# EFFECTS OF COPPER UPON LATERAL LINE NEUROMASTS IN *XENOPUS LAEVIS*

By

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EFFECTS OF COPPER UPON LATERAL LINE

NEUROMASTS IN *XENOPUS LAEVIS*

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Abstract: Fish and aquatic amphibians possess the lateral line, a sensory system used to detect water displacements. Copper is known to inactivate the neuromast organs of the lateral line system. This study investigated copper-induced neuromast loss in *Xenopus laevis*, the African clawed frog. *X. laevis* were exposed to various copper concentrations at three different life stages: newly metamorphosed juveniles, Nieuwkoop-Faber stage 51-54 larvae, and stage 54-55 larvae. Neuromasts counts, stain intensity, and behavior responses were examined. Possible behavioral effects of copper exposure to stage 54-55 larvae were investigated through an assay using air puff stimuli to produce surface waves. No change in neuromast number occurred in juvenile frogs exposed to copper at concentrations up to 3 mg/L. Neuromasts in the 51-54 trial did not photograph well, indicating that the methods of this study are better for imaging tadpoles of higher developmental stages. In the stage 54-55 trial, neuromasts were counted for four body regions: whole body, partial body, head, and tail. Although neuromast number decreased in the tail and partial body methods, this was not significantly different. Intensity of neuromasts showed a stronger concentration-dependent decrease, as a significant effect of copper concentration on intensity was observed in all four body regions. The decrease in intensity but not neuromast number may indicate that although neuromasts are still functioning, they have a decreased number of viable hair cells. Anterior neuromasts may be less sensitive to copper than posterior neuromasts. There was little difference in response to the air puff stimulus between control tadpoles and tadpoles exposed to 400 µg/L of copper. Neuromasts of *X. laevis* appear resistant to copper, as loss of these organs was not observed at sublethal or environmentally relevant copper concentrations. Future research could compare the resistance of neuromasts in *X. laevis* to species that lose the lateral line upon metamorphosis.

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# CHAPTER I

## INTRODUCTION

The ability of organisms to interact with their biotic and abiotic environments is essential for survival and reproduction, and various sensory systems are vital for processing and responding to environmental stimuli. Vertebrates living in aquatic environments use many of the same sensory systems as terrestrial animals, including vision, somatasense, olfaction and hearing (Collin & Marshall, 2008). Some aquatic species possess electroreception (Collin & Marshall, 2008). Additionally, fish, larval amphibians, and some adult aquatic amphibians have a mechanosensory lateral line system (Russell, 1976).

Lateral lines enable animals to sense water displacement and pressure changes (Bleckmann & Zelick, 2009). By sensing water displacement, the lateral line system helps fish and amphibians perform various behaviors, including escaping predators (Stewart *et al*., 2013), capturing prey (Junges *et al*., 2010), schooling (Partridge & Pitcher, 1980), and navigating their surroundings (Burt de Perera, 2004). Lateral lines are composed of organs called neuromasts (Bleckmann & Zelick, 2009). In fish, neuromasts can be located on the skin (superficial neuromasts) or within canals that

channel water over the neuromasts. Amphibians only have superficial neuromasts (Russell, 1976).

Neuromasts contain hair cells covered by a cupula that provides some protection from mechanical damage to the cells (Bleckmann & Zelick, 2009). The part of the hair cell responsible for signal transduction is the hair bundle, located on the apical surface of the cell (Schwander *et al*., 2010). Hair bundles are composed of stereocilia of increasing length; the longer side of the hair bundle ends with the kinocilium (Bleckmann & Zelick, 2009). Mechanotransduction, the conversion of a mechanical stimulus to an electrical signal, takes place at the apices of the stereocilia (Hudspeth & Corey, 1977; Chou *et al.,* 2017). This process occurs when water displacements bend the hair bundle towards the longest stereocilia, opening mechanically gated ion channels located on the apices and allowing cations to move into these channels. As lateral line hair cells function similarly to hair cells in the human ear, studies on neuromast loss and regeneration could have implications to human health (Lush & Piotrowski, 2014).

Sublethal, low micromolar concentrations of certain chemicals can temporarily or permanently disable neuromasts (Faucher *et al.*, 2006; Olivari *et al*., 2008). These chemicals disable neuromasts by competing with calcium ions for fixation sites, which prevents the ion fluxes necessary for signal transduction (Hudspeth, 1983; Faucher *et al.,* 2006). Oxidative stress and the accompanying necrosis or apoptosis may be responsible for killing hair cells of neuromasts (Forge & Schacht, 2000; Olivari *et al.* 2008). One drug known to impair neuromast function are aminoglycosides, a type of antibiotic. In addition to aminoglycosides, various divalent metal cations including cobalt, cadmium,

and copper can also disable neuromasts of fish (Faucher *et al.*, 2006; Olivari *et al.*, 2008).

Copper is an essential trace element, necessary to organisms for survival although excess amounts can cause lethal and sublethal toxicity (Perwak *et al.*, 1980). This is particularly true for aquatic systems where copper toxicity can have adverse impacts on organisms even at low concentrations (Irwin *et al.*, 1997). For example, 96-hour LC50s of fish exposed to copper can be less than 100 µg/L for *Oncorhynchus mykiss* (Howarth & Spague, 1977) and *Pimephales promelas* (Irwin *et al*., 1997). Copper can also interfere with hatching success and post-hatching health in aquatic animals: concentrations of 50 µg/L (Johnson *et al*., 2007) and 24 µg/L (Sonnack *et al*., 2015) slightly reduced hatching rates of zebrafish (*Danio rerio*) embryos. Additionally, for zebrafish hatchlings exposed *in ovo* to copper concentrations of about 100 µg/L, length is 7% smaller and heart rate over 20% higher, suggesting increased stress, compared to unexposed fish (Johnson *et al*., 2007). Copper concentrations of 1 mg/L are toxic to the kidneys and liver of flounder (Baker, 1969). Furthermore, copper can act as a neurotoxicant and is known to interfere with olfaction in fish exposed to only 8 µg/L Cu (Hara *et al*., 1976). In addition to interfering with olfaction, copper also disrupts the function of the lateral line system by killing hair cells within the neuromasts of fish at concentrations as low as 25 µg/L (Linbo *et al.*, 2006). This neuromast damage is not necessarily permanent, as damaged hair cells can regenerate after removing fish from the copper exposure (Hernandez *et al.*, 2006). Sustained neuromast damage likely requires constant exposure to copper as shown by Linbo *et al.* (2006) who found no neuromast recovery among zebrafish larvae in a 77 hour static renewal experiment in which a 50

µg/L copper solution was renewed every 24 hours. However, after five hours of copper exposure and a 72 hour recovery period, zebrafish neuromasts were able to regenerate. Likewise, neuromast loss without regeneration occurred when Johnson *et al*. (2007) exposed zebrafish embryos to copper concentrations of 68 µg/L and 244 µg/L for 120 hours, renewing 50% of the test solution every 24 hours.

The toxicity of copper to aquatic organisms is affected by water chemistry, as this influences metal speciation and thus bioavailability (Klaassen & Watkins, 2015). Specifically, increasing dissolved organic carbon (DOC), pH and hardness reduce toxicity by decreasing the biovailability of copper (Howarth & Spague, 1977; Meador, 1991). In particular, DOC can greatly reduce copper toxicity to neuromasts (Linbo *et al.*, 2009). Water hardness and alkalinity can also decrease copper-induced neuromast toxicity, but to a lesser degree than DOC (Linbo *et al.*, 2009).

Although most studies have examined copper-induced neuromast loss in fish, one study investigated the impacts of copper on the lateral line system of amphibians (Vazquez, 2016). Southern leopard frog (*Lithobates sphenocephalus*) and Great Plains toad (*Anaxyrus cognatus*) tadpoles were exposed to copper for three weeks. *L. sphenocephalus* were exposed to 0-50 µg/L of copper while *A. cognatus* were exposed to 0-30 µg/L. The number of neuromasts in *A. cognatus* decreased by an average of 38% during the first week of the study. Although the number of neuromasts also decreased after one week of copper exposure for *L. sphenocephalus* tadpoles exposed to 50 µg/L, neuromast counts did not significantly differ from the control group. In subsequent weeks of the study for both tadpole species, the number of functional neuromasts in the treatment groups was similar to the control group, indicating neuromast regeneration had

occurred, likely due to loss of copper from the test environment. Among *L. sphenocephalus* tadpoles exposed to 20 µg/L of copper, the number of neuromasts during the third week was 20% higher than the control, suggesting a possible hormetic effect of the metal.

Neuromasts differ in anatomy, distribution, and function. Neuromasts of the head region, called anterior neuromasts, are innervated by the anterior lateral line nerve while trunk (posterior) neuromasts are innervated by the posterior lateral line nerve (Russell, 1976). Another innervation difference for *Xenopus* is that groups of neuromasts, called stitches (Harris & Milne, 1966), are more frequently innervated by more than two fibers in the anterior as opposed to the posterior lateral line (Mohr & Gorner, 1976). The posterior lateral line has more stitches than the anterior lateral line, but stitch density is greater in the head region (Mohr & Gorner, 1976). Due to these differences in anatomy and density, neuromasts of the two body regions have contrasting roles. Head neuromasts are more important than body neuromasts for detecting surface waves in *X. laevis* (Russell, 1976) and in surface feeding fish (Schwartz & Hasler, 1966; Bleckmann & Zelick, 2009). Although anterior lateral line neuromasts are likely more important in prey detection in *Xenopus*, posterior lateral line neuromasts are probably more important in predator avoidance (Mohr & Gorner, 1996). Posterior lateral line neuromasts may be more sensitive to water disturbances, whereas anterior lateral line neuromasts have better spatial resolution due to their higher density (Mohr & Gorner, 1996). As a result of their different anatomy, anterior and posterior neuromasts may also differ in sensitivity to chemicals. This is supported by Hernandez *et al.* (2006), who found differential hair cell regeneration between neuromasts of the head and body in zebrafish. Whereas

regeneration of body neuromasts no longer occurred at copper concentrations greater than 3.18 mg/L, head neuromasts regenerated at all concentrations tested up to 25.42 mg/L. Similarly, posterior neuromasts were more sensitive than anterior neuromasts exposed to caffeine, dichlorvos, 4-nonylphenol and perfluorooctanesulfonic acid, based off a scoring system looking at the number of hair cells stained within a neuromast and fluorescent intensity of mitochondria (Stengel *et al*., 2017). This scoring system also showed posterior neuromasts were more affected than anterior neuromasts after a 30 minute exposure to copper sulfate or neomycin (Stengel *et al*., 2017); however, this was reversed after 96 hours of exposure when anterior neuromasts exhibited greater cellular damage (Stengel *et al*., 2017). The authors speculate differential regeneration or repair mechanisms may account for the contradictory results at 30 minutes and 96 hours.

