The effects of winter cold on acute copper bioaccumulation and toxicity in brook char (*Salvelinus fontinalis*)

by

Julia Macpherson

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Supervisor: Anne Crémazy, PhD, Department of Biological Sciences

Examining Board: Ben Speers-Roesch, PhD, Department of Biological Sciences
Kurt Samways, PhD, Department of Biological Sciences
James McGeer, PhD, Department of Biology, Wilfred Laurier University

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ABSTRACT

Freshwater fish that are acutely exposed to copper (Cu) can experience disturbances of ion regulation and ammonia excretion. Temperature has been shown to affect Cu bioaccumulation and toxicity in fish, but the focus has largely been on warm temperature effects. I investigated how winter cold affects acute Cu bioaccumulation and toxicity in brook char (*Salvelinus fontinalis*). Following acclimation to cold temperature (4 weeks at 3°C) *vs*. warm temperature (14°C) and following cold challenge (24 h at 3°C) *vs*. cold acclimation (13 weeks at 3°C), I measured gill-Cu bioaccumulation, net fluxes of ammonia (NH_3), chloride (Cl^-) and net and unidirectional fluxes of sodium (Na^+) over a 30-h Cu exposure. Contrary to my prediction, I found that Cu bioaccumulation over 30-h was not significantly affected by acclimation temperature and that effects of temperature on Cu toxicity were relatively limited with slightly greater toxic effects observed in warm-acclimated fish.

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1.0 INTRODUCTION

1.1 Copper in the environment

Copper (Cu) is a trace metal naturally occurring in aquatic ecosystems through weathering and erosion of rocks and soils (Environment and Climate Change Canada, 2021). In natural fresh waters, Cu is present at relatively low background concentrations of 0.2-30 µg/L (USEPA, 2007). However, anthropogenic activities, such as mining, combustion of fossil fuels, and agricultural practices, can elevate Cu in surrounding aquatic environments (Grosell, 2012). In fact, Cu concentrations as high as 200 mg/L have been measured in fresh waters near mining sites (Grosell, 2012; USEPA, 2007). Although Cu is essential to various biological processes (Grosell & Wood, 2002), it may become toxic to aquatic life at concentrations greater than background levels (Lim et al., 2015). Notably, Cu has been shown to induce adverse physiological and behavioural responses in freshwater fish exposed to low waterborne Cu concentrations in the µg/L range (Grosell, 2012). Thus, a Canadian Federal Environmental Quality Guidelines (FEQGs) has been developed to provide acceptable Cu concentrations for the protection of freshwater life. This FEQG depends on local water condition, to account for the effect of toxicity-modifying parameters such as pH and dissolved organic carbon (DOC). For example, the FEQG is 0.41 µg/L for a water at 20°C with pH = 7.5, DOC = 0.5 mg/L and hardness = 50 mg/L as $CaCO_3$ (Environment and Climate Change Canada, 2021).

1.2 Effects of copper on freshwater fish

For trace metals such as Cu, the main site of entry into freshwater fish is the gills (Cairns et al., 1975), because of their high surface area and their many transport proteins

allowing uptake of essential Cu ions (Cu^{2+}) (Cairns et al., 1975; Castaldo et al., 2020; Grosell & Wood, 2002). More precisely, uptake of waterborne Cu can occur through specialized Cu transporters (e.g. CTR1) and through sodium (Na^+) channels located at the gill epithelium (Grosell & Wood, 2002; Kamunde et al., 2002). As such, the gills of freshwater fish are known to be the main site of short-term/acute Cu bioaccumulation and toxicity, with the main effects being disruptions of ion regulation and ammonia excretion (Chowdhury et al., 2016; Crémazy et al., 2016; Grosell & Wood, 2002; Laurén & McDonald, 1985, 1987; Lim et al., 2015; Zimmer et al., 2012).

In freshwater fish, the main mechanism of acute Cu toxicity is the disturbance of Na^+ balance, leading to net Na^+ plasma loss and eventual fish death (Wood et al., 2012). In these hypertonic freshwater organisms, passive diffusional loss of plasma ions (e.g. Na^+, Cl^-, K^+, Mg^{2+}) through gill paracellular tight junctions (Griffith, 2017) is balanced by active ion uptake at the gill epithelium (Evans et al., 2005). For instance, the transport of waterborne Na^+ occurs through various pathways involving Na^+/H^+ exchangers (NHE), Na^+ channels, and Na^+-Cl^- co-transporters at the apical membrane and a Na^+/K^+-ATPase at the basolateral membrane of the gill epithelium (Figure 1) (Evans et al., 2005; Zimmer & Perry, 2022). This regulation of ion balance requires energy, and is essential for maintaining constant and high levels of ions in the plasma (Marshall, 2002). Exposure to Cu challenges Na^+ homeostasis by both stimulating passive Na^+ loss and inhibiting Na^+ uptake at the gills (Figure 1) (Laurén & McDonald, 1987). More precisely, during acute exposure to elevated dissolved Cu concentrations, Cu has been reported to compete with Na^+ for entrance through Na^+ transport channels on the apical membrane of epithelial gill

cells (Chowdhury et al., 2016; Grosell & Wood, 2002; Laurén & McDonald, 1987). In addition, Cu can inhibit the Na^+/K^+-ATPase on the basolateral membrane of epithelial gill cells (Chowdhury et al., 2016; Grosell & Wood, 2002; Laurén & McDonald, 1987). Further, Cu has also been reported to inhibit the activity of carbonic anhydrase (CA), which produces a proton used by an apical Na^+/H^+ exchanger. Simultaneously, Cu exposure can displace Ca^{2+} from paracellular tight junctions, leading to an increase in their permeability and thus a stimulation of passive Na^+ loss (Grosell, 2012; Laurén & McDonald, 1985; Matsuo et al., 2005). The combined reduction of Na^+ uptake and stimulation of Na^+ loss reduces plasma Na^+ levels, which can lead to a cascade of events ultimately causing death by cardiovascular failure (Chowdhury et al., 2016; Grosell, 2012).

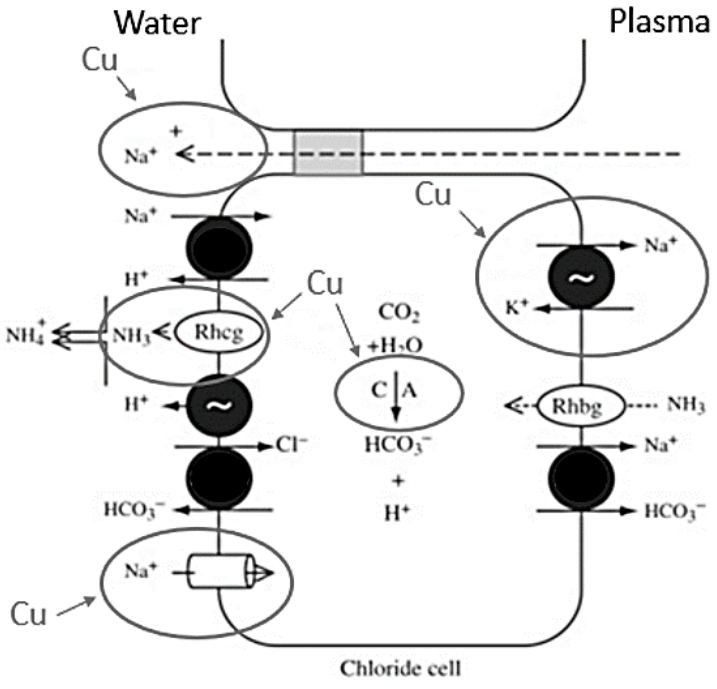

Figure 1. Schematic representation of the transport of sodium and ammonia across the branchial epithelium of freshwater fish, and of the action of copper on these processes

(figure modified from Wood et al., 2012). Carriers marked with ~ perform active transport dependent on ATP. Carbonic anhydrase is abbreviated CA, Rhcg and Rhbg denote rhesus proteins, dashed lines indicate movement via diffusion meanwhile solid lines indicate active transport.

Acute Cu exposure is also known to inhibit ammonia (NH_3) excretion across the gills (Laurén & McDonald, 1985; Lim et al., 2015; Zimmer et al., 2012). Ammonia is a waste product of protein catabolism and is highly toxic to fish (Evans et al., 2005). To avoid high internal levels of NH_3, it is excreted through the gills by facilitated diffusion via rhesus proteins, and this diffusion is dependent on conversion to NH_4^+ in the boundary layer which is maintained via acidification by H^+-ATPase and NHE (Figure 1) (Bucking, 2017; Evans et al., 2005; Zimmer et al., 2012). Yet, Cu is known to inhibit the activity of CA, which supplies protons to H^+-ATPase and NHE for boundary layer acidification (Zimmer et al., 2012). Furthermore, it is suggested that Cu disrupts the enzymes and/or transporters involved in the Na^+/NH_4^+exchange, thus inhibiting Na^+ uptake and NH_3 excretion simultaneously (Laurén & McDonald, 1985; Lim et al., 2015; Zimmer et al., 2012). Altogether, the inability of fish to excrete NH_3 levels in the gills and plasma, may result in harmful effects such as decreased growth, altered tissue structure, and decreased survival (Bucking, 2017).

1.3 Effects of temperature on Cu bioaccumulation and toxicity

The extent to which Cu bioaccumulates and exerts toxicity to freshwater fish depends on various environmental variables, such as water pH, hardness and DOC concentration

(Miller & Mackay, 1980). Specifically, these water chemistry parameters affect the bioavailability of Cu and thus its toxicity to aquatic organisms (Linbo et al., 2009). Although less studied and typically not considered in environmental risk assessment, temperature may be another important variable affecting metal bioaccumulation and toxicity in fish (Cairns et al., 1975). Indeed, as ectotherms, physiological processes (e.g. metabolism) in fish are highly affected by water temperature (Beitinger et al., 2000; Michael et al., 2016). In fact, the effects of elevated temperature on fish biology have received a considerable amount of attention (particularly in the context of global climate change) (Reid et al., 2022). A small proportion of these studies have looked at effects on metal ecotoxicity. Typically, heat tends to increase the toxicity of metals (Sokolova & Lannig, 2008). This increased toxicity seems to be associated with the increased rates of metal uptake and accumulation with warming (Cairns et al., 1975; Sokolova & Lannig, 2008). For example, increased Cu bioaccumulation with elevated temperatures has been observed in *Prochilodus scrofa* (Carvalho & Fernandes, 2006), *Poecilia vivipara* (Dornelles Zebral et al., 2019), *Carassius auratus*, *Oncorhynchus mykiss* and *Ictalurus punctatus* (Smith & Heath, 1979).

