Impact of dissolved copper on the olfactory system of seawater-phase juvenile salmon

Study Summary

Performed for: San Francisco Estuary Institute
                        Regional Monitoring Program

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Background

Copper is a ubiquitous contaminant of aquatic systems in urbanized and agricultural areas throughout the Western United States. In the San Francisco Bay estuary, elevated ambient copper concentrations result from a multitude of anthropogenic sources including, in decreasing order, erosion of buried sediments, inflow from the Sacramento and San Joaquin Rivers, urban and non-urban runoff, anti-fouling marine coatings, urban wastewater, atmospheric deposition, and industrial discharge (CRWQCB 2011). Although the source of copper entering the Bay from the Sacramento and San Joaquin Rivers has not been assessed, one possible source is the application of copper-containing pesticides (data available online from the California Department of Pesticide Regulation Pesticide Use Reporting Program www.cdpr.ca.gov/docs/pur/purmain.htm). Similar sources and processes in marine estuaries throughout the Western United States, e.g. the Puget Sound, make potential impacts of copper exposure on saltwater fish a widespread concern.

In fish, exposures to sublethal dissolved copper concentrations are known to impair peripheral sensory systems (e.g., gustation, mechanosensation, and olfaction). For example, freshwater studies have shown that copper is toxic to fish olfactory receptor neurons (e.g., Baldwin et al. 2003). Furthermore, inhibition of olfactory function is highly correlated with reduced ability to respond to an olfactory alarm cue that triggers anti-predation behavior in juvenile coho salmon (*Oncorhynchus kisutch*, Sandahl et al. 2007). In experiments with predatory cutthroat trout (*Oncorhynchus clarki*), survival of juvenile coho salmon was reduced 3- to 5-fold (McIntyre et al. 2012) following copper exposure. Significantly, these effects on the olfactory system can occur following exposure periods of as little as 30 minutes and increases in dissolved copper concentrations of as little as 3 µg copper/L above background (e.g., Baldwin et al. 2003).

Currently, seawater copper criteria (such as the site-specific objectives [SSOs] for San Francisco Bay) have been derived, in large part, from 96-hour tests on the development of larval *Mytilus edulis* and are higher (e.g., ~10 µg copper/L) than concentrations at which effects on olfactory neurophysiology (Baldwin et al. 2003) and behavior (Sandahl et al. 2007; McIntyre et al. 2012) were seen in juvenile coho salmon. However, published studies on copper neurotoxicity to peripheral sensory systems have thus far been conducted exclusively in freshwater. Given the uncertainty about the protectiveness of seawater from copper olfactory neurotoxicity, it is

difficult to extrapolate effects thresholds to the different water chemistry of marine and estuarine waters. Additionally, differences between the biology of freshwater-phase versus seawater-phase juvenile salmon produce another source of uncertainty. These are issues not unique to salmon within San Francisco Bay, but of concern more broadly to salmon throughout the Western United States. The overall aim of the study was to determine an effect threshold for the olfactory toxicity of copper in seawater-phase juvenile salmon in order to compare these with thresholds measured from freshwater-phase salmon and with marine copper levels and criteria in the Western United States, e.g. the copper SSOs in the San Francisco Bay estuary.

Methods

Animals

Fall Chinook salmon (*Oncorhynchus tshawytscha*) were acquired as fry from the Wallace River Hatchery (Seattle, WA, USA) in March 2012 and transported to the NOAA Fisheries Mukilteo Field Facility in Mukilteo, WA for rearing and experiments. Fish were fed standard commercial salmon pellets (Bio-Oregon, Warrenton, OR, USA) and held in a 1.8-m-diameter circular fiberglass tank on a recirculating freshwater system supplied by carbon-filtered city water (100-300 mg/L total hardness as $CaCO_3$, pH 7.1-7.3, temperature 8-12 °C, oxygen 9-12 mg/L) until smolted over the week of July 18, 2012. Following transition to seawater, fish were held in flow-through, sand-filtered seawater (salinity 30‰, pH 7.8, oxygen 9.5 mg/L, temperature 10-12 °C). This water also served as the background solution for the electrophysiological recordings from the olfactory system (electro-olfactograms; EOGs). Fish used during the experiment were a mean (± SD) weight of 23.5 (± 4.5) g and length of 12.7 (± 0.7) cm.

Neurophysiological recordings (EOGs)

Odorant-evoked EOGs were obtained using established procedures (e.g., Baldwin et al. 2003) adapted to record EOGs in seawater-phase salmonids (Labenia et al. 2007). Briefly, fish were anaesthetized and immobilized, and the skin overlying the olfactory chamber was removed. Fish were placed in an acrylic holder on a vibration isolation table. Stimulus-evoked EOGs were recorded from the sensory epithelium with a pair of glass microelectrodes. The olfactory chamber was continuously perfused with one of several solutions chosen using a computer-controlled valve. Odor-evoked EOGs were produced by switching from background to background containing a model odorant (10^{-3} M L-serine, an amino acid) for 10 seconds. EOG responses were quantified as the amplitude of the negative peak following odor presentation, relative to the pre-odor baseline. Pilot experiments were performed to establish criteria for expected control EOG activity and responses. For the experimental recordings, before treatment with a test solution, 2-3 EOG responses were obtained. If the responses met the criteria, the average amplitude was used as the pre-exposure response. The olfactory chamber was then perfused with either background or background containing copper for 30 minutes. Following the 30-minute exposure period, the perfusion was switched back to background and 2-3 more EOG pulses were obtained. The average of these responses was used as a measure of the post-exposure response.

L-serine and copper test solutions were prepared daily by diluting stock solutions that were prepared weekly.

Analytical Chemistry

Water samples were collected from the holding tanks, the output of the filtration system used to provide filtered seawater (background), and from exposure solutions of nominally 50 and 100 µg copper/L (in background). Samples were analyzed by the Pacific Northwest National Laboratory (Marine Sciences Laboratory, 1529 West Sequim Bay Road Sequim, WA 98382). Sample bottles and collection protocols were provided by the laboratory. Samples analyzed for dissolved concentrations were pre-filtered with a 0.45 µm filter. Analysis for copper concentrations included a combination of a UV digestion followed by flow-injection, dynamic reaction chamber ICP-MS with an isotope dilution calibration method (details in attached report).