Neuromast loss from metals and aminoglycosides can influence animal behavior. Disabling the lateral line system can reduce the ability of fish to escape predation (Stewart *et al*., 2013). Even low toxicant concentrations can impact anti-predator behaviors; for example, exposure to 5 µg/L cadmium interfered with the startle response in sea bass (Faucher *et al*., 2006). Ablation of the lateral line system also effects predators, as when cobalt chloride reduced predation success in marbled swamp eels (Junges *et al*., 2010). Additionally, damage to the lateral line can decrease the swimming abilities of aquatic animals. For instance, cadmium (Baker & Montgomery, 2001) and the aminoglycoside gentamicin (Coombs *et al*., 2001) can interfere with the ability of fish to orientate to currents. Moreover, exposure to streptomycin interfered with schooling in *X. laevis* tadpoles (Lum *et al*., 1982).

The purpose of this study was to examine copper toxicity on neuromasts in a traditional amphibian model. *Xenopus laevis*, the African clawed frog, is commonly used in studies of lateral line function and how this links to behavior (Gorner, 1973; Elepfandt, 1982; Gorner *et al*., 1984; Claas *et al*., 1993; Claas & Dean, 2006). This species has the advantage of retaining the lateral line system after metamorphosis, allowing greater flexibility in time-sensitive experimental design protocols. I also was interested in how neuromast sensitivity to copper differed between larvae and post-metamorphic frogs, and if amphibians would exhibit differential sensitivity of head and body neuromasts. Additionally, I was interested if copper exposure and its possible damage to the lateral line system would impact behavior.

# CHAPTER II

# METHODS

## *Animal Source and Husbandry*

*Xenopus laevis* were obtained from Xenopus1 (Dexter, Michigan). Before the beginning of exposures, animals were communally housed in several 2.5 gallon glass tanks. Juvenile frogs were fed Protec FW Extruded Floating 2.5 mm pellets by Skretting (Tooele, Utah). During the exposure, each was fed three pellets the day before water renewal. Larvae were fed a 60 g/L solution of Micron Growth Food by Sera (Heinsberg, Germany). Each tadpole was fed three drops of this solution twice a day during copper exposure. Animals were kept individually in 3 L glass jars during exposures, filled respectively with 500 mL or 1000 mL of carbon-filtered water for juvenile frogs and larvae. Water temperature was 23-24$^{o}$C, pH was 7.8-8.0, and photoperiod was 12 hours light to 12 hours darkness. Research was performed under Animal Care and Use Protocol AS-18-3 of the Institutional Animal Care and Use Committee (IACUC) of Oklahoma State University.

## *Exposure*

Copper stock solution was prepared by dissolving 196.4 mg copper sulfate pentahydrate (Sigma-Aldrich) into 10 mL 2% nitric acid solution, using ultrapure water

(resistivity 18.2 MΩ-cm). This was further diluted to 50 mL for a stock solution of approximately 1000 µg/mL Cu.

During exposures, 10 mL water samples were collected from each treatment group at intervals of approximately 24 hours. Each sample was acidified with 1 mL of 20% nitric acid. If the copper solution was renewed, samples were taken before and immediately after the water change. A subset of samples were analyzed with the ICP-OES to verify concentrations of copper and other metals. The limit of quantitation for copper was approximately 25 µg/L. The ICP-OES indicated calcium concentrations of about 30 mg/L and magnesium concentrations of 4 mg/L.

Juvenile and larval *X. laevis* were exposed to copper to compare neuromast sensitivity between different life stages. The juvenile exposure used newly metamorphosed individuals that were less than 3.6 cm in snout-vent length. Stage 51-54 tadpoles were initially used to examine larval neuromast toxicity. Since the neuromasts in stage 51-54 larvae were not as well defined as in photographs of more developed tadpoles used in preliminary trials, stage 54-55 tadpoles were subsequently used.

*Juvenile*

Twenty juvenile *X. laevis* were divided among four treatment groups consisting of 0, 1, 2, and 3 mg/L Cu (n = 5). Frogs were exposed to copper for 144 hours. After 72 hours of exposure, the solution was renewed by performing a complete water change and reapplication of the stock solution. Higher copper concentrations were not used because they are unlikely to be encountered in the environment, even the most polluted sites. For example, two studies did not observe copper concentrations exceeding 2.2 mg/L in

mining tailing ponds or drainages with a pH of 5 or greater (Allen *et al.*, 1996; Rosner, 1998).

*Larvae*

Larvae were divided between five treatment groups (n = 16) for both stage 51-54 and stage 54-55 tadpoles. Stage 51-54 larvae were exposed to 0, 200, 400, 600, and 800 µg/L Cu whereas stage 54-55 larvae were exposed to 0, 100, 200, 300, and 400 µg/L copper. All larvae were exposed to copper for 96 hours; the copper-water solution was not renewed. Due to the amount of time needed for the staining and imaging process, dosing was divided between two days. For stage 51-54 tadpoles, half of the glass jars were dosed two days after the tadpole shipment arrived while the remaining forty were dosed the following day. Half of the stage 54-55 tadpoles were exposed the day after arrival; the others were dosed two days after arrival. Tadpoles were divided between sixteen blocks, each containing one individual from each of the five treatment groups. The members of a block were placed adjacent to each other on shelves. The position of the jars within these blocks was assigned with a random number generator (Random.org).

### *Staining and Imaging Procedure*

*Juveniles*

Neuromasts were photographed the day prior to dosing and then after 144 hours of exposure to the copper solution. Frogs were stained, anesthetized and imaged three at a time. For the staining procedure, frogs were placed in a solution of 6.34 µM 4-(4-Diethylaminostyryl)-1-methylpyridinium iodide (DiAsp, from Sigma-Aldrich) for eight minutes. DiAsp is used to selectively label neuromasts as the dye enters live hair cells

through mechanotransduction channels located on the stereocilia (Faucherre *et al*., 2009). Each frog was rinsed 20 times; each "rinse" consisted of pouring approximately 200 mL of carbon-filtered water over the frogs. Frogs were then placed into tricaine mesylate (MS-222) solution, at concentrations ranging from 352-375 mg/L, for at least 20 minutes. The MS-222 solution was prepared the day before imaging the frogs. When preparing MS-222, the anesthetic was dissolved in carbon-filtered water and buffered with sodium bicarbonate to approximately pH 7.8. If frogs displayed a righting reflex or gular pumping, they were kept in anesthesia until these were no longer observed. To prevent photobleaching, the beaker of DiAsp and anesthesia container were covered respectively with aluminum foil or cloth. Aluminum foil was also used when transporting frogs to the cell reader. Anesthetized frogs were placed ventral-side down into a Greiner six-well plate. I chose to photograph the ventral rather than dorsal side because it was flatter and thus a higher percentage of neuromasts were in focus. Stained neuromasts were photographed with a Cytation 5 Imaging Reader by Biotek set on manual mode. The red fluorescent protein (RFP) filter and 4x objective lens was used; the stage distance was set at 1998 µm. To recover from anesthesia, frogs were placed in a 3 L glass jar with 500 mL of water that was oxygenated with an air stone. No mortality or adverse symptoms were observed for any of the frogs.

*Larvae*

Neuromasts were photographed at the end of the 96 hour exposure period. The sixteen blocks of tadpoles were stained and photographed one at a time. Although blocks initially consisted of five tadpoles, some in the stage 51-54 exposure contained fewer due to mortality. Tadpoles were stained in DiAsp for eight minutes and rinsed for two

minutes. After exposure to DiAsp, tadpoles were placed in a petri dish of carbon filtered water until they were rinsed. Immediately after rinsing, larvae were euthanized by exposure to 471 mg/L MS-222 solution for at least five minutes. To prevent misidentification, larvae were stained and euthanized in separate containers, and individually washed. All containers were covered with aluminum foil to prevent photobleaching. Tadpoles were placed one to a well in a Greiner six well plate. Each larva was positioned on its side with the head oriented to the left and the tail to the right.

Tadpoles were individually photographed with the Cytation 5 Imaging Reader by Biotek (cell reader), using the RFP filter, 4x objective, and LED intensity of 10. Images were saved in the TIFF format for further analysis. For stage 51-54 larvae, the cell reader was set on manual mode with the following settings: integration time 100, camera gain 10, and stage distance 1825 µm. For stage 54-55 larvae, a protocol file was created in the Gen5 software to image the tadpoles using the laser autofocus feature. For each of the sixteen blocks, exposure settings were adjusted by focusing on the upper lateral line (Shelton, 1970) or head neuromasts from the tadpole in the 100 µg/L treatment. Ten stage 54-55 larvae, members of blocks 1 and 2, were excluded from data analysis since they were unintentionally photographed at a different LED intensity.

### *Neuromast Quantification*

*Juveniles*

I manually counted the number of neuromasts from images captured before and after exposure to copper. I excluded neuromasts located towards the sides of the frogs that were out of focus or absent due to positioning (Figure 1). The maxillary lateral line

(Shelton, 1970) was not enumerated because the neuromasts were often out of focus or not visible due to the sloping of the frogs' bodies anterior to the mouth. One image, the after exposure picture for frog 2-5 (2 mg/L, individual number 5), had a great amount of non-specific staining on the posterior half of the body of the frog. Consequently, I did not count neuromasts for this individual in that region of the body.

*Stage 51-54 Larvae*

Neuromasts and developmental stage were visually examined. The measure line tool in manual mode of Gen5 software (Biotek) was used to help stage the tadpoles. I had originally hoped to use the "cellular analysis" feature to quantify the number of neuromasts in the images. However, high levels of non-specific stain made this unfeasible. Instead, images were visually evaluated for whether or not they had clearly visible neuromasts. Neuromasts were scored with the following scale: "No clear neuromasts (0)", "few clear neuromasts (1)", "some clear neuromasts (2)" or "clear neuromasts (3)". The criteria for each group are described in Table 1.

