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Copper disrupts alarm chemical response

SMELL NO EVIL: COPPER DISRUPTS THE ALARM CHEMICAL RESPONSE IN A
DIADROMOUS FISH, *GALAXIAS MACULATUS*

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Abstract

Fish, at all life stages, utilize olfactory information in the decision-making processes essential to survival. Olfaction is a sensitive sensory process, and toxicants within urban aquatic environments can have destructive or depreciating effects. In the present study, the authors exposed *Galaxias maculatus*, a native fish commonly found in urban waterways throughout southeastern Australia, to 1 of 5 ecologically relevant copper (II) chloride concentrations (<1 µg/L, 1 µg/L, 6 µg/L, 8 µg/L, 18 µg/L) for 16 h. After exposure, the authors tested the response of individual fish to 1 of 3 stimuli: a conspecific skin extract containing a stress-inducing alarm chemical odor, a conspecific odor, and distilled water as a control. Stress responses were quantified through behavioral assays. The authors found evidence for distinct changes in behavioral response with increasing copper concentration and a marked difference in response between control fish and fish exposed to the alarm chemical odor. Copper, even at relatively low

concentrations, can have a significant effect on the stress response behavior shown by *G. maculatus*.

Keywords: Olfaction, Ecotoxicology, Metal, Fish behavior, Galaxiid, Copper

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INTRODUCTION

Fish rely strongly on their sense of smell for survival. The ability to detect waterborne chemical cues can mediate a fish's ability to migrate, find a mate, recognize conspecifics, find food, and avoid predation [1]. In short, the detection of odors is critical for fish at all life stages to determine a wide spectrum of information about their environment. Recent research has shown that exposure to waterborne contaminants, even at sublethal concentrations, can have a marked disruptive effect on a fish's ability to smell [2]. Metal contaminants are of particular interest because of their disruption of both nervous and endocrine pathways in freshwater organisms [3]. Metals, such as cadmium and copper, have been shown to have deleterious effects on the sensory organs and lateral line in banded kokopu, *Galaxias fasciatus* [4], and zebrafish, *Danio rerio* [5,6], as well as showing marked olfactory degradation in salmonids, *Oncorhynchus* spp. [7,8]. However, relatively little is known about the specific behavioral changes resulting from damage to olfaction and subsequent odor reception caused by increased concentrations of trace metals.

Aquatic systems adjacent to urban environments are often highly contaminated with a variety of potential toxicants. For example, contaminants can enter waterways from point sources, such as industrial discharge, stormwater, and raw or treated effluents from wastewater

[9–11], and be quickly dispersed throughout the catchment [12]. The presence of toxicants in waterways can have dramatic effects on resident species by compromising their abilities to respond appropriately to predation risk, to forage effectively, to reproduce, or to migrate either by causing direct physiological damage or through disruption of fundamental biochemical pathways [13].

In the wild, prey fish utilize 3 main olfactory methods for determining the level of danger from predation: 1) predator odor; 2) stress cues released by a prey fish on being frightened by a predator, prior to attack; and 3) damage release cues exuded from the skin of a prey fish after being attacked by a predator [14]. When a prey fish is bitten by a predator, it will exude a damage release odor that functions as an alarm signal to other conspecifics [15]. If conspecifics (or, in some cases, other closely related species) detect this cue, they will engage in appropriate predator-avoidance behavior, such as reduced foraging and increased shelter seeking [16]. This alarm signaling system serves an important role in reducing predation risk. Alarm chemicals have been found to increase the frequency of an individual's antipredatory behavior when paired with novel predator odors, suggesting that a conspecific alarm chemical plays a role in species learning [14,17]. Any alteration, therefore, of this signaling system would likely increase the risk of predation, with potential for population-level consequences.