The effects of low temperature stress (cold shock) have been much less studied than high temperatures (Reid et al., 2022), especially in ecotoxicology studies. Based on studies on warm temperature by metal interactions, colder temperatures, such as the ones encountered during the winter season, are expected to decrease metal toxicological risk. Yet, there is also rationale for a possibly increased ecotoxicological risk during the winter season of temperate regions. Notably, it has been suggested that low water temperatures

may increase chemical toxicity by slowing down excretion and detoxification processes (Sprague et al. 1970). Furthermore, during winter months, fish typically undergo a seasonal cycle of decreased body condition, lower nutritional status and subsequently a decreased ability to resist metabolic stressors (Lemly, 1996). Lemly (1993, 1996) proposed that fish may be more sensitive to anthropogenic stressors (e.g. Cu) during this time of decreased body condition. This scientist developed the concept of "Winter Stress Syndrome" (WSS), a condition of metabolic stress in warm-water fish that develops when an external stressor causes increased metabolic stress during winter months (Lemly, 1993, 1996). This proposal was made, in part, on the basis of a lab study with juvenile bluegill (*Lepomis macrochirus*) that showed lipid depletion and reductions in feeding resulting in mortality when exposed to selenium (Se) in 4°C water (Lemly, 1993). Lemly (1993) suggested that the combination of lipid depletion due to reduced feeding and Se-induced stress caused bluegill to be drained of energy, resulting in mortality.

The permeability of fish gills and ion transport mechanisms are also sensitive to temperature (Barnes et al., 2014; Toneys & Coble, 1980). Specifically, at cold temperatures, membranes may transition from a liquid to gel phase, which in turn inhibits the functionality of membrane proteins, such as the Na^+/K^+-ATPase (Buhariwalla et al., 2012). To cope with these changes, a greater amount of energy must be dedicated to electrolyte regulation at low temperatures, which may further deplete lipids necessary to combat external stressors (Lemly, 1996; Toneys & Coble, 1980). This observation has been made in bluegill when exposed to Se (Lemly, 1993) and Cu (Smith & Heath, 1979) but not in other fishes from concurrent studies, suggesting a species-specific response.

Furthermore, fish exposed to cold may experience challenges in their Na^+ regulatory capabilities (Gonzalez & McDonald, 2000; Mackay, 1974; Maetz, 1971; Toneys & Coble, 1980), which is the main target of Cu acute toxicity. Cold temperature typically decreases Na^+ outflux, due to decreased gill permeability associated with reduction in gill functional surface area (FSA) and/or increase in blood-to-water diffusion distance (Gonzalez & McDonald, 2000; Mackay, 1974; Maetz, 1971; Onukwufor & Wood, 2020; C. M. Wood & Eom, 2021). Cold exposure also reduces Na^+ active uptake, as reaction rates at transport proteins decrease (Arrhenius law) and as a response of reduced Na^+ efflux rate, so that plasma ions remain at balance (Gonzalez & McDonald, 2000). However, Na^+ imbalance has been observed as a result of cold exposure. More precisely, decreased Na^+ and Cl^- concentrations in the plasma of goldfish (*Carassius auratus*) and Atlantic killifish (*Fundulus heteroclitus*) have been observed after long-term acclimation to cold temperature exposures (Mackay, 1974; Umminger, 1970). Common shiners were also found to have both a reduction in plasma Na^+ levels and Na^+ uptake upon exposure to 5℃ water (2 week exposure) (Gonzalez & McDonald, 2000). This net Na^+ loss might be associated with a reduced Na^+ uptake due to decreased activity of transport protein at cold temperature, while diffusive loss of Na^+ is less affected (Gonzalez & McDonald, 2000; Mackay, 1974; Maetz, 1971; Toneys & Coble, 1980). On the other hand, Gonzalez and McDonald (2000) found that in rainbow trout (a cold-water species) exposed to 5°C, an initial reduction in Na^+ uptake was restored after two-weeks, likely through upregulation of branchial enzymes (Na^+/K^+-ATPase, CA). Thus, compensatory mechanisms of cold-induced ion disturbances are also species-specific.

1.4 Objective, general methodology and hypotheses

Current water quality guidelines for the protection of aquatic life are typically generated from toxicity studies conducted at lab ambient temperatures around 18°C, typical of summer conditions (Environment and Climate Change Canada, 2021). As higher temperatures have typically been shown to increase metal bioaccumulation and toxicity in aquatic organisms, toxicity studies at winter cold temperatures have been scarce (Cairns et al., 1975; Sokolova & Lannig, 2008). Yet, there is theoretical basis for potential exacerbation of Cu toxicity in the winter (WSS and Na^+ imbalance). Therefore, the objective of my MSc project was to explore the effects of winter cold on Cu bioaccumulation and toxicity in a freshwater fish.

I selected brook char (*Salvelinus fontinalis*) as my study species because it is sensitive to Cu (Brix et al 2009), available, and easily reared in laboratory conditions. In the lab, I exposed juvenile brook char to either 14°C or 3°C, the latter temperature being reached with a slow cooling regime mimicking the onset of winter cold temperatures (-2°C/week). After a 4-week acclimation period to 14°C or 3°C, fish were acutely exposed (30 h) to a sublethal Cu concentration (150 µg/L), during which I measured Na^+, Cl^- and NH_3 physiological fluxes, as well as Cu gill accumulation. This Cu exposure was repeated with a second group of fish acutely cooled to 3°C (-3°C/day then 24-hour at 3°C), to compare the effects of long-term cold acclimation versus acute cold challenge on acute Cu toxicity.

My first hypothesis was that there would be a positive relationship between temperature and Cu bioaccumulation in fish gills, so that warm-acclimated fish would accumulate more Cu than cold-acclimated fish. My second hypothesis was that fish

would be more susceptible to Cu during the winter, so that for a same Cu-gill level, Cu toxicity would be greater in cold-acclimated fish.

2.0 METHODS

2.1 Fish husbandry

Juvenile brook char (*Salvelinus fontinalis*) (23.1 ± 1.8 g, n = 275) were obtained from Dr. Benfey's lab at the University of New Brunswick (UNB, Fredericton). They were transported to UNB (Saint John) and placed in 288L fiberglass tanks with recirculating dechlorinated (deCl) freshwater with the following composition (mean ± SEM): pH = 7.03 ± 0.03 (n=4) ; [Ca] = 7.81 ± 0.06 mg/L; [Na] = 15.73 ± 0.14 mg/L (n=20); [Mg] = 0.74 ± 0.01 mg/L (n=20); [K] = 0.65 ± 0.04 mg/L (n=20); [Cl] = 9.34 ± 0.28 mg/L (n=20); [Cu] = <0.001 mg/L (n=20); [DOC] = 1.91 ± 0.13 mg/L (n=9); alkalinity = 34 mg/L $CaCO_3$, hardness = 19.4 mg/L $CaCO_3$ (obtained from certificate of analysis report, RPC, December 2021). Ammonia was filtered from the water using freshwater Biomedia, and ammonia levels were tested every other day using ammonia test kits (API). Fish were fed 1% average body weight every other day with brook trout optimum diet (3mm floating pellets, Corey Nutrition). Fish were kept at about 15°C (14.6 ± 0.12°C, n=20) under a 12D:12L light cycle. Fish were left to acclimate to their new environment for two weeks before starting temperature acclimation. After their initial 2-week acclimation period, they were used in experiments within 9 to 18 weeks.

2.2 Temperature acclimation

Fish were acclimated to various temperature regimes prior to use in Cu experiments, as illustrated in Figure 2. More precisely, acute Cu toxicity was assessed in two successive experiments, respectively comparing the biological response of warm-acclimated fish *vs.* cold-acclimated fish, then of cold-acclimated *vs.* cold-challenged fish.

To this end, fish were separated into three 288L tanks equipped with a recirculating deCl water system and chillers (Artica DBM-250 and EcoPlus ¼ HP Chiller): a tank for a long-term cold acclimation, a tank for a warm-acclimation, and a tank where fish were kept at 14°C (stock tank). For long-term cold acclimation, fish were brought to 3°C at a rate of approximately -2°C per week to mimic the onset of winter (Lemly, 1993). Once the targeted temperature was reached (after 5 weeks), fish were left to acclimate for 4 weeks prior to the first Cu experiment during which the mean water temperature was 3.23 ± 0.052°C, n= 30, alongside the warm-acclimated fish group. For warm-acclimation, water temperature was maintained at 14°C until experimental use, during which time mean water temperature was 14.14 ± 0.092, n=30. Nine weeks after experiment 1, fish in the 14°C stock tank were acutely cooled (for cold challenge) down to use in the second Cu experiment, with the winter-acclimated fish group (at 13 weeks since reaching 3°C). For the cold challenge, fish were brought to 3°C at a rate of ~-3°C per day (i.e. 4-day cooling), then kept at 3°C for 24 h before experimental use.

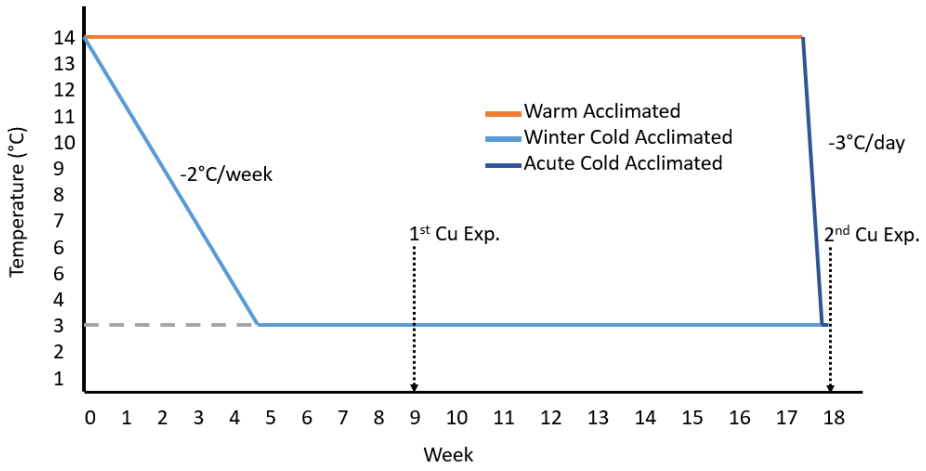

Figure 2. Timeline of the various temperature acclimation regimes undergone prior to the Cu toxicity experiments.