*Stage 54-55 Larvae*

TIFF image files were opened in manual mode of the Gen5 software. The measure line tool was used to verify tadpole stage according to foot and leg morphology (Nieuwkoop & Faber, 1994). Utilizing the cellular analysis feature, the number of neuromasts was quantified using object counts at different thresholds. The threshold is the background intensity level used to detect separation between objects (Held & Banks, 2013). Thresholds that are too low would have artificially high counts by quantifying non-target (i.e. non-neuromast) objects. Additionally, low thresholds may incorrectly count multiple neuromasts as a single object. Although setting thresholds higher reduces

counting of non-target objects and areas of non-specific stain, it also risks excluding target neuromasts. Thus images were tested at various thresholds to find an optimal balance between minimal background interference and neuromast detectability. To determine this optimal threshold, accuracy of neuromast labeling was examined at thresholds of 5000, 7000, 10000, and 15000 relative intensity units (RIU). If higher thresholds were needed, 20000, 25000, and 30000 RIU were also examined. Once the optimal threshold was determined, the neuromast count and the mean intensity of the enumerated objects from each image was recorded.

Four different regions of the body were used to quantify neuromast numbers and intensity. The "whole body" method involved no cropping. An object size of 20-150 µm was used to count neuromasts; "object size" restricts counted objects to the specified minimum and maximum diameters. The "partial body" method involved cropping out areas that commonly possessed high levels of non-specific stain, specifically the bottom of the head, the legs, and the posterior end of the tail (Figure 2). As cropping out these areas reduced background interference, a larger and thus more sensitive 20-200 µm object size range was used. For a few tadpoles, areas of concentrated non-specific stain remained, particularly on the tail, after the plug was performed. These areas of non-specific stain were removed if they could be cropped without excluding neuromasts. The "head" method cropped the image to include an area of the head ventral to the nostrils, dorsal to the eye, and anterior to the stirnorgan (Figure 3). This excluded the bottom of the head, which usually contained significant amounts of non-specific staining. This method also used an object size of 20-200. The "tail plug" method examined an area of

the tail 20000 µm in length by 2000 µm in width (Figure 4) using an object size of 20-200 µm.

Images were excluded from analysis if none of the examined thresholds balanced neuromast detectability with excluding interference from non-target objects. This typically occurred if areas of non-specific stain could not be excluded and had a greater intensity than neuromasts. Five images were excluded from the whole body method, four from the partial body method, two from the head method, and six from the tail method.

***Behavioral Assay***

For the behavioral assay, ten stage 54-55 tadpoles were exposed to 400 µg/L copper for 96 hours three days after arrival. Additionally, ten tadpoles were assigned to a control group receiving no copper. After the exposure period, larval response to surface wave stimuli was quantified in a behavioral assay. Similar to Claas and Dean (2006), pipettes were used to produce an air puff to form surface waves. Whereas adult African clawed frogs orient to air puff stimuli as a predatory behavior (Claas & Dean, 2006), tadpoles were expected to exhibit an anti-predator startle response and required no training. Briefly, an air puff was delivered to a 60 mm petri dish holding the tadpole using a two milliliter plastic pipette attached to a nine-inch glass pasteur pipette (Figure 5). The petri dish was filled to the brim with carbon filtered water. Every tadpole had a five minute acclimation period after being placed in the petri dish before air puffs were delivered. The stimulus was delivered 2, 4, 6, 8, 10, 12 and 14 cm above the surface of the water. As the strength of the stimulus decreased with height, this created a gradient of stimulus intensity. 14 cm was chosen as the maximum height because although the air

puff stimulus still visibly disturbed the water, tadpoles in preliminary trials rarely reacted to the stimulus at this height. The order of heights was randomized for each tadpole. Tadpoles were given 20 seconds after each height adjustment before administering the stimulus. The stimulus was delivered five times at each height, with ten seconds of the tadpole remaining motionless between the stimuli. To ensure that the tadpoles were responding to the air puff and not hand movement, the observer would hold the pipette bulb for at least five seconds before delivering the stimulus. Reaction of the tadpole to the stimulus also had to be scored before the observer removed their hand. Four quadrants, equally dividing the petri dish, were drawn to help evaluate the behavioral response. A circle was drawn in the center of the petri dish to check that the stimulus was being delivered to that point.

The response of tadpoles to each stimulus was scored on a scale of zero to four. Zero indicates no discernible response to the stimulus. A score of one denotes one of the following slight movements: increased tail movements, a more rapid rate of tail vibration, or flaring out of the feet. It also includes a slight forward drift accompanied by no twitching or active swimming movement; this forward drift had to be slow and less than ¼ of the larva's snout-vent length. A score of two denotes movement less than one snout-vent length in distance. This includes swimming forward, a full-body twitch, or a side-to-side rocking movement. A score of three denotes normal reactions in which tadpoles swam greater than one snout-vent length, but remained within two quadrants. Delayed reactions that involved the tadpole swimming across three or more quadrants were also placed in this category. A score of four denotes the tadpole swam across three or more quadrants, without showing a delayed reaction.

***Statistical Methods***

Due to the small sample size of the juvenile trial, only summary statistics were used to analyze the data. The mean and standard error of neuromast counts were calculated for each treatment group. Summary statistics were also calculated for the neuromast scores and stage data for the stage 51-54 trial. Additionally, LC50s were calculated using probit analysis (Finney, 1952).

For the stage 54-55 trial, the neuromast and intensity data were analyzed using the general linear mixed models procedure in SAS 9.4. Because camera exposure settings were adjusted for each of the sixteen blocks, these were treated as blocks using the "random" statement. Whereas neuromast values were modeled with a normal distribution, intensity values were analyzed with an exponential distribution because of their greater variance and departures from normality. The "random residuals" statement and "group = effect" option were also used to account for heterogeneity of variances. Tukey-Kramer pairwise comparisons were performed when the treatment effect was significant. All tests were done at the $\alpha = 0.05$ level.

For the behavioral data, the five response scores of a tadpole at each given height were averaged for the mean reaction of each tadpole at that height. These values were analyzed using general linear mixed models procedure in SAS 9.4. A normal distribution was used and individual tadpoles were treated as blocks. Tukey-Kramer pairwise comparisons were used for significant effects. To calculate the mean response score of a tadpole, the mean reaction at each of the seven heights for that tadpole was averaged together. This was also analyzed in SAS 9.4 using the general linear mixed models, normal distribution, and least square means with the Tukey-Kramer adjustment.

For ICP-OES results from all three trials, the mean and standard deviation were calculated for samples from each copper concentration at the initial and final time-points.

# CHAPTER III

# RESULTS

## *Juvenile Exposure*

Copper concentrations decreased over time (Table 2). Over 72 hour intervals, copper loss ranged from 36% in the 3 mg/L treatment to 10% in the 1 mg/L treatment. Copper in the controls were negligible compared to the treatment groups, with concentrations less than the quantitation limit of 25 µg/L. The measured average copper concentrations for each treatment group at the time points tested remained within 30% of the nominal concentrations.

Neuromasts of juvenile clawed frogs were not sensitive to sublethal copper concentrations of 1-3 mg/L (Table 3). Indeed, counts averaged about 200 neuromasts per frog across all treatment groups, with no change after six days of exposure.

## *Stage 51-54 Larvae Exposure*

ICP-OES results suggest accurate initial concentrations but copper loss over time ranging from 4% for 200 µg/L Cu to 37% for 800 µg/L Cu (Table 4). At 0 hours, the average copper concentration of the treatment groups was within 15% of the nominal concentration. Control group copper concentrations were less than 25 µg/L.

The median and average development stage for all treatment groups was 52-53 (Table 5). Mortality was observed among the various treatment groups for stage 51-54 larvae (Table 6). While no tadpoles died in the control or the 200 µg/L concentration, less than 20% of the tadpoles survived 800 µg/L, the highest concentration. The LC50 for tadpoles in the study was 656 µg/L, with a 95% fiducial confidence interval of 561-766 µg/L.

Many of the tadpoles imaged, including larvae in the control group, did not have well-defined neuromasts. Neuromasts were most clear and numerous in tadpoles exposed to 200 µg/L of copper (Table 7). Neuromasts were less clear in the other treatment groups, in which images received scores of 0 (no clear neuromasts) and 1 (few clear neuromasts). These low scores were more prominent among tadpoles from the two highest treatment groups, 600 µg/L and 800 µg/L, possibly suggesting neuromast loss. Both groups had a median value of 0, denoting no visible neuromasts.

***Stage 54-55 Larvae Exposure***

Some loss of copper from the exposure solutions was indicated by ICP-OES results (Table 8). Immediately after dosing, the measured average copper concentrations of each treatment group were within 12% of the nominal concentration, with the exception of the 200 µg/L treatment. Within the 200 µg/L treatment, one of the three samples read had a copper concentration equivalent to that of the controls. After 96 hours, copper concentrations remained within 30% of the nominal values. Copper loss was the greatest within the 100 µg/L treatment and the 400 µg/L behavioral treatment. Copper concentrations in the control group were under the 25 µg/L limit of quantitation.

Tadpoles were about stage 54-55 upon delivery. After the 96 hour exposure the tadpoles in sets one and two had an average stage of 55 (Table 9). Tadpoles in the behavioral assay averaged approximately stage 57.

Counts of neuromasts did not differ among copper treatments for any of the different body regions (Table 10). Average neuromast counts for the whole body ranged from 175 for the control to 212 for the 100 µg/L and 400 µg/L treatments, a marginal difference ($F_{4,19.83} = 1.81$, $p = 0.17$). For the partial body, the control and 100 µg/L groups had about 140 neuromasts while the 400 µg/L group had about 120, a non-significant difference ($F_{4,18.15} = 0.65$, $p = 0.63$). Head neuromast counts ranged from 29 at 200 µg/L to 37 at 400 µg/L ($F_{4,22.72} = 1.06$, $p = 0.40$). With the tail, neuromast counts ranged from 45 at 400 µg/L to 58 at 0 µg/L ($F_{4,19.71} = 1.20$, $p = 0.34$).