As a contaminant, copper has been shown to cause a range of negative physiological effects in fishes, with the majority of investigations having been conducted on salmonids. It has been demonstrated to cause dose-specific damage to a variety of organs, including the kidneys, liver, brain, and lateral line organ [6,18]. In terms of olfactory disruption, copper has been shown to act generally, rather than specifically [8,9,19,20]. It is believed that the biochemical mechanism of disruption is that copper acts by blocking the cyclic nucleotide gated channel of

olfactory receptor cells, halting the movement of calcium ions into the cell and thus inhibiting the transmission of appropriate signals between olfactory receptors and the brain. This eventually results in either *hyposmia*, the reduction of olfaction, or *anosmia*, the complete loss of olfaction [2].

Galaxias maculatus is a diadromous fish found in Australia, New Zealand, Argentina, and Chile. In autumn, adult *G. maculatus* migrate down to estuaries, where they spawn in and among riverbank vegetation [21]. Given their wide distribution and high abundance in most southeast Australian waterways, including urban systems, they potentially are a good species for monitoring the health of freshwater and estuarine systems. Furthermore, as fish were collected from spawning sites in estuaries, our exposures will give better insight into the role contaminants might play during this sensitive period in the life cycle. Finally, the behavioral effects of predator odors on *G. maculatus* have previously been investigated and can therefore be reasonably used as a basis for behavioral comparison [22,23]. However, aside from these studies, little work has been undertaken with this species to examine either copper's disruptive effects on olfaction or the specific behavioral effects of the conspecific alarm chemical.

Our aim in the present study was to investigate the effect of copper as a disruptor of the olfactory system of *G. maculatus*. We used experimental trials to assess changes in the behavioral response of *G. maculatus* to a waterborne alarm chemical odor following exposure to a range of ecologically relevant copper concentrations. We used alarm chemicals created from skin extracts as the experimental odor as these have been demonstrated to elicit a noticeable behavioral response in other fish species [9]. Two additional stimuli were also used: distilled water to act as a procedural control and a conspecific cue to ensure that responses were the result of stress, rather than just the scent of another galaxiid, as previous studies have also shown that

G. maculatus are attracted to conspecific odor [24].

MATERIALS AND METHODS

Collection of fish

Adult *G. maculatus* were collected between May and July 2014 from the upper reaches of 4 estuaries in Victoria, Australia: Kororoit Creek (37°51'24"S, 144°51'00"E), Maribyrnong River (37°46'21"S, 144°51'08"E), Sheoak River (38°33'59"S, 143°57'59"E), and Cumberland River (38°34'30"S, 143°56'57"E). Fish were collected using fyke nets that were positioned with the mouth facing upstream to catch adults migrating to spawning sites in the upper reaches of estuaries. Nets were set at a variety of times of the day and retrieved between 6 h and 12 h later. Following net retrieval, all fish were sorted, nontarget species were returned to the waterway, and *G. maculatus* were placed into 20-L buckets containing stream water from the site of capture. The retained fish ranged in size from 8 cm to 12 cm (total length) and were placed in insulated containers filled with aerated stream water for transfer back to the University of Melbourne.

Housing and acclimatization

On arrival at the University of Melbourne, fish were transferred to 3 60-L aquaria in the basement aquarium facility of the School of BioSciences. Fish were distributed equally among aquaria, such that it was not possible to determine (during trials) where a given fish had been caught. This was done to remove any possible confounding effect of variation in behavioral responses because of differences in water quality and exposure history of fish among collection sites. Each aquarium had a corner biofilter and was aerated via an individual air stone. To minimize external visual sources of stress, the sides of aquaria were surrounded, as well as separated, by a thin, opaque, plastic screen. To avoid inadvertent copper contamination from tap

water supply, the aquaria were filled with distilled water mixed with a stock solution of standard artificial medium to provide appropriate concentrations of salts ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.044 g/L; NaHCO_3 , 0.010 g/L; MgSO_4 , 0.010 g/L; NaCl , 0.050 g/L; KH_2PO_4 , 0.002 g/L; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.018 g/L [25]). Specifically, the standard artificial medium provided water hardness reflective of concentrations in healthy freshwater environments and in line with previous investigations involving the genus *Galaxias* [4,23]. All water used throughout the experiment was filtered using a Merck-Millipore Elix 35 system, providing source water with low dissolved levels of salts (89 $\mu\text{S}/\text{cm}$ at 19.2 °C) and negligible total organic carbon (<30 ng/g).