Results

EOG responses

EOG responses to a pulse of L-serine consistently showed a biphasic response with a brief positive deflection from pre-odor baseline followed by a longer and larger negative deflection before slowly returning back to the baseline. Figure 1 shows the responses from the same fish before and after a 30-minute exposure to 50 µg copper/L. These responses are typical of the responses from other fish (although the amplitude varies slightly between fish). With respect to copper toxicity, the two responses are similar in timing and amplitude (i.e. there is little difference between EOGs from this fish before and after copper exposure). Slight differences in the amplitudes of the 2-3 responses either before or after copper exposure were seen (data not shown). The dashed lines illustrate the measurement of the EOG amplitude (the difference between the peak of the negative deflection and the baseline). Obtaining robust EOGs from fish during this experiment proved to be more challenging than anticipated based on previous experience, even with seawater EOGs (Labenia et al. 2007). It is worth noting that the fish used in Labenia et al. (2007) were substantially larger (e.g. 117.9 g versus 23.5 g for this study). While all the various sources of the problems are unclear, they likely include increased difficulty with successful dissections and anesthesia and less area of olfactory tissue from which to record a response. Approximately 40% of fish initially tested were excluded from trials. Importantly, all fish that began a trial (i.e. for which an exposure was started) were used for data analysis. Any effects that recording issues might have on the results would be uniformly spread over all the treatment groups.

Effects of Copper

The results of the EOG recordings from fish exposed to background only (no added copper), 50 µg copper/L, and 100 µg copper/L are shown in Figure 2. Table 1 shows the average amplitudes before and after treatment as well as the average change seen in amplitude (post amplitude as a percentage of pre amplitude). No significant effect of treatment was seen in the percent change (ANOVA, p = 0.79).

Analytical chemistry

A summary of the water analyses is shown in Table 2. With respect to copper exposure, the background contained < 1 µg copper/L, while the copper concentrations of the exposure solutions tested were within 92 - 101% of the nominal concentrations (50 and 100 µg copper/L). An important feature of the background water used for the solutions is the low total suspended solids (< 0.49 mg/L, below detection limits) and the low total and dissolved organic carbon (TOC and DOC; both < 1.0 mg/L). These are indications that the exposure solutions had limited ability to form copper complexes that would reduce the bioavailability. This can also be seen in the similarity between the total and dissolved copper concentrations.

Conclusions

The results of this study indicate that copper-induced inhibition of the olfactory system of seawater-phase Chinook salmon requires an exposure concentration of greater than 100 µg copper/L. While this study used a short-term exposure (30 minutes) and only a single odorant (L-serine), the results from freshwater studies imply that the toxicity of copper would not change substantially with either longer exposures or other odorants. Copper-induced olfactory inhibition was observed at <10 µg copper/L following exposures for 30 minutes (Baldwin et al. 2003), 3 hours (Sandahl et al. 2007), and 7 days (Sandahl et al. 2004). Similarly, EOGs evoked by either several amino acids, a bile salt (taurocholic acid), or a skin extract were inhibited to the same degree as EOGs evoked by L-serine following copper exposures (Sandahl et al. 2007).

In contrast to exposure duration and stimulus odor, freshwater studies have shown that increasing the copper complexation ability of water can substantially decrease copper toxicity (e.g., DOC and olfactory toxicity; McIntyre et al. 2008). This potential effect on toxicity can be environmentally relevant, since the DOC in estuaries such as San Francisco Bay can change depending on location. The background seawater used for this study contained very little suspended solids (<0.49 mg/L) and DOC (<1 mg/L). With respect to understanding copper olfactory toxicity in seawater, these values are at the bottom of the ranges seen in estuaries such as San Francisco Bay. Based on the results in freshwater, any increase in DOC in seawater is likely to decrease, rather than increase, copper olfactory toxicity. For this reason, additional copper exposures using elevated DOC levels were not performed as part of this study.

Based on the results of this study and others, the threshold for copper olfactory toxicity in seawater-phase Chinook salmon is greater than 100 µg copper/L and is unlikely to decrease as the DOC varies within the seawater of an estuary such as San Francisco Bay. Copper toxicity thresholds derived from other species (e.g., larval *Mytilus edulis*) and used for determining copper SSOs (which are much lower than 100 µg copper/L) are, therefore, likely to protect the olfactory system of seawater-phase Chinook salmon present in the seawater of San Francisco Bay from copper toxicity.

The dramatic difference in copper olfactory toxicity in freshwater- versus seawater-phase salmon may be the result of water salinity (e.g., the high concentration of various cations and anions in seawater serving to mask copper cations from the olfactory tissue) or salmon physiology (e.g., changes upon smoltification in the proteins involved in olfaction that reduce