Despite the lack of effect on the number of neuromasts, increasing copper concentration did decrease neuromast intensity (Table 11). Whole body intensity values differed among the five concentrations in a dose-dependent fashion ($F_{4,47} = 4.75$, $p = 0.003$). Whole body intensity values for tadpoles exposed to 300 and 400 µg/L Cu were 20-30% less than those in the control group (Figure 6). Intensity values for the partial body, head, and tail also showed a concentration related response to copper exposure similar to that observed for the whole body (Figures 7-9). Average intensity for the partial body was 22 to 37% lower in the 300 and 400 µg/L treatments than the control ($F_{4,48} = 3.74$, $p = 0.01$). Head neuromasts intensity at 400 µg/L was 30% less than the control ($F_{4,50} = 5.66$, $p < 0.001$). With each 100 µg/L increase in copper concentration, head intensity decreased by 6-12%. The average intensity of tail neuromasts was 40% higher for the control group than the 400 µg/L treatment, which was significant ($F_{4,46} =$

3.00, p = 0.03). However, the more conservative Tukey-Kramer pairwise comparisons did not detect any differences in intensity between concentrations (Figure 9).

Behavioral response to air puff stimulus was similar between larvae exposed to 0 µg/L and 400 µg/L copper. The mean response score of individual tadpoles was 1.7 for the control group and 1.6 for treatment tadpoles, which did not differ ($F_{1,18} = 2.15$, p = 0.16; Figure 10). The animals also exhibited a similar response to the stimulus at various heights. As the height of the stimulus increased, the larvae's reaction scores decreased. Although average response scores were lower for treatment group tadpoles at all heights greater than 4 cm, these scores were similar for both groups (Figure 11). The response was only significantly different at the 12 cm height ($F_{1,9} = 5.71$, p = 0.04).

# CHAPTER IV

# DISCUSSION

Neuromasts of *Xenopus laevis* appear partially resistant to acute copper toxicity, as loss of these organs was not clearly observed at sublethal or environmentally relevant concentrations. No neuromast loss was observed in larvae exposed at copper concentrations up to 400 µg/L or in juveniles up to 3 mg/L. Although neuromast loss may have occurred in stage 51-54 tadpoles exposed to 600 and 800 µg/L Cu, mortality was observed at these concentrations, which confounds the loss of neuromasts. Neuromast loss could still occur at copper concentrations higher than those tested in this study but would not be environmentally relevant, as these concentrations are not often observed in nature. For example, 89% of water samples collected from EPA sites across the United States had copper concentrations of 100 µg/L or less, 10% had concentrations of 100-1000 µg/L, and only 1% exceeded 1000 µg/L Cu (Perwak *et al*., 1980). Although copper concentrations exceeding 600 mg/L have been observed in a mining watershed in Montana with a pH below 4.0 (Gammons *et al*., 2005), few amphibian species could inhabit such an acidic environment (Pierce, 1985). Sites in this drainage with a more favorable pH > 6 did not exceed 1.1 mg/L (Gammons *et al*., 2005).

The juvenile life stage appears particularly tolerant of copper, as neuromast loss was not observed at concentrations up to 3 mg/L, which are lethal to *X. laevis* larvae, larvae of several amphibian species in the families Ranidae and Bufonidae (Chiari *et al.*, 2015), and many fish species (Irwin *et al.*, 1997). It is unlikely that neuromasts decreased in number but then regenerated, returning to initial levels prior to imaging. In preliminary trials, I exposed a few animals to copper for 2-3 hours and 72 hours but saw no obvious decrease in neuromast numbers (data not included). Preliminary trials also showed no loss of neuromasts on the dorsal side of the body (data not included). One possible reason for the difference in neuromast sensitivity between juveniles and larvae could be the location of hair cells (Shelton, 1970). In tadpoles, hair cells protrude above the surface of the epidermis, whereas post-metamorphic hair cells are in depressions partially beneath the surface of the skin and between two hillock-shaped sensory organs (Shelton, 1970). This deeper location may serve a protective function, as it could shield the cupulae from stronger water displacements made by the more vigorous movements of adults (Frischkopf & Oman, 1972; Russell, 1976). Copper may enter the apical surface of hair cells via mechanotransduction channels, other membrane channels, or transport proteins (Olivari *et al.*, 2008). By offering physical protection, it is possible the deeper location of post-metamorphic neuromasts could decrease toxicity by reducing copper uptake into the cell through these routes. Furthermore, mucus might help account for differential copper sensitivity between juveniles and tadpoles. Increased mucus production in juveniles could serve as a physical barrier that protects against toxicity, as post-metamorphic clawed frogs have a higher number of mucous glands and secrete more acid mucopolysaccharide (AMPS) than larvae (Shih & Vanable, 1974). In addition to

acting as a physical barrier, mucus can protect against metal toxicity because its glycoproteins and other components can bind metal cations (Pärt & Lock, 1983; Coello & Khan, 1995). Another possible reason for differential sensitivity between juvenile and larval clawed frogs are the numbers of hair cells within a neuromast. Whereas tadpoles have 8-28 hair cells per neuromast (Lannoo, 1987), post-metamorphic individuals have 30-60 (Strelioff & Honrubia, 1978). For neuromast loss to be detectable, more hair cells would need to be damaged in juveniles than for larvae, perhaps requiring greater copper concentrations.

Stage 51-54 larvae appeared more sensitive to copper than the juveniles. Whereas no mortality was observed with juveniles exposed up to 3 mg/L Cu, mortality occurred at 600 µg/L and 800 µg/L for the larvae. Furthermore, neuromasts from tadpoles in the 600 µg/L and 800 µg/L concentrations were not very visible or numerous, suggesting neuromast loss. However, it is difficult to ascertain whether neuromast loss occurred, because many stage 51-54 tadpoles including control individuals did not have well-defined neuromasts. This may be a result of reduced DiAsp uptake in neuromasts of less developed tadpoles because larvae of higher developmental stages, such as those in the stage 54-55 trial, always had clear and well-defined neuromasts. Whereas most groups had some images without clear neuromasts, these organs were always well-defined in the 200 µg/L treatment, suggesting a possible hormetic effect of copper. This effect was unrelated to increased developmental stage resulting in more developed neuromasts, as mean developmental stage of the 200 µg/L larvae was actually less than the 0 µg/L, 400 µg/L, and 800 µg/L groups. Another possible case of hormesis was described by

Vazquez (2016), when neuromast number increased among tadpoles exposed to 20 µg/L copper.

For tadpoles in the stage 54-55 trial, neuromast loss was not evident for any of the four body regions. The similarity of neuromast numbers between treatment groups was likely not due to non-specific label or error with the software enumerating neuromasts. Because analyses were run using the best threshold for each image, the majority of the counted objects were neuromasts. However, the whole body method did contain some non-specific labeling, especially towards the bottom of the head and the limbs, so neuromast counts obtained from the other body regions are more accurate. Although no decrease in the number of neuromasts was observed, this does not indicate that the organs were unaffected by copper. One explanation for the lack of neuromast loss is that neuromasts contain multiple hair cells. For example, a neuromast in a juvenile zebrafish has about 20-30 hair cells (Olt *et al.*, 2014) whereas adult clawed frog neuromasts contain 30-60 hair cells (Strelioff & Honrubia, 1978). Even if some of the hair cells within a neuromast are killed or disabled from copper exposure, the neuromast will presumably still be counted with software as long as other hair cells remain functioning and viable and thus can take up stain.

Despite the lack of effects on neuromast counts, the intensity results support the hypothesis that neuromasts were still affected by copper. For the four body regions, neuromast intensity decreased with increasing copper concentration. These lower intensity values indicate lessened uptake of DiAsp by the hair cells of neuromasts, perhaps suggesting closed mechanotransduction channels (Faucherre *et al.*, 2009). The decrease in intensity could also suggest that although some of the hair cells in the

neuromast are no longer functioning, other viable hair cells that incorporated the dye still remain. To help verify whether the intensity reduction observed in this study was due to decreased hair cell viability, individual neuromast organs could be photographed in addition to imaging the entire tadpole. For example, confocal microscopy has been used to photograph hair cells (Hernandez et *al*., 2006). Additionally, acridine orange stain could be used to determine whether increasing copper concentrations likewise increases cell death of hair cells (Hernandez *et al.*, 2006).

Several studies have described decreased strength of neuromast staining with increasing toxicant concentration similar to the intensity results of this study. For example, the number and intensity of hair cells within zebrafish neuromasts labeled with the dye DASPEI were observed to decrease after exposure to copper (Linbo *et al*., 2006). Decreased brightness of DASPEI stain also occurred in the neuromasts of zebrafish exposed to a variety of different ototoxic compounds, including copper sulfate (Buck *et al*., 2012). In *Xenopus laevis* larvae, the strength of FM-143 dye labeling decreased with increasing concentrations of antibiotics (Nishikawa & Sasaki, 1996). Additionally, scoring fluorescent intensity of hair cells on numeric scales has been used to evaluate damage to neuromasts (Harris *et al*., 2003; Stengel *et al*., 2017).

Although neuromast loss was not evident, the decrease in intensity demonstrates copper still affected these organs and thus behavior could also be influenced by hair cell damage. However, similar responses to air puff stimuli were observed in stage 54-55 tadpoles exposed to 0 and 400 µg/L Cu, despite the 30-40% reduction in neuromast intensity across the four body regions in larvae exposed to 400 µg/L Cu as compared to the controls. This suggests that even though some hair cells were damaged, other hair

cells remain functional and thus a greater percentage of these cells would need to be impaired before the organism's ability to respond to surface waves is reduced. At a height of 12 cm, there was a slight behavioral effect, with control tadpoles exhibiting a stronger response to the stimulus than controls. Just as a person with otic hair cell damage may be able to understand speech at a close distance but not from farther away, the ability of damaged lateral line hair cells to detect water disturbances likely decreases with distance to a greater degree than that of an individual with unimpaired hair cells. Furthermore, response to surface waves may not be a sensitive endpoint as even after great damage to the lateral line system, previous studies have demonstrated that animals can still respond to this stimulus. For example, despite the destruction of all neuromasts, adult clawed frogs still orient to surface waves, but less accurately (Gorner *et al*., 1984). Similarly, after exposure to various ototoxins resulted in complete loss of labeled neuromasts, a startle response was still observed in zebrafish larvae (Buck *et al.*, 2012). The presence of a response despite inactivation of the lateral line suggests that other sensory systems such as the vestibular system or touch may help the animals respond to surface waves (Claas *et al*., 1993). Since adult frogs and larval zebrafish can still detect and respond to stimuli despite extensive damage to the lateral line system, it seems probable that tadpoles would exhibit normal responses to stimuli after the lesser degree of damage observed in this study.