To maintain a constant temperature (18 °C), aquaria were placed on a large flow-through water table such that the bottom 5 cm of each aquarium was submerged. Fish were observed daily and fed dried fish food (Fish Flakes; Fluval) on alternating days. Fish were allowed to acclimatize for a period of no more than 7 d, under a 12:12-h day:night cycle, prior to experimentation.

Experimental design

To test the effect of exposure to copper on olfaction and subsequent antipredator responses, adult *G. maculatus* were exposed to a range of copper concentrations and olfactory stimuli in individual aquaria. To reduce confounding from the potential of a learned behavioral response (and the variation associated with this), each fish was used only once. In Australia and New Zealand, the trigger value for waterborne copper ranges from 1 $\mu\text{g}/\text{L}$ to 2.5 $\mu\text{g}/\text{L}$ in freshwater systems and from 0.3 $\mu\text{g}/\text{L}$ to 8 $\mu\text{g}/\text{L}$ in marine ecosystems [26]. Consequently, the present study used a range of copper exposures that best simulate these trigger values, as well as elevated concentrations such as those that might occur for a short period as part of a stormwater surge. The experiment was treated as a 2-factor fully crossed design, such that each treatment

concentration was combined with each possible stimulus. There were 5 distinct nominal copper concentrations (0 µg/L, 1.5 µg/L, 5 µg/L, 10 µg/L, and 20 µg/L) and 3 possible stimuli (alarm chemical, conspecific odor, distilled water control), resulting in 15 treatment combinations. The effects of treatments were assessed by quantification of behavioral assays. Each block of 15 treatment combinations was repeated 6 times, resulting in 6 replicates of behavioral data per treatment.

Preparation of copper chloride treatments

All copper treatments were prepared from a standard solution of 25 ± 1 mg/L copper (II) (as copper [II] chloride). Working solutions were then prepared by adding a set volume of copper (II) chloride standard to 250 mL of standard artificial medium and then making up to 50 L. Final solutions were measured with a TPS SmartCHEM-LAB[®] Multi-Parameter Laboratory Analyser for temperature (with a range of 18.8–19.4 °C), pH (range 7.6–7.8), and conductivity (range 380–420 µS/cm). The concentration of copper for each treatment was verified for a subset of water samples by an environmental chemistry laboratory (ALS Environmental) through inductively coupled plasma–mass spectrometry according to American Public Health Association method 3125 and US Environmental Protection Agency method 6020A [27,28] (Table 1). To this end, unfiltered water samples were stored in sample containers and acidified to pH 2 with analytical-grade nitric acid. Minimally 1 laboratory control (0.1 mg/L Cu), 1 method blank (<0.001 mg/L Cu), and 1 matrix spike (0.2 mg/L) were made for every 10 samples. Two duplicates were included in every 10 samples, with a relative deviation of 0%. Matrix spikes gave a recovery range of 90.9% to 94%, whereas recovery for the laboratory control standards was between 87% and 105%. For clarity, all further results are presented as measured, rather than nominal, concentrations.

Creation of alarm stimuli

To prepare the alarm chemical, adult *G. maculatus* (of both genders) were killed by a sharp blow to the head. Following death, the skin was removed using a scalpel and forceps, measured with calipers, then immediately placed in a beaker of chilled aquarium water (200 mL) and stored on ice to prevent degradation of the alarm chemical. In total, 85.9 cm² of skin was obtained from 20 adults. The fish skin was then homogenized using a mortar and pestle that had been prechilled with liquid nitrogen. The homogenate was mixed with aquarium water and filtered through a fine (100 µm²) mesh cloth. The resulting solution was made up with aquarium water to 1.0 L. The alarm chemical solution was then measured out in 100-mL aliquots into autoclaved jars, labeled, and frozen at –20 °C.