their sensitivity to dissolved copper). Olfactory toxicity experiments using an intermediate salinity (e.g., 10 ppt) that both freshwater- and seawater-phase salmon can tolerate will help resolve whether either, or both, of these possibilities are involved in the difference in copper olfactory toxicity. There is also an environmental relevance to addressing the unknown olfactory toxicity of copper in intermediate salinities. The salinity within areas of an estuary may vary (e.g., by location, as the tide cycles, or with river flow). While the threshold for salmon olfactory copper toxicity is known to be <10 µg copper/L in freshwater (Baldwin et al. 2003) and >100 µg copper/L in seawater (this study), the thresholds for freshwater- or seawater-phase salmon in an intermediate salinity is an important data gap for understanding copper olfactory toxicity and the risk that copper poses to salmon as they move through estuaries during and after smoltification.
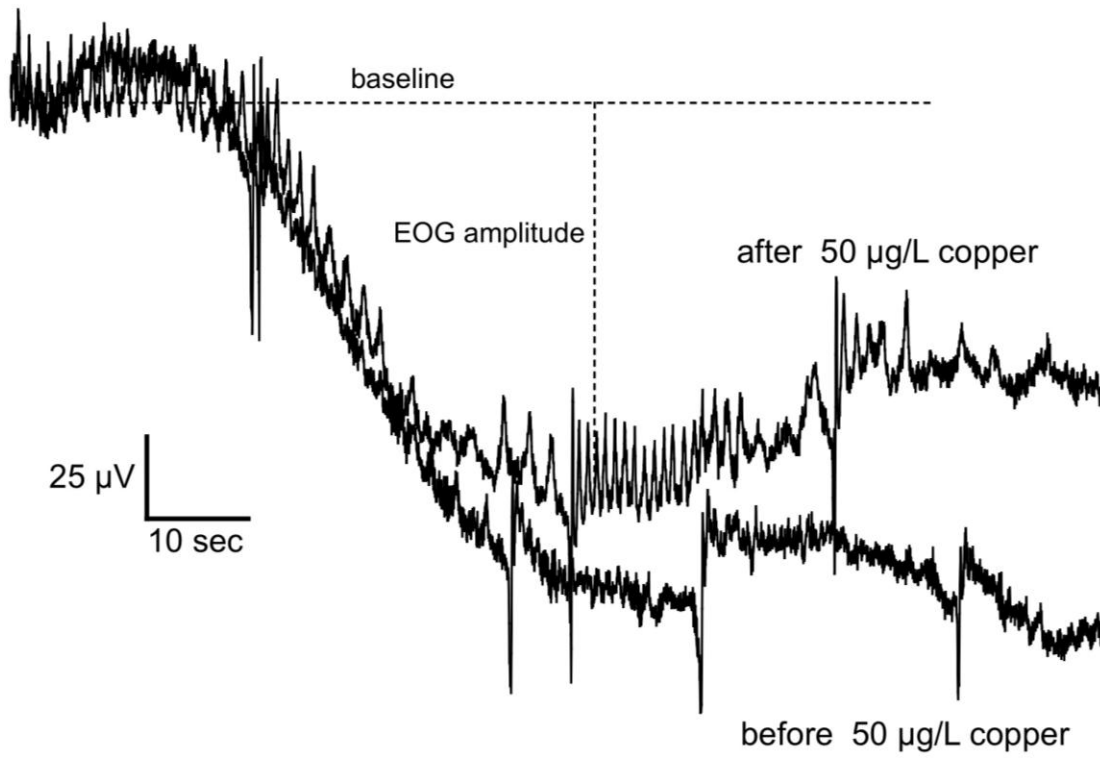
Figure 1: Odor-evoked EOGs elicited by pulses of 10^{-3} M L-serine from the same fish before and after a 30-minute exposure to 50 µg copper/L.
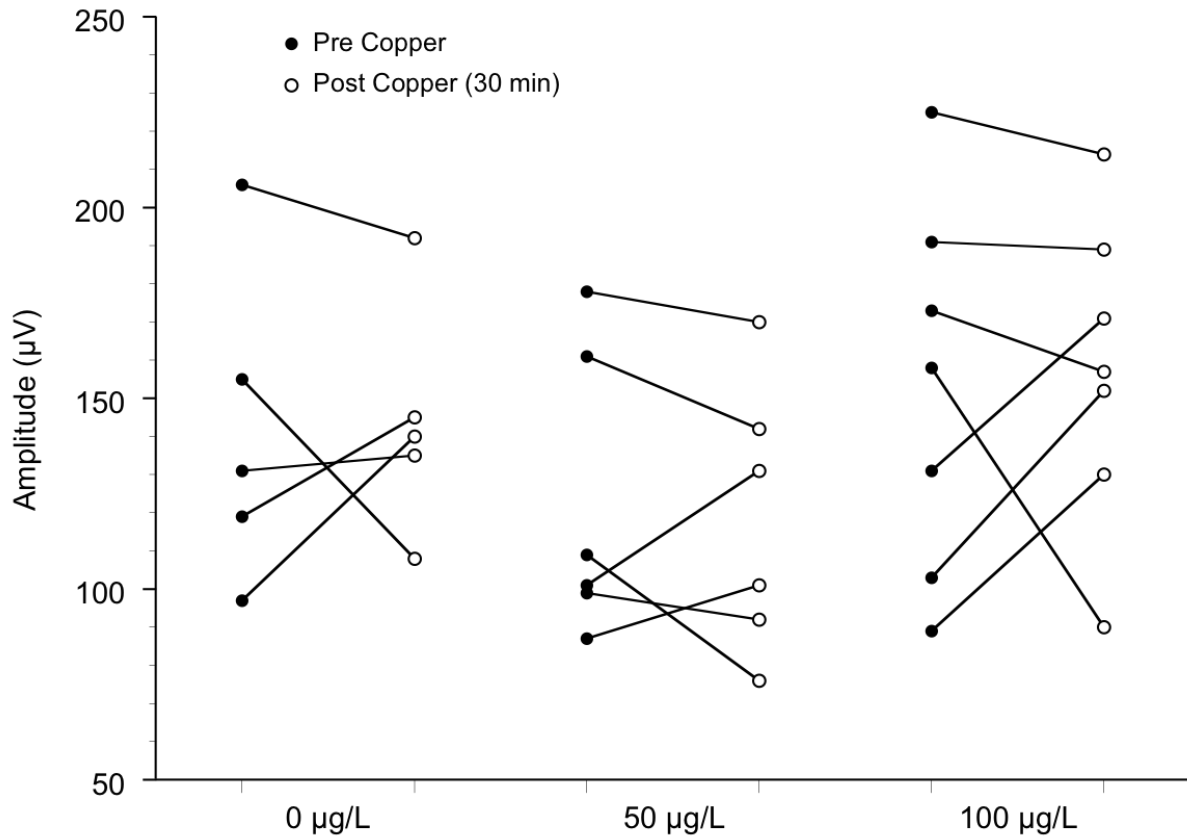
Figure 2: Amplitudes of the EOG responses evoked by 10^{-3} M L-serine before (Pre) and after (Post) 30 min exposures to either control or copper. Each point is the average of 2-3 responses from a single fish. Lines connect the results from individual fish.

Treatment (n)	EOG Pre μV (± sem)	EOG Post μV (± sem)	Post relative to Pre % (± sem)
Control (5)	142 (19)	144 (14)	106 (13)
50 μg/L Copper (6)	123 (15)	119 (14)	99 (9)
100 μg/L Cooper (7)	153 (18)	158 (15)	109 (13)

Table 1: Mean amplitudes of EOG responses evoked by pulses of 10^{-3} M L-serine before (pre) and after (post) 30 min exposure to control or dissolved copper. The number of fish tested is denoted by n.

Sample Units	pH	Salinity PSU	Total Suspended Solids mg/L	Total Organic Carbon mg/L	Dissolved Organic Carbon mg/L	Total Copper μg/L	Dissolved Copper μg/L
Holding Tank	7.74	29.6	< 0.49	0.94	0.93	0.5	0.5
Control Seawater	7.65	29.7	< 0.49	0.84	0.87	0.7	0.7
50 μg/L Copper	NA	NA	NA	NA	0.87	47.2	47.3
100 μg/L Copper	NA	NA	NA	NA	0.94	101	96.3

Table 2: Summary analyses of the waters used during the experiment. NA denotes analyses that were not performed.

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Impact of dissolved copper on the olfactory system of juvenile salmon
Phase II: Effect of estuarine salinity on olfactory toxicity

Study Summary

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Background

Copper is a ubiquitous contaminant of aquatic systems in urbanized and agricultural areas throughout the western United States. In the San Francisco Bay estuary, elevated ambient copper concentrations result from a multitude of anthropogenic sources including, in decreasing order, erosion of buried sediments, inflow from the Sacramento and San Joaquin rivers, urban and non-urban runoff, anti-fouling marine coatings, urban wastewater, atmospheric deposition, and industrial discharge (CRWQCB 2011). Although the source of copper entering the Bay from the Sacramento and San Joaquin Rivers has not been assessed, one possible source is the application of copper-containing pesticides. Copper pesticides were applied to 1.9 million acres of agricultural lands in 2012, with a use of more than 5.4 million pounds of copper pesticide active ingredients (California Department of Pesticide Regulation 2012 report, data available online from the Pesticide Use Reporting Program www.cdpr.ca.gov/docs/pur/purmain.htm). Similar sources and processes in estuaries throughout the western United States, e.g. the Puget Sound and the Lower Columbia River, make potential impacts of copper exposure on fish in estuarine habitats a widespread concern.