Though the ability of tadpoles to respond to surface waves was not drastically reduced, the possible decrease in viable hair cells could still negatively impact other lateral-line mediated behaviors. Even if larvae are still able to detect surface waves, they may have altered behavior regarding more sensitive endpoints. For instance, after

treatment with cobalt chloride and streptomycin, *Xenopus laevis* larvae had less precise rheotaxis (Simmons *et al.*, 2003) and streptomycin exposed tadpoles showed altered schooling behavior (Lum *et al.*, 1982). Perhaps sublethal copper exposure would also affect these behaviors. If this behavioral assay would have indicated a difference in behavior between treatment and control tadpoles, another issue would have been distinguishing whether this difference was actually a result of neuromast loss or instead lethargy due to other effects of copper toxicity. To see if behavioral changes are unique to copper, responses could be compared with those of tadpoles exposed to another substance known to impair lateral line function, such as an aminoglycoside antibiotic. Quantifying neuromasts to check that the two substances decreased the organs to a similar degree could help support that behavioral changes were due to loss of lateral line function; however, at least for copper, concentrations high enough to cause a visible decrease in neuromasts may cause other harmful side effects.

In previous studies, neuromasts of the head and tail of larval zebrafish show differential sensitivity (Stengel *et al*., 2017) and regeneration (Hernandez *et al*., 2006). Most relevant to this study, a greater sensitivity of head neuromasts as compared to tail neuromasts was observed in a similar 96-hour exposure of zebrafish to copper sulfate (Stengel *et al*., 2017). However, the results of this study suggest that the tail neuromasts may be more sensitive to copper than head neuromasts in *X. laevis* larvae, as neuromast count data indicates a non-significant loss of tail neuromasts. No such trend is evident for the head neuromasts because the 400 µg/L group had the highest number of neuromasts. Furthermore, tail neuromast intensity had a larger disparity between the control and 400 µg/L values than the head neuromasts: for the 0 and 400 µg/L groups,

there was a 40% decrease in intensity for the tail neuromasts and a 30% decrease for the head neuromasts. Nevertheless, this should be taken with some caution as the difference between the 0 and 400 µg/L groups for the tail was not significant. Due to the greater role of head neuromasts as compared to tail neuromasts in prey localization and spatial resolution (Mohr & Gorner, 1996), which are important for the survival of predatory post-metamorphic frogs, it is possible the anterior lateral line may have evolved greater resistance to environmental stressors than the posterior lateral line. Future studies could investigate whether *X. laevis* also have a greater regenerative ability in anterior neuromasts than in posterior neuromasts, similar to that observed in zebrafish (Hernandez *et al*., 2006), to further protect their ability to detect and capture prey.

Overall, neuromasts of *X. laevis* appear resistant to copper, with post-metamorphic individuals being less sensitive than larvae. For stage 54-55 larvae, neuromast intensity but not number decreased with increasing copper concentration, indicating that neuromast intensity is more sensitive than neuromast counts. Even though neuromast number remained unchanged, this decrease in intensity suggests the organs were still damaged and experienced loss of hair cells. Future research imaging individual neuromasts in *X. laevis* could verify this link between intensity and the loss of hair cells. The observed reduction of neuromast intensity was not associated with differential response to surface wave stimuli. Future research could compare the sensitivity of *X. laevis* neuromasts to that of other amphibian species. Although environmentally relevant copper concentrations do not appear harmful to the lateral line system of *X. laevis*, this may not be true for all amphibians. For example, two North American anuran species which lose their lateral line system upon metamorphosis were more sensitive than *X.*

*laevis* in terms of neuromast counts, as neuromast loss occurred at concentrations of 50 µg/L or less (Vazquez, 2016). Presumably, these species would also be more sensitive than *X. laevis* regarding neuromast intensity, so we can predict that intensity would be decreased at environmentally relevant concentrations. Future studies could also examine how other heavy metals and aminoglycosides affect *X. laevis* neuromasts to investigate whether the lateral line of *X. laevis* is specifically resistant to copper or to toxicants in general. Perhaps *X. laevis*, as an aquatic species which retains the lateral life throughout its life, has evolved a more robust lateral line system that is better adapted to tolerate environmental stressors as compared to species that only possess the lateral line during a short larval stage.

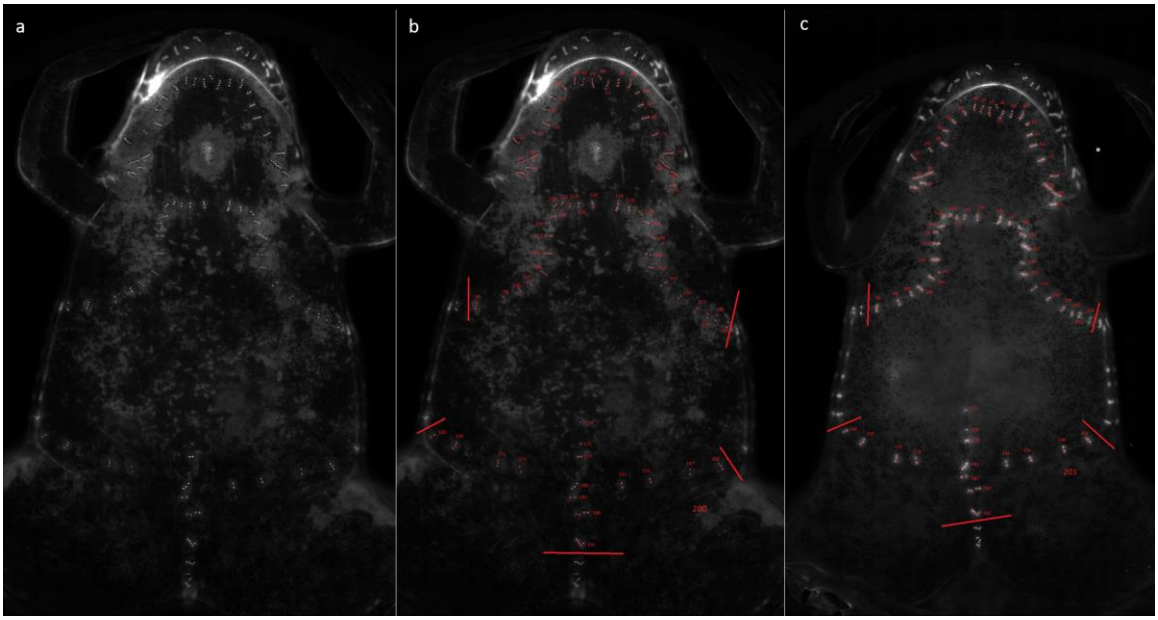


Figure 1. Example of quantifying juvenile frog neuromasts. a) Unedited image of control frog #1 taken before the 144 hour trial period. The bright white dots are neuromasts. b) The same image of control frog #1, now with neuromasts labeled. The red lines indicate cut-offs where neuromasts are out of focus or absent due to the positioning of the frog in one or both of the images; neuromasts were not counted distal to these lines. The numbers in small font are the total number of neuromasts counted up to that stitch. The number at the lower right is the final neuromast count. c) The image of control frog #1 captured after 144 hours.

Table 1. Criteria for scoring neuromast images from NF stage 51-54 *Xenopus laevis* larvae stained with DiAsp after exposure to various copper concentrations.

| Score | Criteria |
|---|---|
| 0 | No clear neuromasts. Some may be present, but are not easily distinguished from non-specific stain. |
| 1 | Few clear neuromasts. Neuromasts are discernible, but few in number, being limited to one or two locations on the body (tail, head, trunk). |
| 2 | Some clear neuromasts. Has more neuromasts than "few", distributed on several locations of the body. Although visible, they are not as bright and easy to see as "clear" neuromasts. |
| 3 | Clear neuromasts. Neuromasts are clearly defined across the body. |

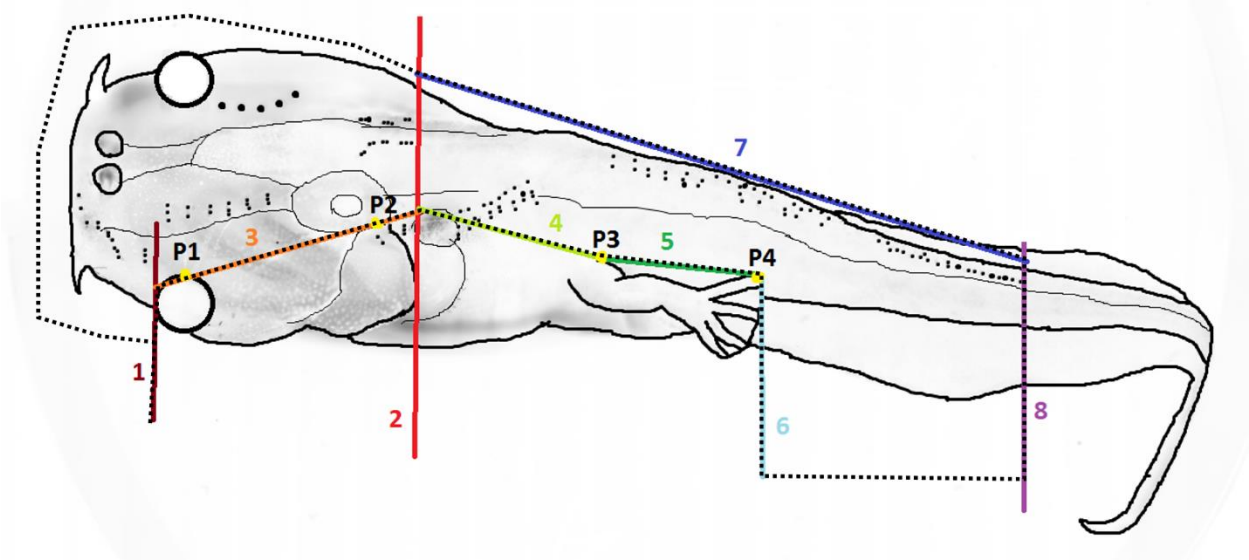


Figure 2. Stepwise instructions on cropping an image for the "partial body" method. Lone numbers represent lines (1 = Line 1) whereas the letter "P" accompanied by a number represents points (P1 = point 1). The dotted line indicates where the image was cropped.