2.3 Copper toxicity experiments

After temperature acclimation, I measured net fluxes of Na^+, Cl^- and ammonia across the gills of individual fish at 14℃ and 3℃ respectively, just before Cu exposure (control/baseline flux), then after 1-7 h (flux 1), 10-22 h (flux 2) and 24-30 h (flux 3) of Cu exposure (Figure 3). These physiological fluxes were measured (Na^+ unidirectional fluxes, and Na^+, Cl^-, and ammonia net fluxes) or calculated (Na^+ outflux) by measuring the concentration of the solute of interest (Na^+, Cl^- or ammonia) in the water at the start and end of set periods. In addition, unidirectional fluxes of Na^+ (influx and outflux) were measured using the radioisotope Na^{22}. As mentioned previously, this Cu toxicity test was repeated twice, with fish at different temperature regimes.

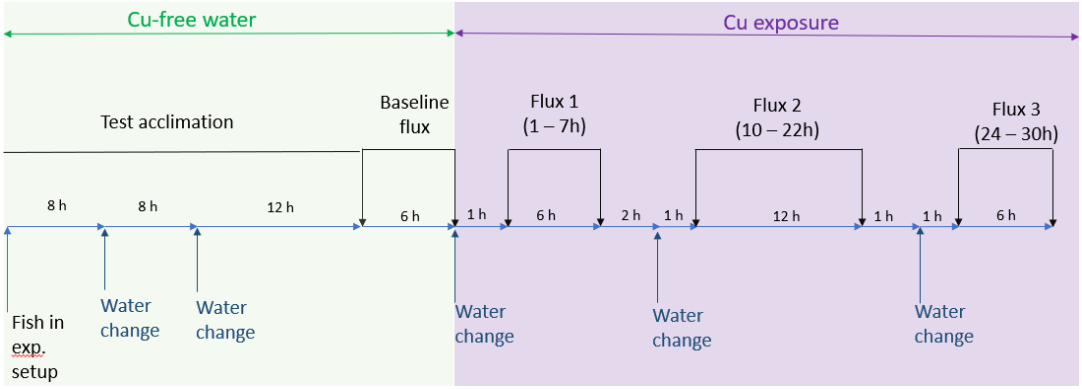

Figure 3. Timeline of the Cu experiments. Fish were exposed to deCl freshwater for the acclimation and baseline flux period, then to 150 µg/L Cu treated water for three subsequent flux periods.

For these Cu exposure tests, we selected one Cu exposure concentration of 150 µg/L total dissolved Cu. This concentration was selected based on a preliminary exposure test, that was found to be sub-lethal to fish. In these preliminary tests, we measured mortality,

NH3, Cl-, and Na+ fluxes of brook char exposed in the range of 10 to 120 µg Cu/L from 24 to 72 h. From the preliminary data (data not shown), I estimated that an exposure to 150 µg Cu/L would lead to substantial effects on NH3, Cl-, and Na+ fluxes, with limited fish mortality. Thus, we decided to use a slightly higher concentration of 150µg/L for our first experiment. Exposure solutions were prepared the day before use, by adding copper sulfate salt ($CuSO_4 \cdot 5H_2O$, ACS grade, Fisher Scientific) into deCl water in 220L polyethylene barrels. Each barrel was equipped with an air diffuser and a chiller to keep water temperature at either 14°C or 3°C. Barrels with Cu-free deCl water at 14°C and 3°C were also prepared for the acclimation and the baseline flux periods.

Feeding was suspended 24 hours prior to fish being transferred to the experimental set-up and the fish were not fed for the acclimated period or duration of the experiment. Fish were placed individually inside 1.2L flux chambers filled with 1 L deCl water at the corresponding temperature, with n=10 fish per temperature (Figure 4). These chambers were placed inside water baths in which water temperature was maintained by pump and chiller systems at either 14°C or 3°C (Figure 4). Individual air lines with diffusers provided constant aeration in each fish chamber throughout the experiment. Fish were left to acclimate to the experimental set-up for 24 hours prior to the baseline flux measurement, for a total of 48 hours without food prior to baselines measurements (cf. Figure 3). Complete water changes were done every 8 – 12 h during this acclimation period, to maintain water quality within the flux chambers. Approximately an hour prior to the start of each flux period, a complete water change was conducted and the volume of water in the flux chamber was reduced to 750mL. Next, each flux chamber was spiked with 0.8 µCi of Na^{22} (NaCl in H_2O, 18.5 MBq, Eckert & Ziegler) and left to mix for 10

minutes before the first water sample was taken (start of the flux period). Precisely, 12mL water samples were collected from each fish chamber, at the start (t = 0) and at the end (t = 6 or 12 h) of the flux period and placed in the -20°C freezer until Cu, Na^{+}, Na^{22}, Cl^{-}, and ammonia analyses. In addition, 10mL water samples were collected from four random flux chambers at the start and end of the flux, in order to determine pH over the flux period. To ensure water temperature was consistent, temperature was measured at the start of each flux in two random flux chambers from each temperature group. Between flux periods, complete water changes were carried out in each flux chamber with Cu water, to maintain water quality (Figure 3).

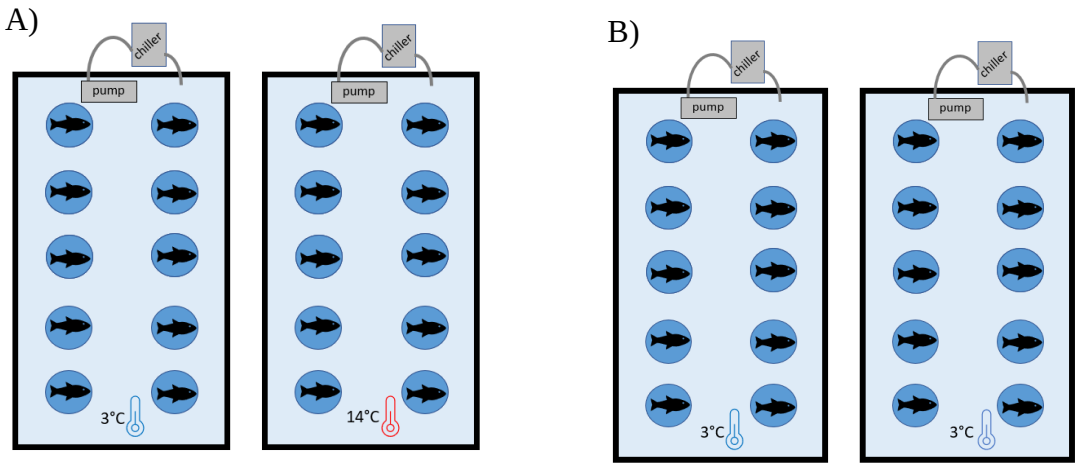

Figure 4. Schematic representation of the experimental set-up for A) flux experiment 1 and B) flux experiment 2. Brook char (n=10) were kept individually inside flux chambers inside of a water bath maintained at either 14°C or 3°C in experiment 1 and in baths at 3°C in experiment 2.

After the final flux period (30 h of Cu exposure), fish were collected and rinsed for 2 minutes in a solution of 1 mM ethylenediaminetetraacetic acid (EDTA, ACS grade, Fisher Chemical) made with deCl freshwater. This rinsing step was conducted to remove Cu bound to the surface of the gills, so that only the internalized Cu was measured. Then, fish were euthanized by overdose in a buffered (0.5 g/L $NaHCO_3$) 0.5 g/L solution of tricaine methanesulfonate (MS-222, Syndel) followed by brain puncture with a razor blade. Immediately after death, fish were blotted dried and measured (weight and total length), then blood was collected from the caudal vein into a heparinized hematocrit tube after cutting the caudal peduncle. The hematocrit tubes were centrifuged at 5000 rpm for 5 minutes (Thermo Scientific Sorvall Legend Micro 17 Microcentrifuge), and the isolated plasma was collected and kept in the -20°C freezer until analysis for plasma-Na^+ levels. Next, livers were dissected and weighed for hepatosomatic index (HSI) calculations. Finally, gill baskets were dissected, weighed, dried in an oven at 60°C for 72 h (until weight stabilized), then placed in the -20°C freezer until Cu analysis. In addition, after concluding the experiment, 10 brook char from the holding tank were euthanized with MS-222 and dissected for their gill baskets to determine a baseline gill-Cu concentration. Moreover, additional water samples were collected from the Cu-free and Cu-spiked barrels for DOC concentration analyses. Specifically, 20 mL water samples were taken from the treatment barrels at the beginning and end of each experiment, filtered (0.45µm, polyethersulfone membrane syringe filter, Cytiva Whatman) and stored in the fridge at 4°C until DOC analysis.

2.4 Chemical analyses

2.4.1 Water samples

Water samples collected for pH analysis were analyzed immediately after collection, using a benchtop pH meter (A214, Thermo Scientific Orion Star). These 10mL samples were spiked with 45µL of 3 M potassium chloride (KCl, ACS grade, Fisher Chemical™) before pH reading, to increase water conductivity and thus the speed and accuracy of the measurement.

Concentrations of DOC were analyzed at the Institut National de la Recherche Scientifique (INRS) (Québec City, Québec) with a total organic carbon (TOC) analyzer (Shimadzu VCPH), using potassium phthalate ($KHC_8H_4O_4$, Millipore Sigma) standards prepared in ultrapure water and analyzed with the non-purgeable organic carbon (NPOC) method (detection limit = 0.05mg/L).

Ammonia concentration was measured immediately after thawing collected water samples from the -20°C freezer at room temperature. Colorimetric plate assays were conducted following the protocol from Verdouw et al. (1978) (Verdouw et al., 1978), using a spectrophotometer (BioTek Epcoh 2 Microplate Spectrophotometer, EPOCH2NS). Specifically, standards for NH_3 concentrations were prepared using ammonium chloride (NH_4Cl, ACS grade, VWR). The standards and water samples were plated as triplicates. After completing the NH_3 assays, the remaining water samples were stored in the fridge at 4°C until the remaining analyses.

A colorimetric plate assay was used to determine Cl^- concentrations in water samples, following the protocol from Zall et al. (Zall et al., 1956) and a spectrophotometer (as above). Specifically, standards for Cl^- concentrations were

prepared using sodium chloride (NaCl, ACS grade, Fisher Scientific) and the standards and water samples were plated as triplicates.