The conspecific cue was prepared by taking water from the aquaria in which *G. maculatus* had been acclimatized and freezing it at –20 °C in 100-mL aliquots, in autoclaved jars. Similarly, the procedural control cue was prepared by freezing 100-mL aliquots of distilled water in autoclaved jars.

Copper exposure

Following acclimatization, fish were randomly allocated to individual treatment tanks (20 cm × 40 cm × 25 cm) containing 15 L of 1 of the 5 possible copper concentrations. Fish were transferred to treatment tanks on the evening before a behavioral trial and allowed 16 h of exposure to the copper treatment. The tanks were surrounded by white Corflute[®] screens on all sides to prevent any potential visual sources of stress. In addition, each tank had a Corflute lid with a small hole for introduction of the alarm stimulus via syringe. The tanks were placed in water baths on a 3-tiered shelf in a controlled-temperature room, and treatments were allocated to tanks randomly. The temperature of the water baths was maintained at 16 °C by circulating

water through a water chiller (Aqua One Arctic). Each shelf had a fluorescent strip light mounted 60 cm above it to provide consistent lighting to the tanks beneath on a 12:12-h day:night cycle. Each tank was aerated with an individual air stone and filled with washed gravel to a depth of 5 cm.

After 16 h of copper exposure, fish were exposed to 1 of the 3 chemical stimuli. To assess changes in individual behavior following the addition of the stimuli, fish were filmed individually both prior to and following introduction of the chemical stimuli. Video footage was later reviewed and fish behavior scored.

Behavioral assay

Fish were filmed using a GoPro Hero 3+ Silver camera affixed to an arm mount attached immediately in front of the tank during each trial. This provided a complete view of the interior of a single tank. Fish were filmed for 5 min prior to and following introduction of 20 mL of the chemical stimulus. Of the 10 min of footage captured per fish, 3 min on either side of stimulus introduction were examined. This interval was chosen to reduce the effect of potential stress caused by the front barrier being removed at the start of filming. Every 15 s, behavior was assessed as being 1 of 4 types: 1) freezing (fish lies motionless on the bottom of the tank, showing only opercular movement), 2) still (fish remains stationary in the water column, showing slow movement of the caudal and pectoral fins), 3) swimming (fish shows distinct, slow, continuous movement either vertically or horizontally and regular movement of pectoral and caudal fins), and 4) dashing (fish moves rapidly and erratically, either vertically or horizontally) [23]. This resulted in a total of 24 behavioral measurements per fish. The total number of observations of each behavior type before and after stimulus was determined and recorded.

Data analysis

Because the experiment was a randomized complete block design, a nonadditive factorial model was employed, with 2 fixed main effects (copper concentration and stimulus type), 1 random blocking factor (date of trial), and all 2-way interactions. (The 3-way interaction was not assessed as it was unreplicated.) This model was tested by analysis of variance performed in R, and a Tukey's honestly significant difference post hoc test was done to determine which treatments differed significantly ($\alpha = 0.05$).

For each fish, each of the behavior types recorded was converted to a prestimulus and a poststimulus frequency. This frequency was expressed as a percentage of time spent exhibiting a given behavior, and the percentage change between poststimulus and prestimulus frequency, for each behavior type, was determined. Given that the 4 observed behavioral types constitute compositional and nonindependent data, we examined the prestimulus behaviors of all subjects combined, as well as just the control subjects, to determine both the most common behavior and the behavior most likely to change following addition of the alarm stimulus. In both cases, the most common behavior type prior to stimulus introduction was "still," with fish on average spending >50% of their time in this behavior. However, "still" can be interpreted as being either resting or a response to stress. Following exposure to an alarm chemical, a given fish may become more "still" than it was prior to introduction of the alarm chemical, making this behavior less useful for assessing behavioral changes. Of the 4 measured behaviors, 2 ("dashing" and "freezing") are distinctly stress responses, whereas "swimming" is an unambiguously relaxed behavior. Consequently, the change in frequency of "swimming" was selected as the response variable. Given the lack of independence, a decrease in frequency of "swimming" behavior was accompanied by an increase in 1 or more of the other 3 behavioral types.