1. Insert a vertical line on the anterior edge of the eye (line 1).
2. Insert a vertical line on the posterior edge of the operculum (line 2).
3. Insert a line connecting the dorsal-most point of the eye (point 1) to line 2, extending this line along or as close as possible to the dorsal most point of the operculum (point 2). This is line 3.
4. From the intersection of lines 2 and 3, draw a line to the dorsal-anterior most point on the leg (point 3). This is line 4.
5. From point 3, draw a line to point 4, the dorsal-posterior most point of the leg; this is line 5.
6. From point 4, draw a vertical line downwards until and off the body of the tadpole. This is line 6.
7. Starting at line 2, draw a new line extending 20000 µm (or as close as possible) posteriorly, so this line runs along the tail. This new line is line 7. Line 7 should be drawn as close as possible to the main body of the tail without crossing onto it; however, the line can be drawn on the tail fin.
8. Starting at the end of line 7, draw a vertical line downwards (line 8).
9. Begin to crop the image. Start with cropping out the bottom of the head by tracing lines 1 and 3.
10. Remove the feet by tracing lines 4, 5 and 6.
11. From the end of line 6, continue to the bottom of line 8
12. Crop along lines 8 and 7. Proceed to go around the outside of the tadpole's body back to the starting point (line 1).

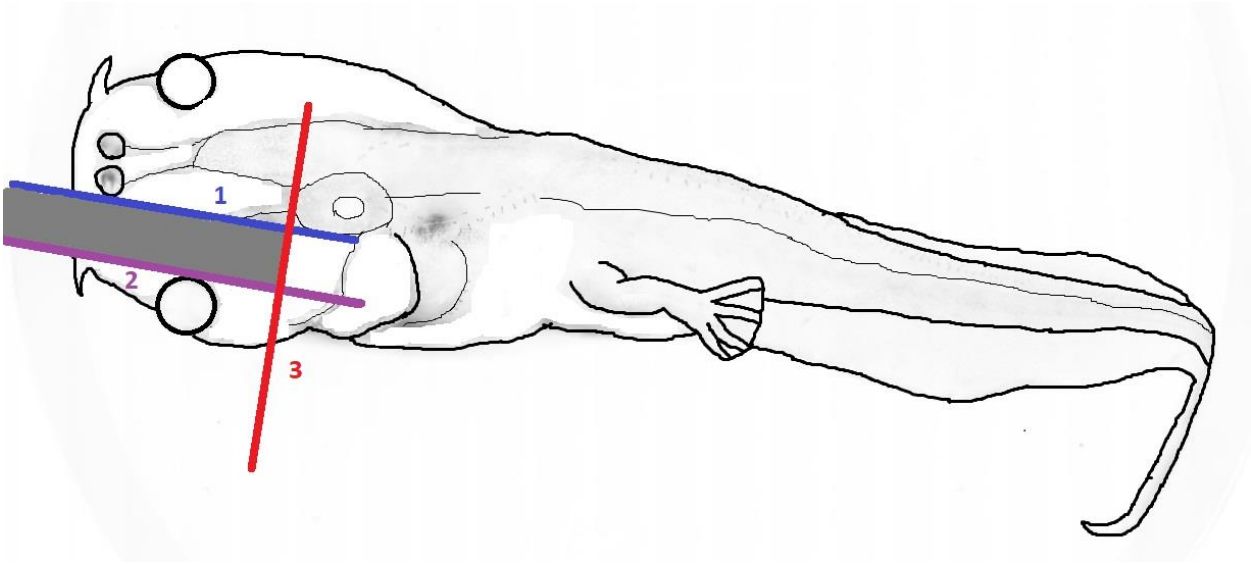


Figure 3. Instructions on creating the "head" plug. The numbers represent lines (1 = line 1). The gray area indicates the area included in the plug; all remaining areas are excluded. To crop the image, first draw a line below the left nostril to the ventral edge of the stirnorgan (line 1). Draw a line parallel to this, touching the dorsal edge of the left eye (line 2). Then create line 3, which extends down from the anterior edge of the stirnorgan. Crop along these lines to evaluate the region inside.

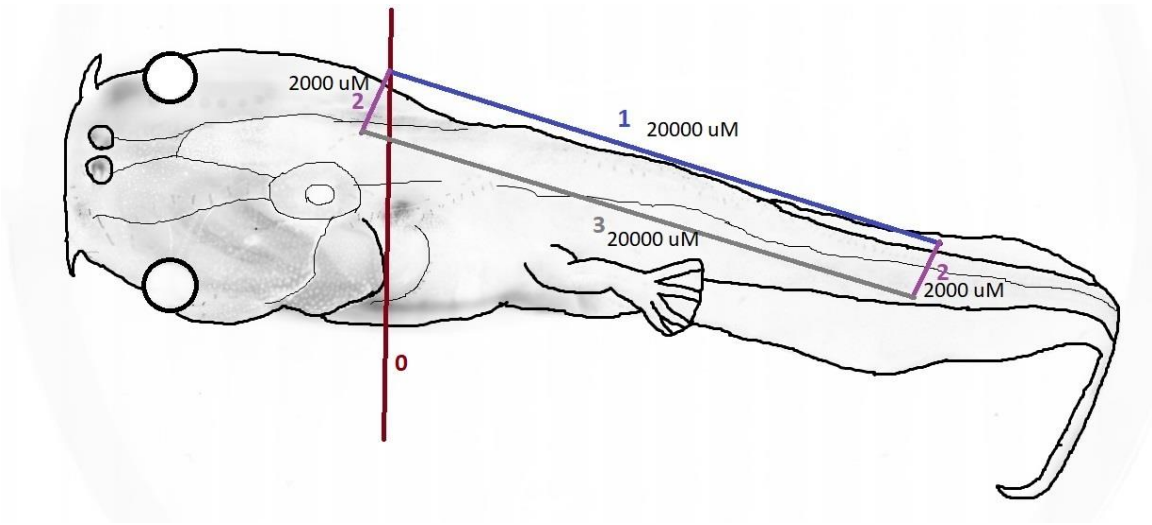


Figure 4. Instructions on creating the "tail" plug. The numbers represent lines (1 = line 1). The lines in this image are not drawn to scale. Start by creating line 0, a vertical line touching the posterior edge of the operculum and extending off the body. Starting at line 0, draw a new line extending 20000 µm (or as close as possible) posteriorly, so this line runs along the tail. This new line is line 1. Line 1 should be drawn as close as possible to the main body of the tail without crossing onto it; however, the line can extend onto the tail fin. At both ends of line one, draw a line extending downwards as close to 2000 µm as possible (lines 2). To ensure the lines are the same length, the second one should be created by copying and pasting. Copy and paste line 1 to create line 3. Connect line 3 to lines 2 to create a rectangle. For some tadpoles located exceptionally high in the well with sloping tails, drawing line 1 above the tail would exclude much of the tail. For these tadpoles, line 1 was drawn as the ventral-most edge of the rectangle rather than the dorsal-most like usual. Lines 2 and 3 were then placed dorsal to line 1. Once the outline is created, crop along the lines to evaluate the area within the rectangle.

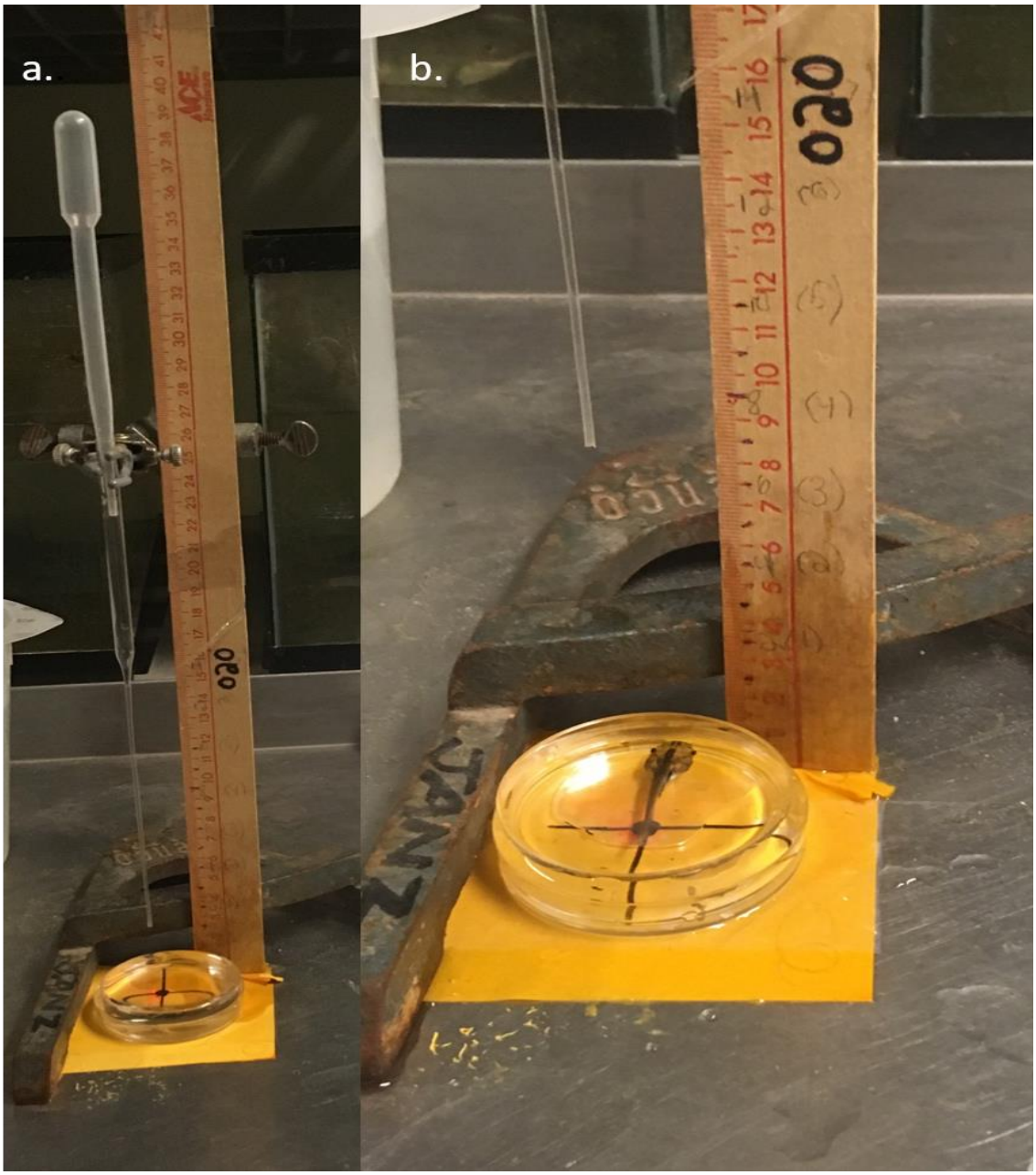


Figure 5. The testing apparatus used for the behavioral assay. a.) A plastic 2 mL pipette attached to a 9 inch glass pasteur pipette was used to produce the air puff stimuli. A clamp stand with an affixed meter stick was used to move the pipettes to various heights. b.) *X. laevis* tadpoles were placed into a 60 mm petri dish. The dish was divided into four quadrants. A circle was drawn in the middle of the dish to check that the pipette was centered. Tadpoles were typically oriented with their heads toward the outside of the dish and tails toward the center.