The concentrations of Cu and major cations (Na, Mg, K, Ca) in the water samples were analyzed at INRS by inductively coupled plasma optical emissions spectrometry (ICP-OES, Agilent 5110 Dual View). For these analyses, water samples were diluted by a factor of 10 with ultrapure water and acidified with to 2% (v/v) nitric acid (HNO_3, trace metal grade, Fisher Chemical). Certified reference water (*Enviro*Mat Drinking water, low EP-L, SCP Science) was analyzed for quality assurance, and we measured percent recoveries of 127%, 126 %, 115 %, 147% and 136% for Cu, Ca, K, Mg, and Na respectively. In addition, instrument signal drift was corrected by analyzing three blanks half-way through sample analysis and at the end. Limits of detection were: Cu = 0.001 mg/L, Ca = 0.001 mg/L, K = 0.002 mg/L, Mg = 0.0003 mg/L, and Na = 0.003 mg/L. Sodium concentrations in collected water samples from experiment 1 were also analyzed using flame photometry (Single Channel Digital Flame Photometer, Sherwood Scientific) at UNB, and results were combined with ICP-OES results, the mean difference between the Na^+ readings from the two instruments was 28.5%.

We used either a gamma counter (5-minute counting time, 433-1417 KeV) (Packard Cobra 2 Gamma Counter) or a beta counter (5-minute counting time, wide window counting) (LS6500, Beckman Coulter) to analyze Na^{22} radioactivity (in counts per minutes, cpm) in collected water samples. Prior to using the beta counter, 2mL of scintillation cocktail (Optiphase HiSafe, PerkinElmer) was added to 200µL of sample water and 3 200µL blank samples (milliQ water) were read.

2.4.2 Biological samples

For Cu analyses in fish gills, dried gill baskets were first removed from the freezer, allowed to thaw at room temperature, then crushed using a clean glass stir bar to homogenize the tissues. Each gill sample was then digested in 1mL of concentrated HNO_3 (trace metal grade, Fisher Chemical) at 70°C for 24 hours, then with the addition of 400µL of concentrated hydrogen peroxide (H_2O_2, ultrapure for trace metal analysis, VWR) at room temperature for another 24 hours. After digestion, the samples were diluted to 10% (v/v) HNO_3 with ultrapure water. One certified reference material (TORT-2; National Research Council Canada) and three blanks (10mL ultrapure water) were also digested and analyzed for Cu concentrations. All digestions were analyzed for Cu concentrations at INRS by inductively coupled plasma mass spectrometry (ICP-MS, Thermo iCAP). The Cu limit of detection was 0.002 µg/L and the Cu percent recovery in TORT-2 was 106 ± 3%.

For Na^+ analyses in plasma samples, samples were removed from the freezer and allowed to thaw at room temperature. A 5µL subsample of plasma was transferred to a 13mL polypropylene tube. Then, 200µL of concentrated HNO_3 (trace metal grade, Fisher Chemical) and 9.8mL of ultrapure water were added for a final 2000 fold dilution and a final matrix of 2% HNO_3. Diluted plasma samples, along with 3 blanks, were analyzed for major cations at INRS using ICP-OES (Agilent 5110 Dual View), following the same method as described above for Na analyses in water samples.

2.5 Data calculations

2.5.1 Fulton condition factor and hepatosomatic index

Fish body condition was assessed using Fulton's condition factor (FCF) with the following equation:

$$FCF = \frac{m}{L^3} \times 100 \qquad \text{(Eq. 1)}$$

where m is the weight of the fish (in g) and L is the total length of the fish (in cm).

In addition, hepatosomatic index (HSI) was calculated to estimate energy reserves, using liver mass (g) and the following equation:

$$HSI = \frac{liver\ mass}{m} \times 100 \qquad \text{(Eq. 2)}$$

2.5.2 Physiological fluxes

Net fluxes of x: Na^+, Cl^- and NH_3 (J^x_{net}, in nmol/g/h) were calculated using the following equation:

$$J^x_{net} = \frac{([x]_i - [x]_f) \times V}{m \times \Delta t} \qquad \text{(Eq. 3)}$$

with $[x]_i$ and $[x]_f$ respectively the initial and final water concentrations of the substance of interest x (in nmol/L), V the water volume in the flux chamber (in L), m the mass of the fish (in g) and Δt the duration of the flux period (in h).

Furthermore, unidirectional fluxes of Na^+ (J^{Na}_{in} and J^{Na}_{out}) were calculated with equations 4 and 5:

$$J^{Na}_{in} = \frac{(R_i - R_f) \times V}{SA \times m \times \Delta t} \qquad \text{(Eq. 4)}$$

$$J^{Na}_{out} = J^{Na}_{net} - J^{Na}_{in} \qquad \text{(Eq. 5)}$$

where R_i and R_f are the activity concentrations of Na^{22} in the water (in cpm/L) at the beginning and end of a flux period respectively, and SA is the specific activity of Na^{22} in the test water (in cpm/nmol of total Na).

2.5.3 Temperature coefficient

For the first flux experiment (warm *vs.* cold-acclimated fish), the temperature coefficients (Q_{10}) of baseline physiological fluxes were calculated using equation 6:

$$Q_{10} = \frac{R_2}{R_1}^{\frac{10}{T_2 - T_1}} \qquad \text{(Eq. 6)}$$

where R_1 and R_2 are the net flux (Na^+, Cl^-, NH_3), influx or outflux (Na^+ only) (nmol/g/h) values and T_1 and T_2 are the two test temperatures (3°C and 14°C).

2.6 Statistical analysis

Statistical analyses were performed using Prism (version 9.4.0 for Windows, GraphPad Software, San Diego, California, USA). Outliers were first identified and removed using the ROUT method, with Q = 2%. Temperature effects on FCF and HSI were assessed using a Welch's t-test. The effects of Cu exposure level (0 and 150 µg/L) and temperature acclimation regime on 30h gill-Cu bioaccumulation were determined with a one-way ANOVA followed by a Tukey's multiple comparison. The effects of Cu exposure duration (baseline, 0-7 h, 10-22h and 24 – 30h Cu exposure), and temperature acclimation regime on physiological fluxes were assessed using a repeated 2-way ANOVA, or a mixed-effects model when values were missing (in the case of mortalities or outliers), followed with Šídák's multiple comparisons. Data are presented as mean ± standard error.

3.0 RESULTS

3.1 Fish condition after acclimation

In experiment 1, two mortalities in the warm-acclimated fish group occurred in the final flux period. The FCF was higher in fish acclimated at 3°C than at 14°C, in both acclimations leading up to experiment 1 (Figure 5a) and experiment 2 (Figure 5b). More precisely, the FCF in the cold-acclimated fish was 19% higher in the first acclimation (from 0.65 ± 0.032 to 0.79 ± 0.04) ($p=0.0086$, Welch's t-test, Fig. 5a) and 41.5% higher in the second acclimation (from 0.83 ± 0.015 to 0.99 ± 0.023) ($p<0.0001$, Welch's t-test, Fig. 5b). A similar effect of temperature was observed on the HSI, which was only measured on fish used in Experiment 2 (Figure 6). Indeed, HSI was 24.5% greater in 3°C acclimated fish (1.84 ± 0.14) than in 14°C acclimated (1.44 ± 0.077) ($p=0.025$, Welch's t-test, Fig. 6).

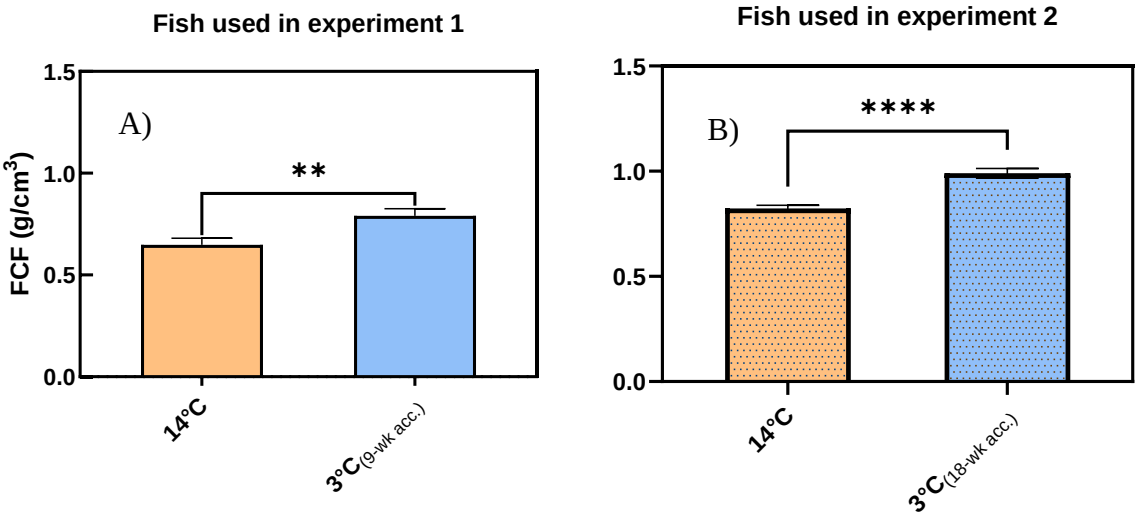

Figure 5. Fulton's condition factor of 14°C or 3°C acclimated fish used in a) Experiment 1 and b) Experiment 2. Cold acclimation lasted 9 weeks for experiment 1 (i.e. 5 weeks of

declining temperature followed by 4 weeks of acclimation) and 18 weeks for experiment 2. (i.e. 5 weeks of declining temperature following by 13 weeks of acclimation). Data is presented as mean ± SEM, n=8-10. Asterisks show significant differences between acclimation regime (Welch's t-test).

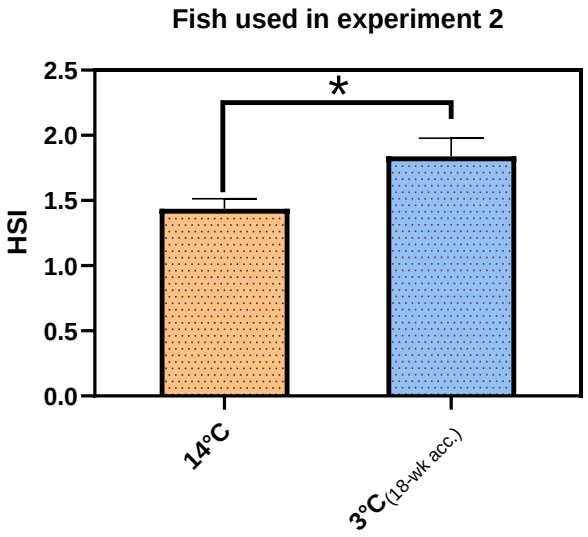

Figure 6. Hepatosomatic index of 14℃ and 3℃ (13 weeks at 3℃) acclimated fish used in Experiment 2 (18-week cold acclimation duration). Data is presented as mean ± SEM, n=9-10. Asterisks show significant differences between acclimation regime (Welch's t-test).