In fish, exposures to sublethal dissolved copper concentrations are known to impair peripheral sensory systems (e.g., mechanosensation and olfaction). For example, freshwater studies have shown that copper is toxic to fish mechanosensory (Hernandez et al. 2006; Linbo et al. 2006) and olfactory (Hansen et al. 1999; Baldwin et al. 2003) receptor neurons. Furthermore, inhibition of olfactory function is highly correlated with reduced ability to respond to an olfactory alarm cue that triggers anti-predation behavior in juvenile coho salmon (*Oncorhynchus kisutch*, Sandahl et al. 2007). In experiments with predatory cutthroat trout (*Oncorhynchus clarki*), survival of juvenile coho salmon (as prey) was reduced 3- to 5-fold (McIntyre et al. 2012) following copper exposure. Significantly, these effects on the olfactory system can occur following exposure periods of as little as 30 minutes and increases in dissolved copper concentrations of as little as 3 µg copper/L above background (e.g., Baldwin et al. 2003).

Current site-specific copper objectives (SSOs) for San Francisco Bay were derived, in large part, from 96-hour tests on the development of larval *Mytilus edulis* (USEPA 2003). As per USEPA (United States Environmental Protection Agency) guidance, toxicity tests in site water and standard laboratory waters were compared to account for the local influence of water chemistry on the bioavailability of copper to relevant ligands in this organism. When considering copper neurotoxicity to peripheral sensory systems of sensitive fish taxa, it is important to realize that water chemistry may not have the same influence as on the development of larval *Mytilus edulis*. In tests on peripheral sensory systems in freshwater fishes, hardness and alkalinity provided little to no protection from copper neurotoxicity and dissolved organic carbon (DOC) provided some protection (McIntyre et al. 2008; Linbo et al. 2009). This differs from the traditional understanding of the influence of water quality on copper toxicity derived from data on copper toxicity to fish gills (e.g., as used to develop the Biotic Ligand Model [BLM] used by USEPA for BLM-based copper criteria). These results are likely due to differences in the properties of the fish sensory epithelia relative to the fish gill (e.g., binding affinities of ligands for copper or sensitivity to copper; Meyer and Adams et. al. 2010).

Currently, the copper SSOs are higher (e.g., ~10 µg copper/L) than concentrations at which effects on olfactory neurophysiology (Baldwin et al. 2003) and behavior (Sandahl et al. 2007; McIntyre et al. 2012) were seen in juvenile coho salmon (e.g., 3-5 µg/L). However, published studies on copper neurotoxicity to peripheral sensory systems have thus far been conducted in freshwater. Given the uncertainty about the influence of salinity on copper olfactory neurotoxicity, it is difficult to extrapolate effects thresholds to the different water chemistries of marine and estuarine waters. Additionally, differences between the biology of freshwater-phase versus seawater-phase juvenile salmon produce another source of uncertainty. For example, the process of smoltification may alter the ligands for copper present in the olfactory epithelia in ways that affect copper toxicity. These are issues not unique to salmon within San Francisco Bay, but of concern more broadly to salmon throughout the Western United States as they mature and potentially encounter dissolved copper while migrating from freshwater, through estuaries, to seawater and then the reverse.

A recent study found that 30-minute exposures to dissolved concentrations of copper of 50 or 100 µg/L in seawater were insufficient to produce reductions in the olfactory sensitivity of seawater-phase Chinook salmon (Baldwin, 2012). What is uncertain is whether this substantial decrease in copper olfactory toxicity is due to the increase in salinity relative to freshwater or the change in salmon physiology upon smoltification. Additionally, both freshwater and seawater-phase salmon will encounter intermediate salinities in estuaries as they undergo their normal migrations. The current SSOs do appear to be protective of copper olfactory toxicity in seawater. Whether they are still protective for salmon in estuarine situations is unknown. The olfactory toxicity of copper may depend on the physiology of the salmon or the salinity of the water. For example, the olfactory toxicity of copper in 10 ppt salinity water may differ between freshwater- and seawater-phase salmon if the physiological state affects copper toxicity. Alternatively, salinity may be the important factor and the olfactory toxicities are similar and 10 ppt salinity may or, importantly, may not be sufficient to protect the olfactory system from exposure to 50 µg copper/L. Specifically, does an increase in salinity from freshwater to 10 ppt salinity reduce

the olfactory toxicity of copper in freshwater-phase salmon? Conversely, does a decrease in salinity increase the olfactory toxicity of copper in seawater-phase salmon? The aim of this study is to assess whether a change in salinity to estuarine conditions (10 parts per thousand [ppt] for this study) alters the olfactory toxicity of copper to freshwater-phase and seawater-phase juvenile salmon.

Methods

Animals

This study relied on ongoing coho salmon rearing at the Northwest Fisheries Science Center's (NWFSC) hatchery facility. Freshwater coho were maintained on recirculating dechlorinated and buffered municipal water (10 °C, pH 7.2, alkalinity ~150 mg/L, hardness 85-100 mg/L). Seawater coho were obtained by transitioning freshwater coho over the course of a week to seawater from a recirculating seawater system (11 °C, pH 8-8.2, alkalinity ~205 mg/L). Freshwater coho used in the study were (mean ± sd) 13.7 ± 1.2 cm and 30.8 ± 8.0 g. Seawater coho used in the study were 16.9 ± 1.4 cm and 56.7 ± 17.4 g.

Neurophysiological recordings

Odorant-evoked electro-olfactograms (EOGs) were obtained using established procedures (e.g., Baldwin et al. 2003) adapted to record EOGs in seawater-phase salmonids (Labenia et al. 2007). Briefly, fish were anaesthetized and immobilized, and the skin overlying the olfactory chamber was removed. Fish were placed in an acrylic holder on a vibration isolation table. Odorant-evoked EOGs were recorded from the sensory epithelium with a pair of glass microelectrodes. The olfactory chamber was continuously perfused with one of several solutions chosen using a computer-controlled valve. EOGs were evoked by switching from the current background solution to the background containing an odorant for 10 seconds. For freshwater-phase salmon, the odorant was 10^{-4} M L-serine (an amino acid). For seawater-phase salmon, the odorant was an amino acid mixture of 10^{-2} M L-serine, 10^{-2} M L-leucine, and 10^{-2} M L-arginine. The stronger odorant was used with the seawater-phase salmon since the increased conductivity of seawater reduces the detectability of electrophysiological responses (independent of any biological change in odorant sensitivity). Previous studies on freshwater-phase salmon have shown that copper reduces the olfactory responses evoked by a single amino acid, a mixture of amino acids, and over a range of odorant concentrations (Baldwin et al. 2003; Sandahl et al. 2007).