RESULTS

Galaxias maculatus displayed a range of behavior types, depending on choice of stimulus and copper concentration (Tables 2 and 3). Regardless of the odor stimulus delivered, *G. maculatus* showed a significant alteration in its swimming behavior with increased copper concentration (Table 4 and Figure 1). Fish that were exposed to the lowest concentration of copper (<1 and $1 \mu\text{g/L}$) showed the smallest overall change in frequency of swimming (increases of $2.8 \pm 4.9\%$ and $1.4 \pm 3.9\%$, respectively), whereas those that had been exposed to $6 \mu\text{g/L}$ or $8 \mu\text{g/L}$ copper showed decreases in frequency of swimming ($-6.9 \pm 3.6\%$ and $-9.7 \pm 6.7\%$, respectively). This is in contrast to those exposed to $18 \mu\text{g/L}$ copper, where an overall increase of $12.5 \pm 5.3\%$ in swimming was observed (Figure 1). There was a significant difference between the highest copper concentration when compared with the middle concentrations but not in comparison with both the lowest concentration and the control. There was also no difference between the lowest concentration and the control or between the 2 middle concentrations.

Galaxias maculatus showed a decreased frequency of swimming when exposed to alarm chemical, independent of the copper treatment exposure concentration (Figure 2 and Table 4). On average, fish exposed to alarm chemical decreased swimming by $8.3 \pm 3.4\%$, in contrast to an increase in swimming of $4.2 \pm 4.7\%$ in fish exposed to distilled water (controls) and a $4.2 \pm 3.6\%$ increase in swimming in fish exposed to conspecific odor (Figure 2). There was a significant difference in the change of behavior elicited by the alarm chemical when compared with that elicited by both distilled water and conspecific odor. There was no significant interaction between copper concentration and stimulus type (Table 4).

DISCUSSION

The results of the present study indicate that *G. maculatus* responds to the presence of a

conspecific alarm chemical by adjusting its behavior through devoting less time to “swimming” behavior. In fish exposed to environmentally relevant concentrations of dissolved copper, close to the Australian and New Zealand trigger value range (i.e., treatments $<1\text{ }\mu\text{g/L}$ and $1\text{ }\mu\text{g/L}$), there was little variation in the frequency of their swimming behavior; exposure to higher concentrations (i.e., $>6\text{ }\mu\text{g/L}$), however, caused distinct, yet not statistically significant, changes in behavior. We propose that in the event of increased copper concentrations in estuaries (such as following storm events), normal locomotive behavior in common galaxiids is likely to be influenced, which could potentially lead to negative effects on an individual fish’s ability to respond appropriately to predation risk. A previous study on behavioral responses of *G. maculatus* to predator odors suggests that its most common response to the threat of predation is to remain motionless in the water column [23]. However, as the behavior of *G. maculatus* in that study was only observed in the period following the introduction of an alarm cue, it is unclear whether the observed behavior represented a change in antipredatory tactics or simply the continuation of an already established behavior. By observing behaviors prior to and following exposure to the alarm cue, our results confirm that reduction in the frequency of swimming is a likely response by *G. maculatus* to a perceived threat of predation. The reduction in movement as a behavioral response to an alarm chemical has also been observed in other prey species such as fathead minnow (*Pimephales promelas*) and zebrafish [29,30]. A reduction in movement likely increases the crypsis of prey species, allowing them to more easily blend with their environment and thus avoid visual detection by predators. McIntyre et al. [31] demonstrated that copper-exposed juvenile coho salmon were both less responsive to chemical predation cues and more active in the presence of predators, consequently being more likely to be eaten. Considering that olfaction acts in concert with other sensory systems to warn prey fish of

impending danger, the presence of an olfactory cue, coupled with the absence of visual evidence of a predator (or, indeed, an injured conspecific) further suggests a behavioral response of caution [32].

Interestingly, exposure to copper had a varying effect on the change in frequency of swimming, causing reduced swimming in individuals exposed to moderate concentrations of copper, yet showing an increase in swimming behavior in the highest copper exposures, irrespective of the type of stimulus introduced. One possible explanation for this result is that copper may be impairing more than 1 sensory system.