Table 2. Copper concentrations (µg/L) from exposure solutions of juvenile *X. laevis* collected at various time points after initial exposure. "Pre-water change" refers to water samples collected before the water change 72 hours and 144 hours. "Post-water change" refers to samples taken immediately after the water change at 72 hours.

| | Pre-Water Change | | Post-Water Change | |
|---|---|---|---|---|
| Nominal | Mean | N | Mean | N |
| 0 | < 25 | 2 | < 25 | 2 |
| 1000 | 1010 | 2 | 1120 | 2 |
| 2000 | 1619 | 3 | 2297 | 2 |
| 3000 | 2292 | 4 | 3412 | 2 |

Table 3. Mean (± SE) number of neuromasts for juvenile *X. laevis* before and after 144 hours of copper exposure.

| Cu (µg/L) | N | Before | After |
|---|---|---|---|
| 0 | 5 | 193 ± 13 | 193 ± 14 |
| 1000 | 5 | 203 ± 22 | 203 ± 21 |
| 2000 | 5 | 185 ± 23 | 186 ± 23 |
| 3000 | 5 | 207 ± 11 | 207 ± 11 |

Table 4. Copper concentrations (µg/L) from exposure solutions of NF stage 51-54 *X. laevis* larvae sampled at 0, 24 and 96 hours.

| | 0 hours | | | 24 hours | | | 96 hours | |
|---|---|---|---|---|---|---|---|---|
| Nominal | N | Mean | SD | N | Mean | SD | N | Mean | SD |
| 0 | 4 | < 25 | 2.3 | 3 | < 25 | 4.9 | 6 | < 25 | 2.7 |
| 200 | 1 | 190.8 | -- | 1 | 167.0 | -- | 4 | 172.2 | 13.2 |
| 400 | 1 | 370.3 | -- | 1 | 333.3 | -- | 4 | 371.0 | 26.7 |
| 600 | 1 | 575.0 | -- | 2 | 566.4 | 23.6 | 3 | 441.0 | 156.1 |
| 800 | 3 | 823 | 25.1 | 2 | 768.3 | 88.1 | 2 | 501.8 | 245.8 |

Table 5. Nieuwkoop-Faber developmental stage of surviving *X. laevis* larvae from the NF stage 51-54 copper exposure trial.

| Cu (µg/L) | Median | Average | N | Min | Max | SE |
|---|---|---|---|---|---|---|
| 0 | 53 | 53 | 14 | 51 | 54 | 0.3 |
| 200 | 53 | 52 | 13 | 51 | 54 | 0.2 |
| 400 | 54 | 53 | 12 | 52 | 54 | 0.2 |
| 600 | 52 | 52 | 10 | 50 | 54 | 0.4 |
| 800 | 53 | 53 | 4 | 52 | 53 | 0.3 |

Table 6. Survival of NF stage 51-54 *X. laevis* larvae after 96 hours of copper exposure. Each group initially had 16 animals.

| | Copper (µg/L) | | | | |
|---|---|---|---|---|---|
| | 0 | 200 | 400 | 600 | 800 |
| Number Surviving | 16 | 16 | 15 | 12 | 3 |
| Percent Survival | 100 | 100 | 93.75 | 75 | 18.75 |

Table 7. Qualitative neuromast scores for *X. laevis* larvae (NF stage 51-54) exposed to various copper concentrations. 0 = no clear neuromasts; 1 = few clear neuromasts; 2 = some clear neuromasts; 3 = clear neuromasts. A more detailed explanation of the ranking system is provided in Table 1.

| Cu (µg/L) | Median | Average | N | Min | Max |
|---|---|---|---|---|---|
| 0 | 1.5 | 1.5 | 16 | 0 | 3 |
| 200 | 3 | 2.8 | 15 | 2 | 3 |
| 400 | 2 | 1.8 | 15 | 0 | 3 |
| 600 | 0 | 0.4 | 12 | 0 | 2 |
| 800 | 0 | 0.3 | 3 | 0 | 1 |

Table 8. Copper concentrations (µg/L) from exposure solutions of NF stage 54-55 *X. laevis* larvae collected at the beginning (0 hours) and end (96 hours) of the trial.

| | 0 hours | | | 96 hours | | |
|---|---|---|---|---|---|---|
| Nominal Cu | Mean | N | SD | Mean | N | SD |
| 400 | 410.5 | 4 | 17.7 | 375.8 | 4 | 90.0 |
| 300 | 296.3 | 4 | 6.3 | 256.3 | 4 | 16.7 |
| 200 | 127.8 | 3 | 114.4 | 167.8 | 4 | 14.5 |
| 100 | 93.4 | 4 | 1.3 | 70.3 | 4 | 6.2 |
| 0 | < 25 | 4 | 1.2 | < 25 | 4 | 0.2 |
| Behavior 400 µg/L | 351.3 | 3 | 37.9 | 295.2 | 3 | 162.4 |
| Behavior 0 µg/L | < 25 | 2 | 2.0 | < 25 | 2 | 0.8 |

Table 9. Nieuwkoop-Faber stage of larvae in the stage 54-55 trial after 96 hours of copper exposure. "Neuromast" refers to tadpoles in for which neuromasts were quantified; "behavior" refers to tadpoles used in the behavioral assay. The stage of one of the 400 µg/L larvae in the behavioral assay was not recorded.

| | Treatment | N | Mean | SD | Min | Max |
|---|---|---|---|---|---|---|
| Neuromast | 0 µg/L | 14 | 55 | 0.5 | 55 | 56 |
| | 100 µg/L | 14 | 55 | 0.5 | 55 | 56 |
| | 200 µg/L | 14 | 56 | 0.7 | 55 | 57 |
| | 300 µg/L | 14 | 55 | 0.5 | 55 | 56 |
| | 400 µg/L | 14 | 55 | 0.8 | 55 | 57 |
| Behavior | 0 µg/L | 10 | 58 | 0.7 | 57 | 59 |
| | 400 µg/L | 9 | 57 | 0.6 | 56 | 58 |

Table 10. Mean (± SE) counts of neuromasts for NF stage 54-55 *X. laevis* larvae at different body regions quantified following 96 hours of copper exposure.

| | Concentration (µg/L) | | | | |
|---|---|---|---|---|---|
| | 0 | 100 | 200 | 300 | 400 |
| Whole Body | 175 ± 10 (n = 13) | 212 ± 11 (n = 12) | 203 ± 19 (n = 13) | 198 ± 11 (n = 14) | 212 ± 18 (n = 13) |
| Partial Body | 142 ± 9 (n = 12) | 136 ± 8 (n = 13) | 126 ± 11 (n = 14) | 132 ± 8 (n = 14) | 123 ± 11 (n = 13) |
| Head | 33 ± 1 (n = 13) | 32 ± 2 (n = 14) | 29 ± 2 (n = 14) | 31 ± 2 (n = 14) | 37 ± 4 (n = 13) |
| Tail | 58 ± 4 (n = 13) | 56 ± 5 (n = 14) | 46 ± 6 (n = 12) | 51 ± 9 (n = 14) | 45 ± 8 (n = 11) |

Table 11. Mean (± SE) neuromasts intensity for NF stage 54-55 *X. laevis* larvae at different body regions quantified following 96 hours of copper exposure.

| | Concentration (µg/L) | | | | |
|---|---|---|---|---|---|
| | 0 | 100 | 200 | 300 | 400 |
| Whole Body | 17346 ± 1594 (n = 13) | 14294 ± 1119 (n = 12) | 15377 ± 1467 (n = 13) | 13742 ± 1244 (n = 14) | 11727 ± 1013 (n = 13) |
| Partial Body | 18009 ± 1727 (n = 12) | 14707 ± 971 (n = 13) | 14987 ± 1487 (n = 14) | 13979 ± 1288 (n = 14) | 11426 ± 1266 (n = 13) |
| Head | 24772 ± 2332 (n = 13 ) | 23256 ± 2000 (n = 14) | 21836 ± 2287 (n = 14) | 19705 ± 2076 (n = 14) | 17400 ± 2119 (n = 13) |
| Tail | 14000 ± 1889 (n =13) | 11843 ± 745 (n = 14) | 11762 ± 1356 (n = 12) | 8701 ± 1356 (n = 14) | 8161 ± 1138 (n = 11) |

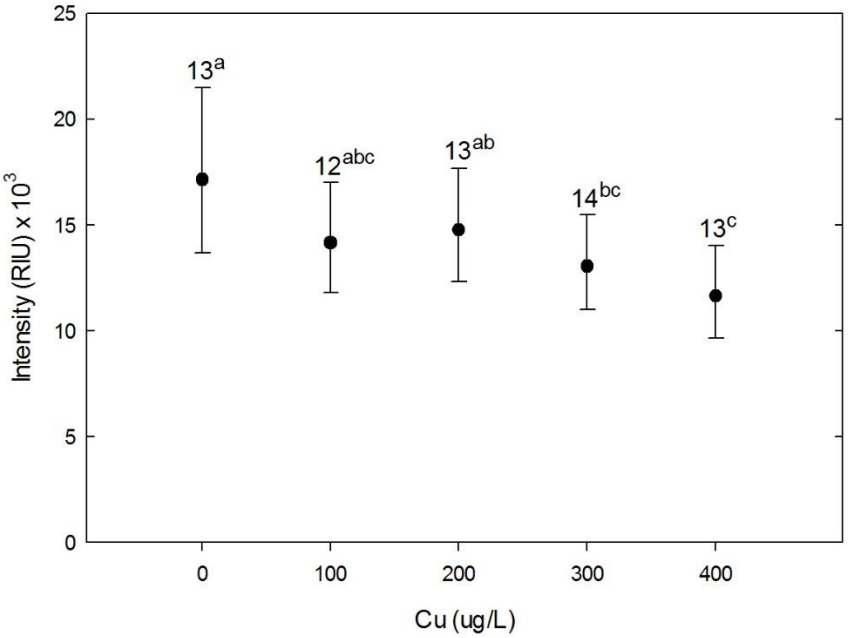


Figure 6. Mean neuromast intensity values of NF stage 54-55 *X. laevis* larvae by copper concentration for the whole body enumeration method. Error bars represent the 95% confidence interval. The number above each data point gives the sample size. Data points with the same letter are not significantly different ($\alpha = 0.05$). RIU is a measure of relative intensity.