EOG responses were quantified as the amplitude of the negative peak following odor presentation, relative to the pre-odor baseline. Pilot experiments were performed to establish criteria for expected control EOG activity and responses. Criteria included a stable pre-odorant baseline and small background noise that allowed the EOG peak to be measured. For the experimental recordings, before treatment with a test solution, 2 or 3 EOG responses were obtained using freshwater or seawater as the background (depending on fish). If the responses met the criteria, the average amplitude was used as the pre-exposure response. The olfactory chamber was then perfused with the test solution for 30 minutes. Following the 30-minute exposure period, the perfusion was switched back to the background and 2 or 3 more EOG

pulses were obtained. The average of these responses was used as a measure of the post-exposure response.

Test solutions consisted of either freshwater or a 10-ppt salinity water (for freshwater-phase salmon) or seawater or a 10-ppt salinity water (for seawater-phase salmon). The 30-minute exposures were performed with the test solution only (nominally 0 µg copper/L) or the test solution with 50 µg copper/L (nominal). During the 30-minute exposure, EOGs were elicited every 5 minutes by a 10-sec switch to an odorant-containing test solution. The 10-ppt test solution was made daily by mixing the appropriate amounts of freshwater and seawater. Salinity was checked with a refractometer (~10 ppt) and the pH checked with a pH meter (~7.4). Amino acid and copper test solutions were prepared daily by diluting stock solutions that were prepared weekly. An intermediate salinity of 10 ppt was used since it is osmotically neutral for salmon and should not cause any osmoregulatory stress. Exposure durations of 30 minutes were chosen because they are known to be sufficient to produce olfactory toxicity (e.g., Baldwin et al. 2003). The short test durations were used to avoid triggering smoltification processes in the freshwater-phase salmon.

Analytical Chemistry

Water samples were collected from the freshwater and seawater backgrounds and the 10 ppt test solution (with and without 50 µg copper/L for all three). Samples were analyzed by the Pacific Northwest National Laboratory ([PNNL] Marine Sciences Laboratory, 1529 West Sequim Bay Road Sequim, WA 98382). Sample bottles and collection protocols were provided by the laboratory. Samples analyzed for dissolved concentrations were pre-filtered with a 0.45 µm filter. Analysis for copper concentrations included a combination of a UV digestion followed by flow-injection, dynamic reaction chamber ICP-MS with an isotope dilution calibration method (details in attached report).

Results and Discussion

Analytical chemistry

A summary of the water analyses is shown in Table 1. The nominally 0 µg/L solutions were found to contain 0.3 – 2.5 µg dissolved copper/L (depending on solution). This reflects some background sources of copper (e.g., within the seawater recirculating system). The total copper concentrations of the 50 µg /L exposure solutions were 47 – 52 µg/L, while the dissolved were slightly lower (38 – 47 µg/L). The pH of the test solutions ranged from 6.8 – 7.9, while alkalinities would likely range from 150 – 205 mg/L (see Methods). Importantly, based on pH and alkalinity data from 20 µg copper/L exposures to freshwater salmon (McIntyre et al. 2008), the olfactory toxicity of the copper exposures used here would not be expected to change appreciably over these ranges of water chemistries. For example, a pH change of 1 unit produced no change in the olfactory toxicity of 20 µg copper/L (McIntyre et al. 2008). All of the test solutions contained little total suspended solids (≤ 0.6 mg/L), indicating limited ability for particulates to reduce copper bioavailability. The three solutions differed noticeably in their dissolved organic carbon concentrations, ranging from 0.5 mg/L for the freshwater to 4.0 mg/L for the seawater. While increases in organic carbon would be expected to reduce copper

bioavailability, even at the highest concentration of 4 mg/L freshwater salmon exposed to 20 µg copper/L still show significant olfactory toxicity (McIntyre et al. 2008).

Freshwater-phase salmon

    Examples of EOGs obtained from four different freshwater-phase salmon are shown in Figure 1. The upper-left panel shows EOGs from a fish exposed to no copper without a change in salinity. Here, repeated EOGs obtained every 5 minutes before, during, and after the 30-minute exposure period display a normal response-to-response variation in amplitude. The lower-left panel shows EOGs from a fish exposed to copper without a change in salinity. With this fish, EOGs obtained every 5 minutes before, during, and after the 30-minute exposure display reductions in the EOG amplitudes expected due to copper olfactory toxicity (e.g., Baldwin et al. 2003).

    The right column of Figure 1 shows data from two fish where the salinity was increased to 10 ppt during the 30-minute exposure. As seen in the upper-right panel, the increase in salinity alone produced an instant change in the amplitude and shape of the EOG. This is due, at least in part, to an abiotic effect of the increase in conductivity of the solution on the electrophysiological recording. There may also be an effect on the olfactory transduction process due to the sudden increase in the concentrations of specific ions (e.g., Na^{++}, K^{+}, and Cl^{-}) outside the olfactory epithelium. Importantly, these effects are immediate and reverse upon return to freshwater as can be seen by comparing the 'pre' and 'post' responses. The slight reduction in amplitude seen in the 0 µg/L exposure may be due to the slight increase in copper concentration in the 10 ppt salinity water compared to freshwater (see Table 1). The lower-right panel of Figure 1 shows responses from a freshwater-phase fish exposed to both copper and an increase in salinity. Qualitatively, the responses show the same pattern as fish not exposed to copper.

    For each fish the effect of exposure was quantified as the relative EOG amplitude (the 'post' EOG amplitude divided by the 'pre' EOG amplitude). Figure 2 summarizes the data from all of the freshwater-phase fish as box plots. The results showed a significant two-way ANOVA (copper concentration and salinity, $F=12.077$, $p<0.0001$) with a significant effect of copper ($p<0.0001$), without an effect of salinity ($p=0.203$) and with a significant interaction ($p=0.04$). A significant difference was seen between the 0 µg/L and 50 µg/L copper exposures in freshwater (Tukey post-hoc, $p<0.05$). No significant difference was seen between the exposures in 10 ppt salinity water (Tukey post-hoc, $p>0.05$).