Generally, studies into the role of copper as an olfactory disruptor have used short exposure periods, typically no longer than 4 h [8,19,20,33]. However, in the natural environment, copper exposures would generally be longer; therefore, it is likely that other sensory organs could also be compromised [18]. Long periods of exposure to high concentrations of contaminants, including copper, have been shown to have a negative effect on the lateral line organ by causing damage to the sensory hairs located in the lateral line pits [6,34,35]. In those studies, as the concentration of copper increased, the levels of overall stress also increased.

Through the neuromasts of the lateral line, fish can easily detect slight changes in both flow (rheotaxis) and temperature (thermosensation) of water [36,37]. Consequently, it is possible that fish showed an increased level of caution (i.e., a reduction in frequency of swimming) independent of the stimulus type, the result of both the mechanical disturbance of the tank water when the stimulus was added and the thermodynamic change associated with adding a low-temperature (i.e., thawed) stimulus. This cautionary behavior was likely exacerbated by the distress associated with increasing concentrations of copper. In other words, at the highest concentration of copper exposure, the lateral line may have become significantly compromised,

causing an inability to detect the direction of disturbance and therefore inducing a state of confusion and an increase in swimming in *G. maculatus*. Introducing the stimulus at the same temperature as that of water in the experimental tanks would potentially eliminate this as a confounding factor.

The present study did not detect the expected interaction between copper concentration and stimulus type. This is most likely because of the high level of variability among replicate blocks (Figures 1 and 2). Furthermore, because fish were sourced from a variety of rivers, and thus had different exposure histories with regard to water quality, it is reasonable to suppose that individual responses to treatments might vary. However, as a result of fish being randomly allocated to housing aquaria, it was not possible to link differences in the observed behavioral response with the source location of each fish.

Copper can induce a stress response in fish through both the impairment of organ systems and the disruption of neural pathways [18,31,34,35]. It is therefore difficult, in olfactory testing, to determine whether a given individual's stress response is the result of detection of a conspecific alarm chemical or of widespread copper-induced organ and neural impairment. To combat this particular difficulty, we adopted a before–after control impact design, which allowed us to isolate the behavioral response from an alarm chemical from any behavioral alteration caused by copper toxicity. The results indicate that *G. maculatus* is able to detect a waterborne alarm chemical and adjust its behavior in response to it, irrespective of whether it has been exposed to copper at the concentrations investigated in the present study.

An emerging class of contaminant in urban waterways is the engineered metal nanoparticle. Copper nanoparticles particularly have been shown to have antibacterial qualities and are often used in pesticides [38]. Interestingly, copper atoms at the surface of a nanomaterial

can oxidize easily, readily converting to the more stable ionic form and thus being able to block the cyclic nucleotide gated channel in olfactory receptor cells [39]. Unsurprisingly, copper nanoparticles have been the source of much recent investigation with regard to their role as olfactory and nervous system disruptors [18,35,40]. The present work is largely consistent with the findings of these studies, suggesting that copper ions have a noticeable effect on the behavior of test fish. *Galaxias maculatus* tend to reduce movement when presented with a nonvisible “threat” cue, a response that was not seen in the highest concentrations of copper, suggesting that their ability to respond appropriately to olfactory cues has been compromised. This lack of crypsis resulting from exposure to high copper concentrations is likely to impact on *G. maculatus* by increasing the risk of predation. Furthermore, because olfaction is used by teleosts as the sole way of detecting pheromones [41,42], the reduction or removal of olfactory sensitivity would hinder a wide range of life events. For a diadromous species such as *G. maculatus*, olfactory impairment could have undesirable effects not only on threat avoidance, foraging, and species recognition but also on migration [24], resulting in the disruption of both spawning and juvenile recruitment. As catchments and urban waterways continue to experience an influx of metals (and other contaminants) from industrial processes and urbanization, efforts to identify and manage their impacts will need to consider the ecological consequences of the sublethal and indirect effects that this contamination is likely causing within aquatic ecosystems.