3.2 Physico-chemistry of test waters

The average water physico-chemistry data from experiments 1 and 2 are presented in Table 1. The pH and concentrations of Na^+, Ca^{2+}, Mg^{2+}, K^+ and DOC did not vary significantly between test waters and experiments (Welch's t-test). The overall mean water temperature during Cu exposure was 3.48 ± 0.29°C (n=15) in the cold-water treatment (target temp = 3°C) and 14.58 ± 0.18°C (n=6) in the warm-water treatment

(target temp = 14°C), with no significant variation between test waters and experiments (Welch's t-test). Mean Cu concentration was below the method limit of detection (< 10µg/L) in the control water of either experiment. On the other hand, Cu concentration in Cu-treated water (target conc. = 150 µg/L) varied between experiments, as it was 30.1% higher in experiment 2 (149.57 ± 0.0044 µg/L) than in experiment 1 (115 ± 1.3 µg/L at both temperatures) ($p<0.0001$, Welch's t-test). Moreover, we also found that Cu concentration in the 14°C test water (118.38 ± 2.47) had significantly higher Cu than in the 3°C test water (112.73 ± 0.19) ($p= 0.27$, Welch's t-test).

Table 1. Mean water physico-chemistry in Cu-free water (used in baseline fluxes) and Cu-spiked water (used in subsequent flux periods). Data is presented as mean ± standard error (n).

	Cu-free water		Cu-treated water	
Exp 1	14°C	3°C	14°C	3°C
Temp (°C)	14.53 ± 0.07 (3)	3.50 ± 0.17 (3)	14.63 ± 0.15 (3)	3.47 ± 0.23 (3)
Cu (µg/L)	< 10 (40)	< 10 (40)	118.38 ± 2.47 (55)	112.73 ± 0.19 (60)
Na^+ (mg/L)	14.99 ± 0.10 (19)	14.92 ± 0.12 (19)	14.96 ± 0.40 (56)	14.64 ± 0.12 (60)
Ca^{2+} (mg/L)	7.73 ± 0.07 (20)	7.77 ± 0.07 (20)	8.65 ± 0.19 (56)	8.96 ± 0.10 (60)
Mg^{2+} (mg/L)	0.74 ± 0.01 (20)	0.74 ± 0.01 (20)	0.76 ± 0.02 (56)	0.78 ± 0.01 (60)

K^+ (mg/L)	0.64 ± 0.03 (20)	0.71 ± 0.06 (20)	0.75 ± 0.05 (56)	0.85 ± 0.05 (60)
DOC (mg/L)	1.46 ± 0.40 (5)	1.67 ± 0.80 (5)	1.56 ± 0.11 (5)	1.59 ± 0.15 (5)
pH	6.84 ± 0.12 (4)	6.90 ± 0.09 (4)	7.08 ± 0.02 (12)	7.11± 0.02 (11)
Exp 2	3°C		3°C	
Temp (°C)	3.48 ± 0.13 (6)		3.50 ± 0.15 (3)	
Cu (µg/L)	< 10 (38)		149.57 ± 0.0044 (113)	
Na^+ (mg/L)	13.95 ± 0.12 (38)		14.32 ± 0.40 (113)	
Ca^{2+} (mg/L)	7.00 ± 0.06 (38)		6.63 ± 0.03 (113)	
Mg^{2+} (mg/L)	0.77 ± 0.01 (38)		0.71 ± 0.01 (113)	
K^+ (mg/L)	0.50 ± 0.02 (38)		0.81 ± 0.04 (113)	
DOC (mg/L)	1.99 ± 0.18 (6)		1.50 ± 0.10 (6)	
pH	7.42 ± 0.06 (9)		7.54 ± 0.02 (9)	

3.3 Physiological effects of copper and temperature

3.3.1 Sodium plasma concentrations

There was no effect of acclimation temperature (Figure 7a) or duration of acclimation (Figure 7b) on plasma Na^+ levels in brook char (p=0.13 and 0.16 respectively, Welch's t-test). From the experiment 2 plasma Na^+ data set, 2 outliers were removed from the acutely cooled fish group and 1 was removed from the cold-acclimated group.

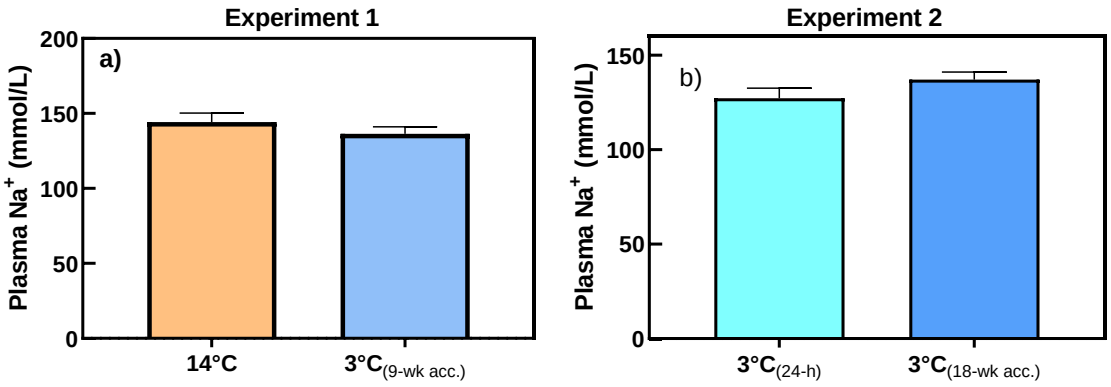

Figure 7. Plasma Na^+ levels in fish a) acclimated to 14°C or 3°C (4 weeks at 3°C, experiment 1) or b) challenged or acclimated to 3°C (13 weeks at 3°C, experiment 2). Data presented as mean ± SEM (n=7-10).

3.3.2 Sodium fluxes

- Warm *vs.* cold acclimation (4 weeks at 3°C)

Sodium fluxes of fish from experiment 1 are presented in Figure 8a, 1 outlier was identified and removed from the 14°C Na^+ influx and outflux data set. Overall, the mixed effects analyses found no effect of temperature on net flux, influx and outflux of Na^+ (p>0.1). On the other hand, Cu slightly affected net Na^+ flux (p=0.044) in a temperature-independent manner (interaction p>0.1), and strongly affected influx (p<0.000) in a

temperature-dependent manner (interaction $p<0.0001$), but had no effect on efflux ($p>0.1$).

Baseline net fluxes were close to zero in both 14°C and 3°C acclimated fish, indicating fish were close to Na^+ balance. This balance was achieved with Na^+ gain almost matching Na^+ loss. This balance was achieved with a Na^+ turnover that appears about 3-fold faster in the 14°C than in 3°C acclimated fish, although a temperature effect was only detectable on Na^+ influx ($p=0.0048$, Sidak's test). The Q10 values for Na^+ influx, outflux and net flux in the baseline flux period were 3.49, 2.01 and 0.34, respectively.

In the presence of Cu, mean Na^+ net flux became more negative in both warm and cold-acclimated fish, although there was no significant difference with baseline fluxes ($p> 0.05$, Dunnett's test). Sodium influx in warm-acclimated fish decreased sharply with Cu exposure until complete inhibition ($p=0.0063$, 0.0002 and 0.0349 after 1-7, 10-22 and 24-30 h respectively, Dunnett's test). On the other hand, Na^+ influx in cold-acclimated fish remained stable over time. Despite this difference in Cu effect in warm and cold-acclimated fish, there was no significant difference between influxes of cold and warm-acclimated fish at any given Cu exposure time (Sidak's test). Finally, Cu exposure had no significant effect on Na^+ outflux, despite an apparent promotion of Na^+ loss at the beginning of Cu exposure in both the 14°C or 3°C acclimated fish.

- Cold challenge *vs.* cold acclimation (13 weeks at 3°C)

Sodium fluxes of fish from experiment 2 are presented in Figure 8b, 5 outliers were removed from the cold challenged Na^+ influx data set and 1 was removed from the cold acclimated data set. Additionally, 5 outliers were removed from the cold challenged

group and 3 were removed form the cold acclimated Na^+ outflux data set. Overall, two-way ANOVAs showed very small effects of the cooling regime on Na^+ net flux and outflux ($p = 0.042$ and 0.046 respectively). Furthermore, Cu affected Na^+ influx ($p<0.0001$) and efflux ($p=0.0052$), but not the net flux ($p>0.1$). There was no interaction between the temperature and the Cu effects.

As in experiment 1, baseline Na^+ fluxes indicated that both the cold-challenged and cold-acclimated fish were close to ion balance. Indeed, the slightly negative net flux values were close to zero, thanks to Na^+ gain almost matching Na^+ loss.

No significant effect of Cu was observed on Na^+ net flux, although net fluxes were systematically negative in the presence of Cu in both cooling treatments. A small effect of duration of acclimation to cold was observed on net fluxes ($p = 0.042$), with fish appearing to lose more Na^+ after the 18-week cold exposure compared to the acute cold exposure. Yet, no significant difference was observed between cooling regime at any given Cu exposure period ($p>0.1$, Sidak's test). As in experiment 1, Cu exposure affected Na^+ influx ($p<0.0001$), and the effect was not dependent on acclimation (interaction $p= 0.110$). Indeed, Na^+ influx was greatly decreased over the course of the Cu exposure in both fish groups, to reach ~ zero at the end of the exposure. A significant effect of Cu exposure was observed on Na^+ outflux ($p=0.0057$) and this effect was not dependant on acclimation (interaction $p= 0.787$). Indeed, slight decreases of Na^+ outflux values were observed in the acutely cooled fish after 10-22h ($p = 0.0132$, Dunnett's test) and 24-30h ($p = 0.0246$, Dunnett's test) of Cu exposure (compared to the baseline). A very small temperature effect was also observed on Na^+ outflux ($p=0.0468$), with efflux appearing more important in the cold-acclimated fish compared to the acutely cooled

fish. However, a significant difference was only observed between the two fish groups after 10-22h of Cu exposure (p=0.0006, Sidak's test).