Seawater-phase salmon

    Examples of EOGs obtained from six different seawater-phase salmon are shown in Figure 3. The left column shows EOGs from fish exposed without a change in salinity. Exposure to 50 µg copper/L for 30 minutes had no apparent effect on the EOG amplitude. Specifically, the amplitude and shape of the responses before and after exposure show little difference. All fish showed a prolonged negative period following the initial response that varied in duration but eventually returned to the baseline. Qualitatively, this is consistent with the results from Chinook salmon from a previous study (Baldwin 2012). Conversely, the EOG responses from fish exposed in 10 ppt salinity water showed a dramatic change in shape. The middle column of

Figure 3 shows responses in seawater recorded before and after exposures in 10 ppt salinity water. The EOGs shift from an entirely negative response to a bi-phasic response with an initial negative peak followed by a positive peak. The transition in shape is seen regardless of whether there was copper present in the 10 ppt salinity water. Over time, repeated EOGs in seawater showed signs of the shape beginning to return back to the negative-only peak (data not shown). Collectively, this could reflect an effect on the EOG of an osmoregulatory response in the olfactory epithelium triggered by the decrease in salinity that reverses with the return to seawater. Recordings from a fish were not typically maintained for more than 15 minutes following the 30-minute exposures, so how long this process required was not determined.

The effect of 10 ppt salinity on EOG shape seen in seawater-phase fish is quite distinct from that seen in freshwater-phase fish. In freshwater-phase fish there was an immediate change in shape upon the change in salinity and the change in EOG shape immediately reversed upon return to freshwater (right column of Figure 1). The right column of Figure 3 shows that, in contrast, the change in shape seen in the seawater-phase fish in 10 ppt salinity water occurs gradually over the course of the 30 minutes. Presumably, the decrease in salinity is causing dynamic changes in processes underlying the olfactory recordings. Exactly which processes are unclear, but could include adaptation mechanisms within the olfactory epithelium of seawater-phase fish. The dynamic nature of these changes to the EOG introduces a major source of uncertainty and variability in quantifying the amplitudes of responses after 10 ppt salinity exposures. Because these changes were observed whether fish were exposed to copper or not, qualitatively, there was no apparent effect of the copper exposure on the EOGs. The presence of robust responses indicates that there was no obvious reduction in the olfactory sensitivity of copper-exposed fish.

Figure 4 shows that for the seawater-phase coho exposed in seawater there was no significant difference in the relative EOGs following exposure (t-test, $p > 0.05$). Both unexposed and exposed fish showed no change in amplitude following 30 minutes. This is consistent with recent results from Chinook (Baldwin, 2012). For reasons mentioned above, a similar quantitative comparison was not performed for the seawater-phase fish exposed in 10 ppt salinity.

Conclusions

The aim of this study was to extend the results of previous studies on the olfactory toxicity of copper (e.g. Baldwin et al. 2003 and Baldwin 2012). Those studies found that, based on EOG recordings, the thresholds for copper olfactory toxicity were ~5 µg/L in freshwater-phase salmon and >50 µg/L in seawater-phase salmon when tested without a change in the salinity (and with low DOC values of <3 mg/L). The results of this study are consistent with these findings. Exposure to 50 µg copper/L produced a significant decrease in olfactory responses from freshwater-phase salmon but no significant change in the responses from seawater-phase salmon.

This study extended these results by assessing the olfactory toxicity of copper under an intermediate salinity (10 ppt for this study) that both freshwater- and seawater-phase salmon might encounter as they migrate through estuaries such as the San Francisco Estuary, Lower Columbia River Estuary, or Puget Sound. The results of this study provide evidence that the threshold for copper olfactory toxicity following short-term exposures in 10 ppt salinity water is

≥ 50 µg copper/L for both freshwater- and seawater-phase coho salmon. In essence, the increase in salinity to 10 ppt substantially reduced the olfactory toxicity of copper to freshwater-phase salmon and did not increase the toxicity to seawater-phase salmon. One possible explanation is that the 10 ppt estuarine water contains a sufficient level of ionic compounds such that competition with cations and complexation with anions reduces the bioavailability of copper to the olfactory epithelium. While studies in freshwater have shown that relatively small increases in these compounds (e.g. varying hardness and alkalinity) have little effect on copper olfactory toxicity (e.g. McIntyre et al. 2008), an increase in salinity to 10 ppt represents a substantial increase outside the range typically tested in freshwater studies. Of note, for freshwater-phase salmon this result is quantitative but for seawater-phase salmon this result is qualitative and complicated by a response to the change in salinity alone. Further work with a modified experimental design would be needed to confirm this result. Using exposure durations of 4 hours done prior to EOG recordings (e.g. Sandahl et al. 2007), for example, might allow for the change in EOG shape to stabilize. However, this exposure duration is too long to reliably maintain a fish for EOG recordings.

While this study used a short-term exposure (30 minutes) and only amino acids as odorants, the results from freshwater studies imply that the toxicity of copper would not change substantially with either longer exposures or other odorants. Copper-induced olfactory inhibition was observed at <10 µg copper/L following exposures for 30 minutes (Baldwin et al. 2003), 3 hours (Sandahl et al. 2007), and 7 days (Sandahl et al. 2004). Similarly, EOGs evoked by either several amino acids, a bile salt (taurocholic acid), or a skin extract were inhibited to the same degree as EOGs evoked by L-serine following copper exposures (Sandahl et al. 2007). Of note, all of theses studies used similar water chemistries (e.g. low DOC values of < 3 mg/L). Additionally, while this study used coho salmon, studies in other freshwater-phase salmonids using EOGs (e.g., Sandahl et al. 2006 for chum salmon and Baldwin et al. 2011 for steelhead trout) have found similar copper thresholds (i.e. ~5 µg/L) using similar water chemistries as the above studies and the recent study in seawater-phase Chinook salmon (Baldwin 2012) found copper thresholds to be > 50 µg/L. These results indicate that species difference may not be a major factor for copper olfactory toxicity.