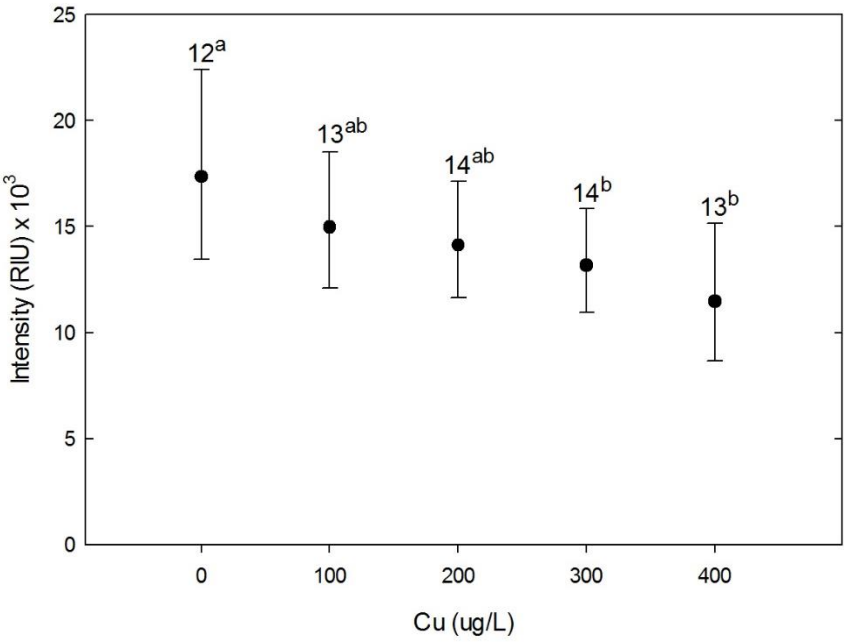


Figure 7. Mean neuromast intensity values of NF stage 54-55 *X. laevis* larvae by copper concentration for the partial body enumeration method. Error bars represent the 95% confidence interval. The number above each data point gives the sample size. Data points with the same letter are not significantly different ($\alpha = 0.05$). RIU is a measure of relative intensity.

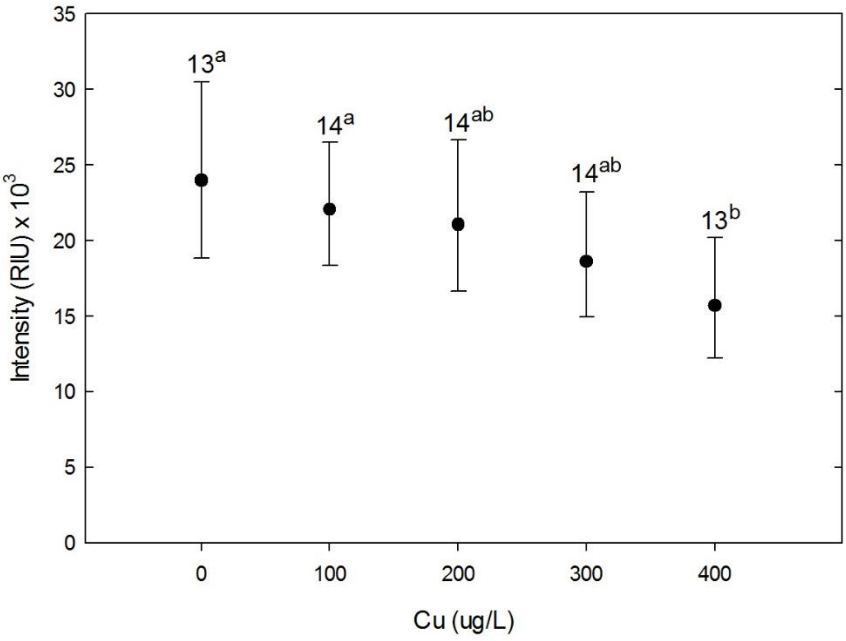


Figure 8. Mean neuromast intensity values of NF stage 54-55 *X. laevis* larvae by copper concentration for the head enumeration method. Error bars represent the 95% confidence interval. The number above each data point gives the sample size. Data points with the same letter are not significantly different ($\alpha = 0.05$). RIU is a measure of relative intensity.

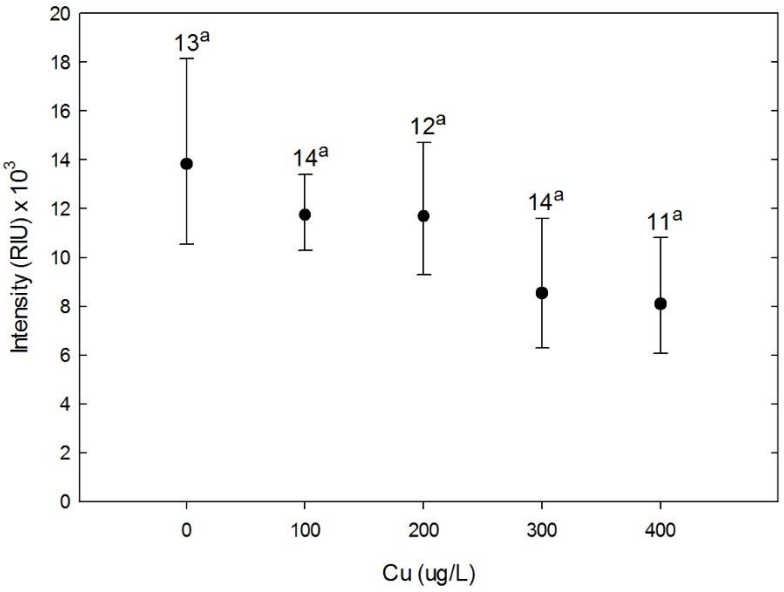


Figure 9. Mean neuromast intensity values of NF stage 54-55 *X. laevis* larvae by copper concentration for the tail enumeration method. Error bars represent the 95% confidence interval. The number above each data point gives the sample size. Data points with the same letter are not significantly different according to Tukey-Kramer groupings ($\alpha$ = 0.05). RIU is a measure of relative intensity.

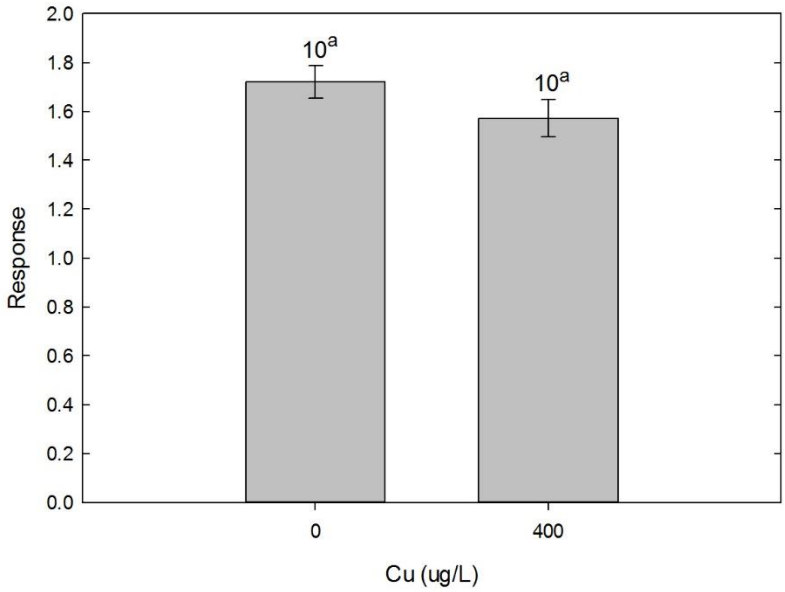


Figure 10. Mean (± SE) individual response score of NF stage 56-59 *X. laevis* larvae to an air puff stimulus averaged for all the tadpoles in each copper treatment group. The mean response score for each tadpole is its average response for all heights. The number above each bar gives the sample size. Bars with the same letter are not significantly different ($\alpha = 0.05$).

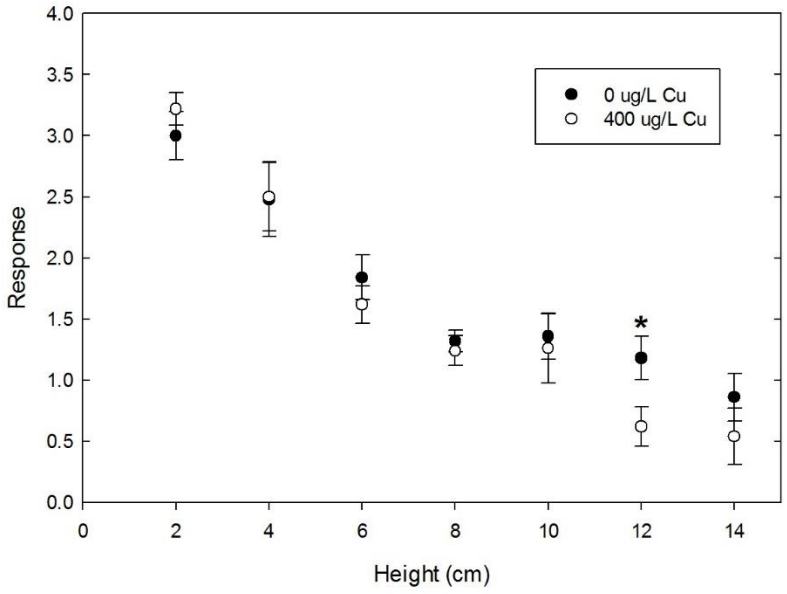


Figure 11. Mean (± SE) response score of NF stage 56-59 larvae to an air puff stimulus at seven different heights (n = 10). An asterisk denotes a significant difference in Tukey-Kramer groupings between the control and 400 µg/L Cu treatments at a particular height ($\alpha = 0.05$).

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