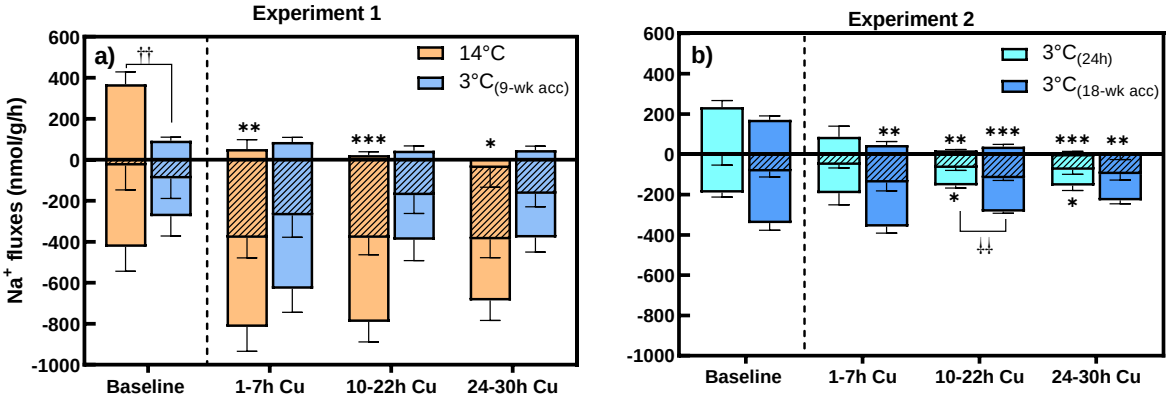

Figure 8. Sodium fluxes a) in fish acclimated to 14°C or 3°C (4 weeks at 3°C, experiment 1) and b) in fish at 3°C after an acute cooling (24h at 3°C) or an 18-week cold-acclimation (13 weeks at 3°C, experiment 2). Plain bars above and below the x-axis represent influx and outflux respectively, and hatched bars represent the net flux. Asterisks indicate a difference from the baseline flux for a given temperature treatment (Dunnett's multiple comparisons test). Daggers indicate an effect of temperature for a given flux period (Sidak's multiple comparisons test). Data presented as mean ± SEM, n=6-10.

3.3.3 Chloride net fluxes

- Warm *vs.* cold acclimation (4 weeks at 3°C)

Chloride net fluxes of fish from experiment 1 are presented in Figure 9a, 2 outliers were identified and removed from the 3°C data set. Overall, the mixed-effects analysis revealed that these fluxes were not affected by temperature (p>0.1, mixed-effects

analysis), but were affected by Cu (p=0.0002, mixed-effects analysis), and this Cu effect was not temperature dependent (interaction p=0.38, mixed-effects analysis).

Baseline net fluxes of the 14°C and 3°C acclimated fish were close to zero but slightly negative, suggesting potential small Cl^- loss across the gills.

The 14°C and 3°C acclimated fish then followed a similar trend of an initial loss of Cl^- at the start of the Cu exposure (p=0.023 and p=0.0097 in 14°C and 3°C fish respectively, Dunnett's test), followed by a gradual recovery near baseline levels by the end of the Cu exposure. The Q10 value for Cl^- flux was 1.49.

- Cold challenge *vs*. cold acclimation (13 weeks at 3°C)

Chloride net fluxes of fish from experiment 2 are presented in Figure 9b. Overall, Cl^- net fluxes were not affected by temperature (p>0.1, two-way ANOVA), but a significant effect of Cu was observed (p=0.0052, two-way ANOVA), and this effect was dependent on cooling treatment (interaction p=0.013, two-way ANOVA).

As in experiment 1, baseline net fluxes of the two fish groups were similarly slightly negative, suggesting potential slight Cl^- loss across gills.

Copper exposure gradually promoted Cl^- loss in cold-acclimated fish, with net Cl^- fluxes about 4-fold (p=0.0228, Dunnett's test) and 9-fold (p=0.0128, Dunnett's test) more negative respectively after 10-22 h and 23-30 h of Cu exposure (compared to the baseline). On the other hand, in the cold-challenged fish, Cl^- net flux appeared unaffected by Cu, although an initial Cl^- loss was almost significant after 1-7 h of Cu exposure (p=0.051, Dunnett's test).

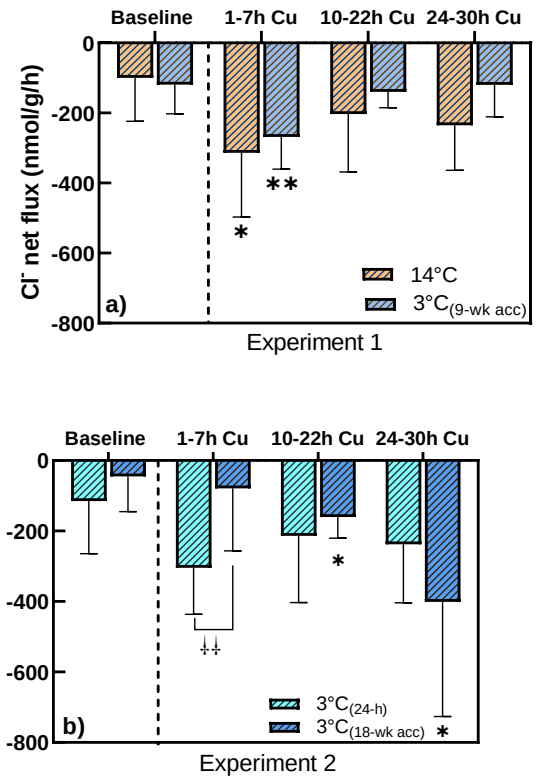

Figure 9. Net chloride fluxes a) in fish acclimated to 14°C or 3°C (4 weeks at 3°C, experiment 1) and b) in fish at 3°C after an acute cooling (24h at 3°C) or an 18-week cold-acclimation (13 weeks at 3°C, experiment 2). Asterisks indicate a difference from the baseline flux for a given temperature treatment (Dunnett's multiple comparisons test). Daggers indicate an effect of temperature for a given flux period (Sidak's multiple comparisons test). Data presented as mean ± SEM, n=8-10.

3.3.4 Ammonia net fluxes

- Warm *vs.* cold acclimation (4 weeks at 3°C)

Ammonia fluxes of fish from experiment 1 are presented in Figure 10a, 2 outliers were identified and removed from the 3°C data set. Overall, the mixed-effects analysis

revealed that ammonia excretion was significantly affected by temperature ($p<0.0001$) and Cu ($p=0.0002$), with an interaction between both variables ($p=0.0012$).

Both the 14°C and 3°C acclimated fish had negative NH_3 net fluxes, indicating NH_3 excretion. Warm-acclimated fish excreted systematically more NH_3 than cold-acclimated fish. Notably, fish excreted about 4-fold more ammonia in the baseline period ($p=0.0001$, Sidak's test), with a corresponding Q10 for NH_3 net flux was 3.21.

In the presence of Cu, net NH_3 fluxes decreased then returned to near baseline levels in the warm-acclimated fish ($p=0.011$, 0.018 and 0.99 after 1-7h, 10-22h and 24-30h respectively, Dunnett's test). This trend was not observed in cold-acclimated fish exposed to Cu ($p=0.54$, 0.056 and 0.072 after 1-7h, 10-22h and 24-30h respectively, Dunnett's test).

- Cold challenge *vs*. cold acclimation (13 weeks at 3°C)

Ammonia fluxes of fish used in experiment 2 are presented in Figure 10b. One outlier was removed from the cold acclimated NH_3 flux data set. Overall, the mixed-effects analysis revealed that ammonia excretion was significantly affected by the cooling regime ($p=0.0002$) and Cu exposure ($p<0.0001$), with an interaction between both variables ($p<0.0001$).

As in experiment 1, all fluxes were negative, indicating NH_3 excretion. Fish that were rapidly cooled excreted about twice less NH_3 than the 3°C-acclimated fish. Notably, in the baseline, we found that the cold-acclimated fish were losing 1.8x more NH_3 than the cold-challenged fish ($p=0.0015$).

While a Cu effect was detected, there was no clear trend as NH_3 excretion appeared to decrease, increase, then decrease again over the course of the Cu exposure, in both fish groups.

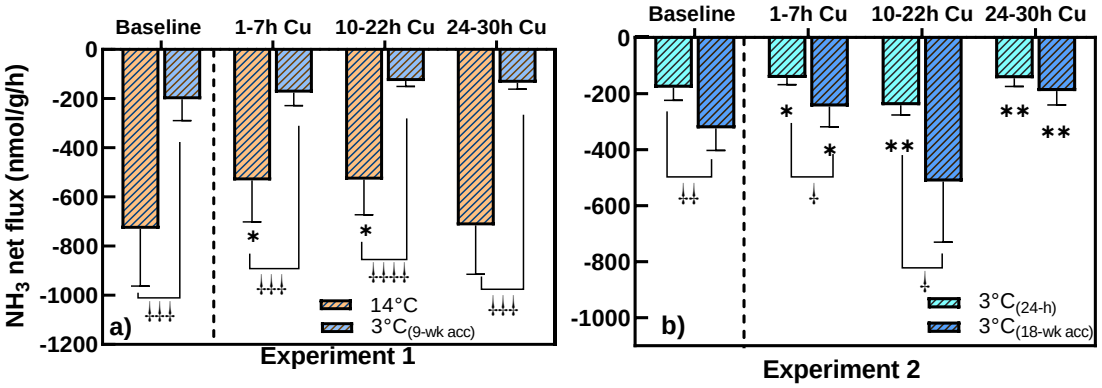

Figure 10. Net ammonia fluxes a) in fish acclimated to 14°C or 3°C (4 weeks at 3°C, experiment 1) and b) in fish at 3°C after an acute cooling (24h at 3°C) or an 18-week cold-acclimation (13 weeks at 3°C, experiment 2). Asterisks indicate a difference from the baseline flux for a given temperature treatment (Dunnett's multiple comparisons test). Daggers indicate an effect of temperature for a given flux period (Sidak's multiple comparisons test). Data presented as mean ± SEM, n=8-10.

3.4 Gill copper concentrations

Copper concentrations in the gills of fish from experiments 1 and 2 are presented in figure 11a and 11b, respectively.

In experiment 1, the 30 h Cu exposure had an effect on Cu accumulation in the gills ($p=0.001$, one-way ANOVA), such that Cu-exposed fish had higher gill-Cu levels

than the control fish, ($p<0.0001$ in the two fish groups, Tukey's test). There was no significant effect of temperature on gill-Cu level ($p=0.97$, Tukey's test).

In experiment 2, the same Cu effect was observed ($p<0.0001$, one-way ANOVA), with Cu-exposed fish having higher gill-Cu levels than the control fish ($p<0.0001$ in the two fish groups, Tukey's test). Furthermore, cold-acclimated fish accumulated slightly more Cu than the acutely cold fish ($p=0.018$, Tukey's test).