Based on the results of this study, the threshold for copper olfactory toxicity in both freshwater- and seawater-phase salmon (including Chinook) is greater than 50 µg copper/L for fish in estuarine waters of 10 ppt salinity. The copper toxicity thresholds derived from other species (e.g., larval *Mytilus edulis*) and used for determining copper SSOs (which are much lower than 50 µg copper/L) are, therefore, likely to protect the olfactory system of juvenile salmon present in the estuaries of San Francisco Bay from copper toxicity regardless of their smolt status. Therefore, maintaining the copper concentrations within the estuaries below the current SSOs will likely prevent exposures to copper from impairing the olfactory ability of juvenile salmon. An uncertainty still to be addressed is how sensitive the olfactory system of adult salmon is to copper exposures as they return to estuaries and ultimately to freshwater. However, the potential for copper exposure is greater for juveniles, since adults typically spend relatively little time, relative to juveniles, in estuaries as they migrate back to spawning habitats.
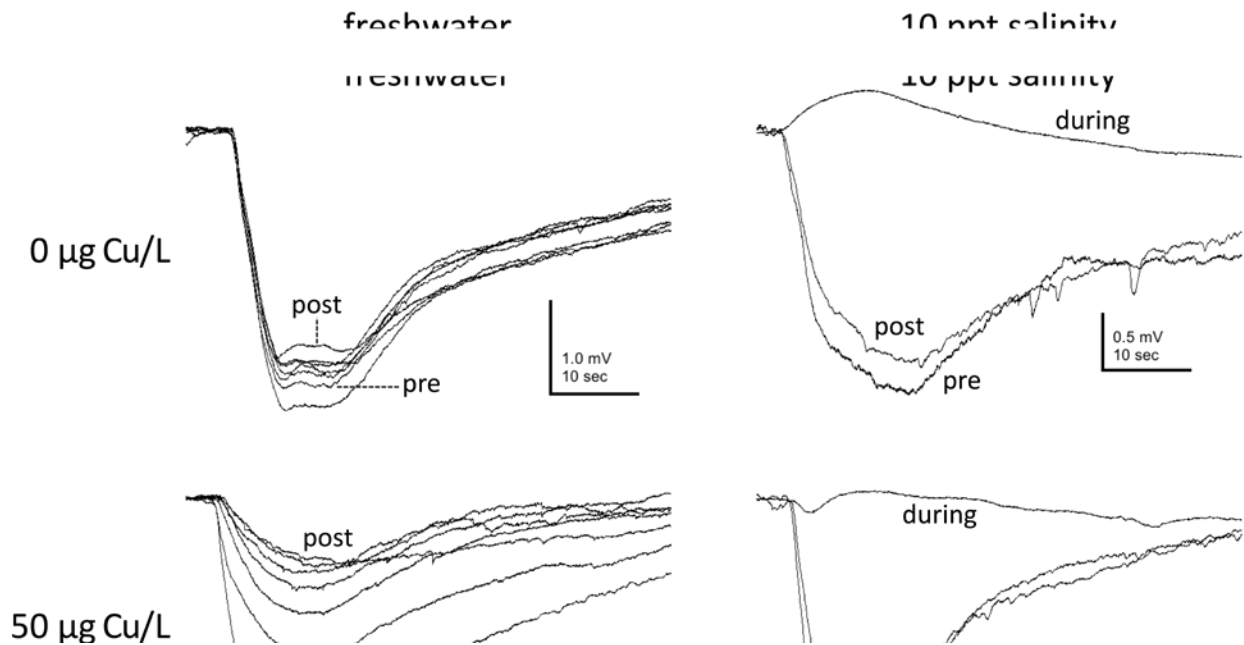
Figure 1: Odor-evoked EOGs from freshwater-phase salmon elicited by pulses of 10^{-4} M L-serine from four different fish before ('pre'), during, and after ('post') 30-minute exposures to either 0 µg or 50 µg copper/L (rows) in either freshwater or 10 ppt salinity water (columns). All of the 'pre' and 'post' response were obtained in freshwater. The remaining responses are from during the 30-minute exposure and were either in freshwater (left column) or 10 ppt salinity water (right column). Only single responses during the 10 ppt salinity exposures are shown. Qualitatively, no change in the shape or amplitude of the responses during the 10 ppt salinity exposures were observed either in the 0 or 50 g copper/L exposures.
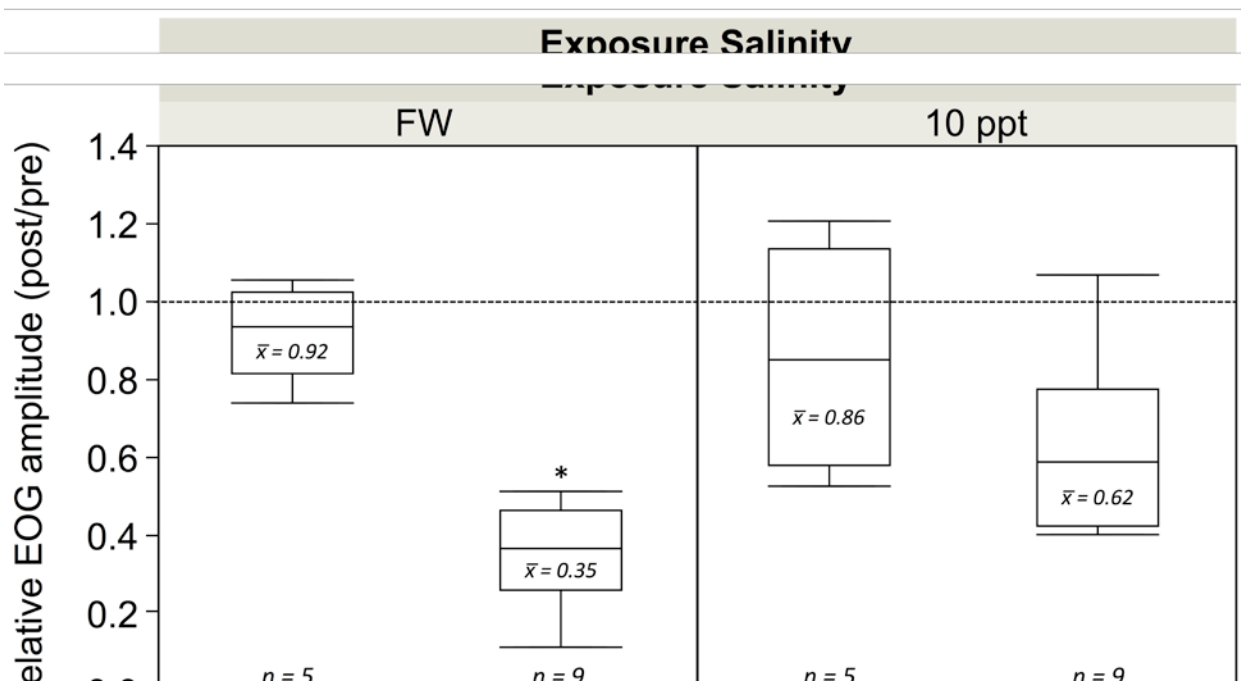
Figure 2: Summary of the effects of exposures on the EOG responses of freshwater-phase salmon. Box plots showing the relative EOG amplitudes evoked by 10^{-4} M L-serine following 30-min exposures (post amplitude/pre amplitude). A relative value of 1 would mean no change in amplitude between the 'pre' exposure responses and the 'post' exposure responses. The mean for each treatment group is shown within the box. The number of fish tested is shown below each box plot. A significant effect of copper exposure was observed comparing the responses in freshwater, while no significant effect was observed between the responses in 10 ppt salinity water (two-way ANOVA, Tukey's post hoc, $p < 0.05$).
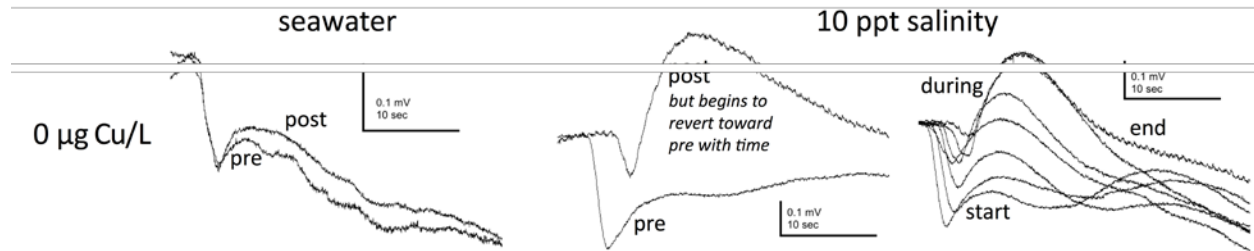
Figure 3: Odor-evoked EOGs from seawater-phase salmon elicited by pulses of an amino acid mixture from six different fish before ('pre'), during, and after ('post') 30-minute exposures to either 0 µg or 50 µg copper/L (rows) in either seawater or 10 ppt salinity water (columns). All of the 'pre' and 'post' responses were obtained in seawater. The remaining responses (rightmost column) are from during the 30-minute exposure and were obtained in 10 ppt salinity water. The initial response during the 30-minute exposure is denoted with 'start' and the final response is denoted with 'end'. A gradual transition in the shape of the responses can be seen in both the 0 and 50 µg copper/L exposures.
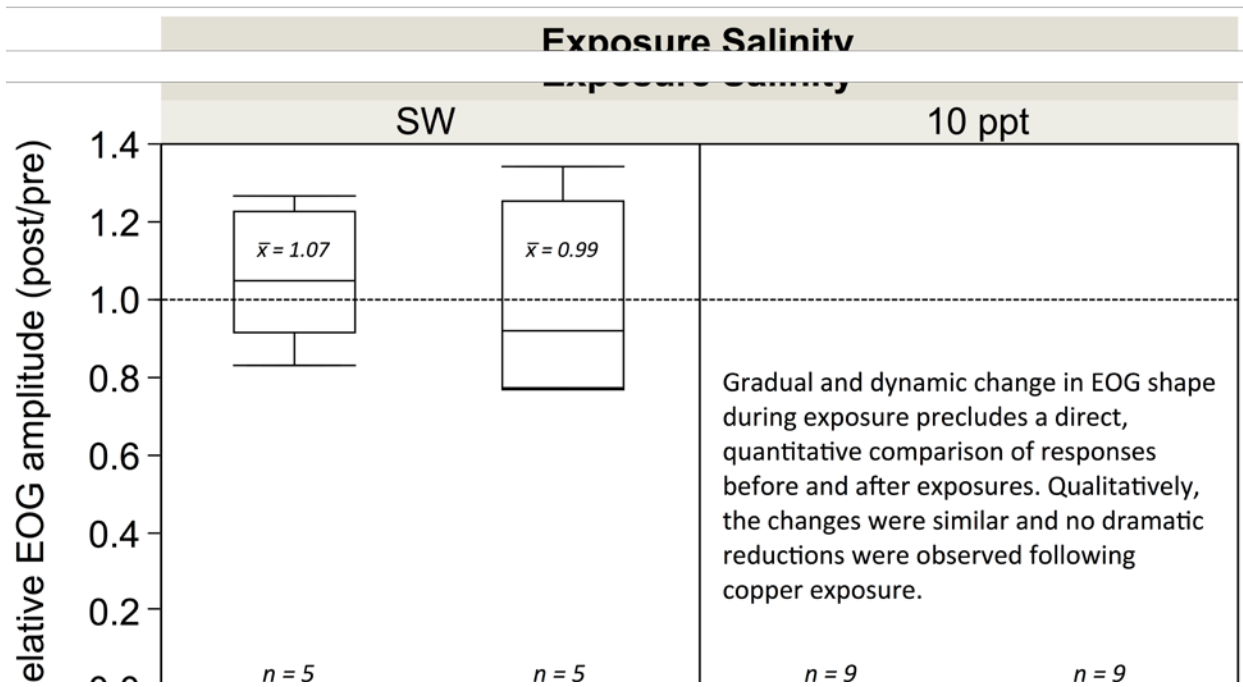
Figure 4: Summary of the effects of exposures on the EOG responses of seawater-phase salmon. Box plots showing the relative EOG amplitudes evoked by an amino acid mixture following 30-min exposures (post amplitude/pre amplitude). A relative value of 1 would mean no change in amplitude between the 'pre' exposure responses and the 'post' exposure responses. The mean for each treatment is shown in the box. The number of fish tested is shown above the X-axis. No significant effect of copper exposure was observed comparing the responses in seawater (t-test, p<0.05). The dynamic change in shape in the EOGs caused by exposure to 10 ppt salinity alone precludes a quantitative assessment of the relative amplitudes.