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Data availability—Data, associated metadata, and calculation tools are available by contacting O. Thomas (oliverrbthomas@gmail.com). All statistical analyses were done in R.

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Figure 1. Percentage change in frequency of swimming of *Galaxias maculatus* exposed to a range of copper concentrations (error bars = ± 1 standard error). (All odor cues combined.) Treatments that do not share a letter are significantly different.

Figure 2. Percentage change in frequency of swimming of *Galaxias maculatus* following addition of 3 odor cues, distilled water (control), conspecific odor, or alarm chemical (error bars = ± 1 standard error), averaged across copper exposure treatments. Treatments that do not share a letter are significantly different.

Table 1. Nominal and actual (measured by inductively coupled plasma–mass spectrometry) concentrations of copper (II) chloride across treatments

Nominal concentration ($\mu\text{g/L}$)	Actual concentration ($\mu\text{g/L}$)
0.0	<1
1.5 ± 0.1	1 ± 1
5.0 ± 0.4	6 ± 1
10.0 ± 0.7	8 ± 1
20.0 ± 0.1	18 ± 1

Table 2. Mean percentage change in frequency of each of the 4 observed behaviors exhibited by *Galaxias maculatus* exposed to different copper concentrations (5 levels) and stimulus types (3

levels)^a

Copper concentration ($\mu\text{g/L}$)	Stimulus	% Change freeze	% Change still	% Change swim	% Change dash
<1	Alarm	19.44	-9.72	-8.33	-1.39
<1	Conspecific	-1.39	-16.67	16.67	1.39
<1	Water	15.28	-16.67	0.00	1.39
1	Alarm	15.28	-9.72	-11.11	5.56
1	Conspecific	0.00	-11.11	9.72	1.39
1	Water	0.00	-2.78	5.56	-2.78
6	Alarm	-11.11	18.06	-2.78	-4.17
6	Conspecific	0.00	11.11	-18.06	6.94
6	Water	-1.39	1.39	0.00	0.00
8	Alarm	9.72	1.39	-19.44	8.33
8	Conspecific	-9.72	8.33	1.39	0.00
8	Water	0.00	8.33	-11.11	2.78
18	Alarm	0.00	-1.39	0.00	1.39
18	Conspecific	0.00	-5.56	11.11	-5.56
18	Water	0.00	-26.39	26.39	0.00

$n = 6$ fish for each of the 15 treatment combinations.

Table 3. Range, mean, and standard deviation for percentage change in frequency of

“swimming” behavior exhibited by *Galaxias maculatus* under different copper concentrations (n

= 18 fish/factor level) and stimuli ($n = 30$ fish/factor level)^a

Factor	Mean	Range	Standard deviation
Copper concentration			
<1 µg/L	2.78	−41.67 to 33.33	20.61
1 µg/L	1.39	−41.67 to 41.67	16.48
6 µg/L	−6.94	−50.00 to 16.67	15.46
8 µg/L	−9.72	−75.00 to 33.33	28.33
18 µg/L	12.50	0.00 to 91.67	22.62
Stimulus			
Distilled water	4.17	−75.00 to 91.67	25.87
Conspecific odor	4.17	−50.00 to 41.67	19.91
Alarm chemical	−8.33	−66.67 to 8.33	18.70

Because both factors were crossed, the total number of fish was 90, with 6 fish for each of the 15 treatment combinations.

Table 4. Results from 2-factor randomized complete block analysis of variance on the change in frequency of swimming^a

Source	Degrees of			
	freedom	Mean square	<i>F</i>	<i>p</i>
Copper concentration	4	1388.9	6.881	0.001*
Stimulus type	2	1562.5	6.285	0.017*

Copper concentration $\times$ stimulus	8	604.7	1.168	0.342
Block	5	683.3		
Copper concentration $\times$ block	20	201.9		
Stimulus type $\times$ block	10	248.6		
Residuals	40	517.7		

No F and p values are reported for those factors that include the randomized block term because there are no tests for these.

*Significant difference at $p < 0.05$.

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