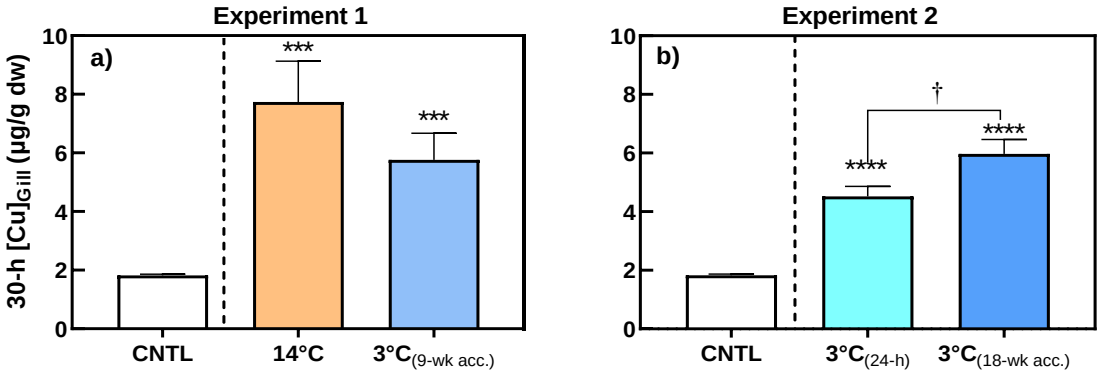

Figure 11. Cu concentrations in the gills a) of fish acclimated to either 14℃ or 3℃ (5 weeks at 3℃, experiment 1) and b) of fish at 3℃ after an acute cooling (24h) or an 18-week cold-acclimation (13 weeks at 3℃, experiment 2). Asterisks indicate an effect of Cu, relative to the control (CNTL) fish (one-way ANOVA). Daggers indicate an effect of cooling treatment (Tukey's multiple comparison test). Data are presented as mean ± SEM, n=8-9.

4.0 DISCUSSION

I investigated the effects of acclimation temperature (3°C [5 weeks at 3°C] or 14°C) and of an acute temperature drop (from 14°C to 3°C [24 hours at 3°C]) on fish condition, ion regulation, ammonia excretion with and without exposure to copper, which is a known disruptor of ion regulation and ammonia elimination. I found that acclimation temperature affected fish metabolism and physiology. More precisely, cold temperature acclimation enhanced fish condition, decreased NH_3 excretion and decreased Na^+ turnover rate across the gills. The latter physiological effects occurred rapidly following cold exposure, as evidenced by the cold-challenge data (Fig. 8 & 9). Acute Cu exposure inhibited Na^+ influx (Fig. 8) and NH_3 excretion (Fig. 10), as expected. Contrary to my first hypothesis, I did not find an effect of acclimation temperature on gill-Cu bioaccumulation. However, acutely cooled fish accumulated slightly more Cu than cold-acclimated fish (Fig. 11). Finally, contrary to my second hypothesis, I found that Cu effects on Na^+ influx and NH_3 were slightly higher at warmer temperature. These latter effects were relatively limited and equivocal, likely as a result of limited temperature effects on Cu-gill bioaccumulation, the main driver of toxicity.

4.1 Temperature effects on brook char

4.1.1 Fish condition

In my study, fish condition and hepatosomatic index were higher in fish acclimated to 3°C for both 9 weeks and 18 weeks compared to fish acclimated to 14°C. Previous studies have also reported increased condition factor and/or hepatosomatic index in fish acclimated to low temperatures, notably in common carp (*Cyprinus carpio*) (Castaldo et al., 2021), zebrafish (*Danio rerio*) (Vergauwen et al., 2010) and rainbow

trout (*Oncorhynchus mykiss*) (Bouchard & Guderley, 2003). Cold temperatures lead to reduced metabolic rate and reduced activity in ectotherm fish (Castaldo et al., 2021; Reeve et al., 2022; Vergauwen et al., 2010), these physiological changes being beneficial during the food-limited winters in temperate to high latitudes (Speers-Roesch et al., 2018). In our study though, both the 14℃ and 3℃ fish groups were fed at the same rate (in terms of % body weight/day), so that these adaptive seasonal changes likely led to a net surplus of energy available for growth in the cold-acclimated fish, relative to the warm-acclimated fish.

4.1.2 Ammonia excretion

I found significant effects of acclimation temperature and duration of cold acclimation on NH_3 excretion. First, fish at 3°C excreted less NH_3 than 14°C acclimated fish (Q10 = 3.21), regardless of the duration of cold acclimation. Many previous studies have reported this positive relationship between temperature and NH_3 excretion in fish (Bucking, 2017; Kieffer et al., 1998; Maetz, 1971). Ammonia excretion is known to be driven by metabolic rate, and thus to be highly dependent on temperature (Bucking, 2017; Maetz, 1971). More precisely, elevated (Bucking, 2017; Kieffer et al., 1998; Maetz, 1971). Temperatures result in increased metabolic rate and thus greater rates of deamination of amino acids, subsequently producing increased nitrogenous metabolic waste that must be excreted at the same rate it is produced to avoid toxic NH_3 build up (Bucking, 2017; Evans et al., 2005). Second, acutely cooled fish had slightly lower NH_3 excretion rates than cold-acclimated fish. The stress associated with a cold challenge has been shown to cause fish to reduce, or even cease feeding (Reid et al, 2022), which could explain the lower NH_3 excretion in the acutely cold fish in my study.

4.1.3 Sodium and chloride fluxes

In my study, Na^+ and Cl^- net fluxes were rather unresponsive to acclimation temperature, as observed in many previous studies (Evans 1969; Motais and Isaia 1972; Onukwufor and Wood 2018). It was expected that Na^+ and Cl^- net fluxes would follow similar trends, as the transport of both ions is known to be coupled at the gills (Preest et al., 2005). Overall, net Na^+ and Cl^- baseline fluxes were close to zero, indicating a close match between active ion uptake and diffusive ion loss. Although close to zero, the net Na^+ and Cl^- fluxes across the gills were always slightly negative. This observation does not necessarily mean that fish were not at balance, as additional salts are also acquired from the diet, and fish in my study were only starved 48 prior to baseline flux measurement (Onukwufor and Wood, 2018). Contrary to my observations on Na^+ fluxes and plasma levels, other studies have shown that cold leads to Na^+ plasma loss. For example, while Gonzalez & McDonald (2000) found that rainbow trout maintained plasma Na^+ levels at cold temperatures, they showed that common shiners experienced a 20% decrease in plasma Na^+ when acclimated to cold. Sodium and Cl^- losses have also been previously observed in cold acclimated killifish (Umminger, 1970), and goldfish (Mackay, 1974). In my study though, brook char maintained ionic balance throughout long-term cold acclimation, with a reduction of Na^+ uptake, that closely matched reduced Na^+ diffusive loss, as discussed below.

Na^+ influxes and outfluxes were responsive to temperature in my study, with sodium turnover rate about 3-fold faster in the 14℃ than in the 3℃ acclimated fish. Sodium uptake was strongly inhibited by cold temperature, with a Q10 of 3.49. As Na^+ influx is an active process in freshwater fish, this drop in Na^+ uptake rate was expected.

Indeed, Na^+ uptake is a highly temperature sensitive process (Gonzalez & McDonald, 2000; Maetz, 1971), with reduced Na^+ uptake at low temperatures notably reported in goldfish (Maetz, 1971), common shiner and rainbow trout (Gonzalez & McDonald, 2000). The temperature sensitivity of Na^+ uptake rates, and more precisely the existence of compensatory mechanisms, is species specific. For example, Gonzalez & McDonald (2000) observed Na^+ uptake rate in rainbow trout recovered to warm-temperature levels after 2 weeks of cold exposure, but this recovery was not observed in common shiner. The upregulation of branchial transporters (e.g. increase NKA activity and numbers) is likely the mechanism behind the restoration of Na^+ uptake in some fish (Gonzalez & McDonald, 2000). In our study though, Na^+ influx in cold-acclimated brook char remained significantly lower than in warm acclimated fish. In agreement with my study, a lack of compensation was reported in brook char by Schwarzbaum et al. (1992), as the fish did not demonstrate elevated Na^+/K^+ pump activity upon acclimation to cold temperatures. To achieve ionic balance, this species seems to use another strategy than the upregulation of Na^+ uptake: the reduction of Na^+ diffusive loss (Onukwufor & Wood, 2018; Schwarzbaum et al., 1992), as discussed below.

Similar to Na^+ uptake, Na^+ diffusive loss decreased with lowered acclimation temperature, with a Q10 of 2.01. The effect of temperature on Na^+ loss was less evident than on Na^+ influx, which could be due to differences in analytical measurement. Indeed, Na^+ uptake rate was measured with very a sensitive radiotracing technique, while Na^+ loss was calculated from the influx and net flux, the latter being measured with a relatively less sensitive spectrometry technique. Overall, it is likely that a concurrent reduction of Na^+ outflux enabled the cold-exposed fish to compensate the reduction of

Na^+ influx, and thus to maintain ionic balance. Findings from other studies suggest that this drop in Na^+ efflux during cold exposure could be attributed to a reduction in functional surface area (FSA) of the gills (Gonzalez & McDonald, 2000). The FSA can be reduced with rapid development of cell masses between the branchial lamellae, where gas exchange takes place (Gilmour & Perry, 2018; Sollid et al., 2005). This reversible gill remodeling occurs when O_2 demand surpasses O_2 supplies in fish, which typically occurs when metabolic rate is suppressed during the winter. In accordance with the "osmorespiratory compromise", corresponding to the conflict between gas transfer and ion regulation in gills of most fish, a reduced FSA at low temperatures is expected to limit the costs of osmoregulation, and preserve energy which can then be stored for winter survival (Sollid et al., 2005). Another possible mechanism for reduced ion loss is via a reduction of epithelial electrolyte permeability (Gonzalez & McDonald, 2000), notably via homeoviscous adaptations (Buhariwalla et al., 2012; Raynard & Cossins, 1991). These different morphological changes at the gills are known to occur very quickly (Gilmour and Perry, 2018), which is probably the reason why similar temperature effects were observed during the acute temperature drop and the chronic cold acclimation in my study.

4.2 Copper effects on brook char

In my study, acute Cu exposure resulted in Cu gill accumulation and disturbances of Na^+ uptake, Cl^- balance, and ammonia excretion in brook char. The latter two disturbances were transient and did not persist past 7 h of exposure. These observations are in agreement with known effects of Cu in freshwater fish following an acute exposure

(Chowdhury et al., 2016; Crémazy et al., 2016; Grosell & Wood, 2002; Laurén & McDonald, 1985, 1987; Lim et al., 2015; Zimmer et al., 2012).

