uptake of copper from the water had not yet translated into a detectable increase in hepatic copper due to high endogenous background levels in this tissue. However, after a week of exposure to copper (experiment 3), subordinate fish in the stream tank did display higher liver copper accumulation than more dominant ranks of fish, even though the differences in gill copper concentrations had disappeared.

Fish exposed to 30 μg/L for a period of one week in the stream tank experiments demonstrated both higher gill and liver copper concentrations than those fish in the control treatments. However, there was no significant difference in kidney copper concentration between control and experimental treatments. No significant change in total copper concentrations in the kidneys of trout exposed to the same concentration of copper for 28 d has also been previously demonstrated by Grosell et al. [33,34]. This result may be due to a rapid turnover of copper in the kidney. Grosell et al. [34] also demonstrated a high turnover of copper in the gills of rainbow trout exposed to 20 μg/L copper for 28 d. A high turnover of copper in the gills after 7 d of exposure may explain why no significant difference was found among the ranks of fish exposed to 30 μg/L of copper in experiment 3 of the present study.

Explanation of higher copper uptake in subordinate fish

Subordinates within pairs of fish have been shown to have higher standard metabolic rates [22]. Whether this higher standard metabolic rate is a direct result of subordination or reflects the chronic elevation of cortisol seen in subordinate fish [17,35,36] is not known, but it is possible that higher metabolic rates lead to increased copper uptake across the gills by increasing ventilation volume and gill permeability. Fish of earlier life stages were shown to be more sensitive to lead as a toxicant due to a higher metabolic rate [1]. Carpenter [37] also demonstrated that smaller fish were more sensitive to lead than larger fish due to their higher metabolic rate.

Stress results in ion loss across the gills, which in turn can stimulate elevated concentrations of catecholamines as a physiological mechanism to recover from ionic disturbances (see review [38]). Cortisol, another hormone involved in the stress response and associated with subordinance, is also known to alter the ion transport mechanisms across the gills [39]. It is possible that increased copper accumulation in subordinate fish is due to cortisol-mediated increases in transport of ions across the gills. Perry et al. [39] demonstrated an increase in branchial chloride cell area in the gill epithelia associated with injections of cortisol, which was correlated with increased ion uptake. In the present study, subordinate fish also demonstrated elevated uptake of sodium across the gills (experiment 2). Recent

work investigating the transport of sodium and copper across the gill epithelia has suggested that copper may be transported partly via the sodium uptake pathway [40].

It is apparent that subordinate fish have an elevated uptake of copper; however, it is also possible that increased accumulation of copper in subordinates is associated with a decreased excretion rate. Grosell et al. [25] demonstrated that 9 d of starvation caused reduced excretion of copper via the bile in European eels (*Anguilla anguilla*). Segner [41] also demonstrated that nutrient-related alterations of physiological status could affect copper accumulation in roach (*Rutilus rutilus*). Starved yearling roach showed much higher accumulation of copper in the liver than fed fish, and it was suggested that food-deprived fish lack the ability to regulate transfer of copper within the body [41]. Subordinate fish generally acquire less food than dominant fish, and within the stream tank environment, subordinate fish demonstrated a lower specific growth rate compared with dominant ranks of fish. While differences in food intake may in part explain the higher liver copper concentrations seen in subordinate fish, even the lowest ranks of subordinate fish exhibited a mean positive specific growth rate ($1.23 \pm 0.17\%$ [mean $\pm$ standard error of the mean]), so complete starvation did not occur.

Growth dilution has recently been illustrated as an important consideration in tissue copper accumulation; fish with higher specific growth rates accumulate the same amount of copper as fish with lower specific growth rates but grow more, thus diluting tissue copper burdens [42]. However, within the present study, there are several factors that suggest that higher liver copper content of subordinate fish cannot be explained by growth dilution alone. First, the effect is seen in the copper-exposed fish but not in the control treatments. Second, each replicate trial fish was initially size matched. Although dominant fish showed higher specific growth rates, the difference in specific growth rates between dominant and subordinate fish was only between 1 and 2% change in weight per day. Third, subordinate fish among size-matched pairs took up significantly more whole-body copper from the water over the 2 h of exposure to ^{64}Cu, thus strongly supporting the inference that higher liver copper contents of subordinate fish held in stream tanks are a function of subordinance.

In conclusion, the characteristic social behavior of stream-dwelling salmonid fish does not appear to be influenced by sublethal waterborne copper. However, the social status of a fish influenced the physiological responses that the fish displayed as indicated by copper uptake, e.g., subordinate fish accumulated more copper (and sodium) from the water than dominant fish. Dominance and social interaction are integral parts of any fish population; thus, it can never be assumed that all individuals within a population will be affected equally by the presence of waterborne copper. Individual variation in copper uptake by fish is mediated in part by social competition. Such physiological and social factors may have significant ecological consequences in the environment.

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