Sample	pH	Salinity	Total Suspended Solids	Total Organic Carbon	Dissolved Organic Carbon	Total Copper	Dissolved Copper
Units		PSU	mg/L	mg/L	mg/L	µg/L	µg/L
Freshwater w/o Cu	6.8 (0.5)	0.07 (0.05)	0.6 (0.2)	0.6 (0.1)	0.50 (0.04)	0.5 (0.3)	0.3 (0.3)
Freshwater w/ Cu	NA	NA	NA	0.6 (0.1)	0.50 (0.02)	48 (6)	38 (8)
10 ppt water w/o Cu	7.4*	10*	NA	1.6 (0.4)	1.6 (0.4)	2.9 (0.6)	2.5 (0.3)
10 ppt water w/ Cu	NA	NA	NA	1.7 (0.3)	1.6 (0.3)	52 (1)	42 (2)
Seawater w/o Cu	7.9 (0.1)	31.5 (0.4)	0.3 (0.2)	4.0 (0.7)	4.0 (0.7)	1.8 (0.3)	1.7 (0.2)
Seawater w/ Cu	NA	NA	NA	3.35 (0.05)	3.36 (0.06)	47 (3)	47 (3)

Table 1: Summary analyses (mean with sd) of solutions used during the experiment. NA denotes analyses that were not performed. * denotes measurements made at the NWFSC to check the 10 ppt solution.

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