Chloride net loss in warm acclimated fish was slightly promoted in the first hours of Cu exposure, then appeared to recover. The strongest effect of Cu was on Na^+ influx, which became completely inhibited after one day of exposure in the 14°C acclimated and in the 18-week 3°C acclimated fish. It is well known that Cu affects the regulation of Na^+ and Cl^- in freshwater fish, with resulting Na^+ plasma loss being the main reason for mortality (Grosell, 2012; Laurén & McDonald, 1986; Laurén and McDonald 1985). Mechanisms for Cu-induced reduction of Na^+ uptake include the competition between Na^+ and Cu^{2+} ions at apical Na^+ gill transporters, the inhibition of basolateral NKA activity and the inhibition of CA activity (cf. Figure 1) (Chowdhury et al., 2016; Grosell & Wood, 2002; Laurén & McDonald, 1987). Sodium outflux is also generally affected by acute Cu exposure, with a promotion of Na^+ diffusive loss attributed to increased gill permeability from the displacement of Ca^{2+} from paracellular tight junctions by Cu (cf. Figure 1) (Grosell, 2012; Laurén & McDonald, 1985; Matsuo et al., 2005). Yet, I did not detect such effect in my study, suggesting that Na^+ influx inhibition was the principal mechanism of Cu toxicity in brook char under the tested exposure conditions. This finding agrees with some previous reports that the threshold of Cu concentrations for stimulation of Na^+ outflux are greater than those of Na^+ outflux (Chowdhury et al., 2016; Laurén & McDonald, 1986, 1987).

We also found that Cu affected NH_3 excretion in the 14°C acclimated fish. Disruption of NH_3 clearance, and resulting elevated plasma NH_3 levels, is one of the most commonly observed effects of Cu exposure in freshwater fish (Grosell, 2012; Zimmer et

al., 2012). Similar patterns of transient disruption of NH_3 excretion, followed by full recovery, have been observed in freshwater fish acutely exposed to Cu (Laurén & McDonald, 1985; Zimmer et al., 2012). This toxic effect appears to be linked to the ability of Cu to inhibit CA activity in the gills.

4.3 Effects of temperature on Cu bioaccumulation and toxicity in brook char

4.3.1 Copper gill bioaccumulation

In this study, we did not find a significant effect of temperature on Cu bioaccumulation in fish gills (experiment 1). Indeed, the slightly higher Cu bioaccumulation observed in warm-acclimated fish was not significant. However, we did find a slightly significant effect of cooling treatment on Cu accumulation in fish gills (experiment 2). Namely, cold-acclimated fish accumulated about 1.3-fold more Cu in their gills than the cold-challenged fish.

The lack of effect on Cu levels in the gills of 3°C acclimated compared to 14°C acclimated was surprising because acclimation to warmer temperatures typically results in increased metal accumulation in fish (Cairns et al., 1975; Sokolova & Lannig, 2008). Indeed, branchial Cu uptake is an active process and thus it is expected to be sensitive to temperature. However, bioaccumulation is a function of both uptake and elimination rates, so it is possible that increased Cu elimination rates might have counteracted the increased Cu uptake rates at high temperatures. This explanation was proposed by (Carvalho & Fernandes, 2006), who found no difference in the 96h-LC_{50} value for fish acclimated at 20 or 30°C. However, Cu elimination might have been relatively limited during the short 30 h Cu exposure in my study. An additional explanation for the limited

temperature sensitivity could be associated with homeostatic mechanisms aimed at maintaining sufficient Cu levels in the fish, during their temperature acclimation period in low-Cu water. Indeed, while Cu is toxic at high concentrations, this metal is also an essential element that is notably involved in many enzymatic processes (Grosell & Wood, 2002). Thus, fish might respond to a cold-induced inhibition of Cu uptake by upregulating their branchial Cu transporters, as seen for Na^+ uptake in rainbow trout after two weeks of cold exposure (Gonzalez & McDonald, 2000). This upregulation might have limited the difference in metal accumulation between the warm- and cold-acclimated fish, after the acute Cu exposure in experiment 1. On the other hand, it is possible that the acutely cold fish might not have had sufficient time to upregulate their Cu branchial transport to the same level as the chronically cold fish, leading to slightly lower metal uptake following the Cu exposure in experiment 2.

4.3.2 Copper toxicity

Warm-acclimated fish appeared generally more affected by Cu exposure, but these temperature effects were relatively limited. Likewise, duration of cold acclimation had limited effects on Cu toxicity in brook char.

A small and transient effect of temperature was observed on Cu inhibition of ammonia excretion. Indeed, ammonia excretion was slightly decreased at the start of Cu exposure in warm-acclimated fish, but not in cold-acclimated fish. This small temperature effect might be the result of a slightly higher Cu bioaccumulation in the gills of warm-acclimated fish, although this latter difference was not significant. No clear effect of cooling speed was observed on Cu inhibition of ammonia excretion, which

might reflect the relatively close (albeit significantly different) accumulation of Cu in the gills of the two fish groups.

Copper strongly inhibited Na^+ influx in warm acclimated fish, but not in the concomitantly tested cold-acclimated fish (in experiment 1). This temperature effect was much stronger than the temperature effect on Cu gill accumulation, which was somewhat surprising considering that metal toxicity is usually a function of metal tissue bioaccumulation. Yet, in their study Laurén and McDonald (1987) found that Cu in the gills of rainbow trout were not significantly elevated at the greatest point of inhibition of Na^+ uptake. It has been suggested that, during a short-term Cu exposure, Cu does not accumulate in gills at levels high enough to strongly inhibit the basolateral NKA (Grosell et al, 2002). Thus, competition between Na^+ and Cu^{2+} ions at Na^+ apical channels, which might occur without actual Cu internalization, could be an important mechanism behind Na^+ influx decrease in 14°C acclimated fish in my study. Indeed, Laurén and McDonald (1987) showed that a large portion of apparent Cu uptake during acute exposure is due to surface binding. Conversely, Cu effects on Na^+ influx were not significant in cold-acclimated fish exposed in parallel to the warm-acclimated fish (experiment 1), despite a two-fold decrease in Na^+ influx between the beginning and end of Cu exposure. This temperature difference could reflect the existence of different Na^+ apical transporters at cold temperature, that might be less sensitive to Cu exposure, such as Na^+/H^+ exchange (Evans et al., 2005). Nevertheless, inhibition of Na^+ influx was observed in cold-acclimated fish in experiment 2. Fish in experiment 2 were exposed by mistake to a 1.3-fold higher Cu concentration (Table 1), which might be the reason why Cu effects were more pronounced in cold-acclimated fish in this second experiment. In this latter

experiment, Cu effects were quite similar between the cold-acclimated and the cold-challenge fish, possibly due to relatively close Cu gill bioaccumulation levels. Overall, temperature effects on Na^+ influx did not translate into important and potentially toxic effects on the net Na^+ flux. Indeed, while Na^+ net losses appeared systematically more important in warm-acclimated fish *vs*. cold-acclimated fish, a significant difference could not be detected. Likewise, effects of temperature on Cl^- net loss were small and equivocal.

Overall, the collected data reject my second hypothesis, that fish would be more susceptible to Cu during the winter. However, my findings are consistent with how brook char responded to the different temperature regimes in my study. First, contrary to some other studies, acute and chronic cold exposure did not alter net flux of ions in brook char, as this fish species may have rapidly adapted their gills to limit Na^+ loss and remain at balance despite reduced Na^+ uptake. Furthermore, fish condition was enhanced rather than reduced following long-term cold acclimation. Altogether, these observations suggest that cold-exposed brook char should not be more sensitive to Cu at cold temperature. Notably, Grosell et al. (2002) proposed that Cu sensitivity in freshwater species is a function of their Na^+ turnover rate, which was lower in cold-exposed fish in our study. Overall, WSS does not seem to apply to brook char, at least not under the conditions tested in my study. The WWS is likely more relevant in the field where food is scare during the winter (Lemly, 1996). Yet, in my study, fish were fed every day, which would not adequately mimic the decrease in food availability that this fish likely experiences during the winter. Stronger effects of temperature might have also been observed using a fish species that undergoes dormancy, as opposed to a cold-water

species like brook char (Reeve et al., 2022). For instance, the juvenile bluegill as used in the Lemly (1993) become semi-dormant during cold weather, causing rapid lipid depletion and mortality after one-third of the fish exposed to selenium (Lemly, 1993).

5.0 CONCLUSION

Our study investigated the effects of winter cold on Cu bioaccumulation and toxicity in brook char. Contrary to my first hypothesis, acclimation temperature had limited effects on acute Cu bioaccumulation in fish gills. I propose that this lack of temperature effect could be the result of similar temperature effects on Cu uptake and elimination rates and/or of homeostatic mechanisms to maintain optimal Cu levels in fish. Additional studies are needed to understand how acute and long-term temperature changes affect Cu accumulation in fish gills.

As expected from the limited temperature effects on Cu bioaccumulation, temperature effects on Cu toxicity were also limited. Overall, slightly greater Cu effects were observed on Na^+ uptake (inhibition) and ammonia excretion (transient reduction) in warm-acclimated fish, which could reflect a slightly higher (albeit not significant) Cu gill bioaccumulation, or a slightly higher fish sensitivity at warmer temperature. Thus, my second hypothesis, that fish would be more susceptible to Cu during the winter, was also rejected. Indeed, brook char were not challenged by cold, as demonstrated by their higher condition during cold-acclimation, and the absence of ion disturbance due to decreased ion turnover rate across the gill. Future studies could continue this investigation by decreasing food availability in cold-acclimated fish and adjusting the photo period, which would more realistically reproduce winter conditions. How fish respond to cold is also species-specific, and I suggest that future studies investigate warm-water species and/or species undergoing complete winter dormancy.

Overall, this study shows that temperature effects on Cu bioaccumulation and sublethal physiological effects are not straightforward. It also suggests that brook char are

not more at risk of Cu effects during the winter, such when they are in good condition, so that environmental quality guidelines derived at warm lab-ambient temperatures also would be protective during the winter season. Rather, our data suggest that heat might be a more concerning problem than cold in this fish. Finally, our study highlights the importance of investigating toxic responses at a wider range of environmentally relevant temperatures in temperate fish species.

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CURRICULUM VITAE

Candidate's full name: Julia Macpherson

Universities attended:

-University of Guelph – BSc. Marine and Freshwater Biology, 2016-2020

-University of New Brunswick (Saint John) – MSc Biology, 2021-2023

Publications: N/A

Conference Presentations:

-CRI Days (virtual), March 2021

-Canadian Ecotoxicology Workshop (CEW, Halifax, NS), October 2021

-Canadian Ecotoxicology Workshop (CEW, Winnipeg, MB), October 2022