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Effects of copper on a reconstructed freshwater rainbow trout gill epithelium: Paracellular and intracellular aspects

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ABSTRACT

The barrier properties and intracellular responses of a primary cultured trout gill epithelium (containing both mitochondria-rich and pavement cells) were examined over 24 h of copper (Cu) exposure (0, 200 and 1000 μg/L) in apical fresh water. Transepithelial resistance (TER) and mRNA abundance of tight junction proteins zonula occludens-1, occludin, cingulin, claudin-8d and -28b were examined as endpoints of barrier function and the paracellular pathway. Intracellular endpoints analyzed were Cu accumulation, Na^+ content, carbonic anhydrase activity and mRNA abundance of carbonic anhydrase (*ca-II*) and Na^+/K^+ ATPase (*nka α1a* and *nka α1b* isoforms). After a brief initial drop in TER in the 1000 μg Cu/L treatment, Cu at both levels increased TER over the first 6 h of exposure but there were no differences among groups from 12 h onwards. After 24 h of Cu exposure, there were no differences in mRNA abundance of any of the tight junction proteins examined. Cu accumulation occurred at 1000 μg Cu/L (5.5-fold increase), but no depletion of Na^+ content. Carbonic anhydrase activity decreased significantly (by 76%), however Cu exposure did not alter the transcript abundance of *ca-II, nka α1a, or nka α1b*. This study provides a first report of carbonic anhydrase sensitivity to Cu exposure in a cultured model gill epithelium. We conclude that Cu impacts the permeability of this model during the early stages of exposure and that the use of carbonic anhydrase inhibition as an endpoint of metal toxicity in this model preparation may be useful for future mechanistic investigations and environmental monitoring.

1. Introduction

A primary cultured freshwater rainbow trout gill epithelium model on filter inserts was first developed by Wood and Pärt (1997). This model, composed exclusively of gill pavement cells (PVCs), generates a polarized epithelium that tolerates freshwater exposure at the apical surface. Over the last 20 years, the preparation has undergone some modifications (*e.g.* Kelly et al., 2000; Wood et al., 2002a; Walker et al., 2007; Minghetti et al., 2014; Schnell et al., 2016), but a major advance (termed the double-seeded insert preparations, DSI) was the modification of protocol so as to allow the incorporation of gill ionocytes (mitochondria-rich cells, MRCs) (Fletcher et al., 2000). The DSI epithelium not only comprises different cell types- MRCs, PVCs and mucus cells - at similar percentages as found in the intact gill (Kelly et al., 2000), but also generates a high transepithelial resistance when exposed apically to fresh water (Bury et al., 2014). All these characteristics mimic the intact gill, simulating the *in vivo* state, and supporting the use of this preparation as means to facilitate a reduction in the use of fish for experimental purposes. Indeed primary cultured trout gill models have been used in a variety of studies to provide insight into gill function that would have been difficult to attain using the intact gill – for example the molecular physiology of the tight junction (TJ) complex (*e.g.* Chasiotis et al., 2010; Kolosov et al., 2014), occurrence of channels (O'Donnell et al., 2001), transepithelial transport of ammonia (Kelly and Wood, 2001; Tsui et al., 2009) and pharmaceuticals (Stott et al., 2015), and environmental monitoring (Bury et al., 2014; Minghetti et al., 2014). However, only a few studies have directly evaluated the effects of metals with this preparation (Zhou et al., 2004; Walker et al., 2007; Walker et al., 2008). Notably Smith et al. (2001) using cultured epithelia comprising only PVCs showed that exposure to 1600 μg/L Cu inhibited O_2 consumption rate. More recently, Smith et al. (2017) constructed Cu titration curves in gill cell suspensions and cultured epithelia with and without MRCs. They concluded that the DSI epithelium containing both MRCs and PVCs best mimicked Cu-binding in the intact gill, and that the MRCs were the major site of Cu uptake.

Copper is an essential metal for aquatic animals, but exposure to

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elevated concentrations may exert toxic effects. The key mechanism of acute Cu toxicity is ionoregulatory disturbance due to competition for binding sites on the gills (especially those for Na^+ uptake), increases in diffusive permeability for ion losses, and inhibition of the activities of key enzymes involved in Na^+ transport (reviewed by Grosell, 2012). When MRCs isolated from rainbow trout gills were exposed *in vitro* to a range of Cu concentration s (6–600 μg/L) for only 1 min, inhibition of Na^+ uptake occurred (Goss et al., 2011). In intact fish, effects include inhibition of Na^+/K^+-ATPase (Laurén and McDonald, 1987; Li et al., 1998) and carbonic anhydrase activities (Zimmer et al., 2012). This latter enzyme is recognized as a good biomarker for metal exposure (Lionetto et al., 2012; Lionetto et al., 2016; Ali et al., 2017). Cu effects on carbonic anhydrase have been implicated in both *in vitro* experiments with isolated gill cells from a marine bivalve (Nogueira and Bianchini, 2018), and *in vivo* experiments on freshwater and marine bivalves (Nogueira et al., 2017, 2018) and freshwater fishes (Zimmer et al., 2012; de Polo et al., 2014).

Moreover, metals can increase branchial permeability, thereby increasing the diffusive loss of Na^+ and other ions (Laurén and McDonald, 1985; Chowdhury et al., 2016). It is commonly believed that Cu acts by displacing surface-bound Ca^{2+} ions which are important in maintaining the integrity of TJs in vertebrate epithelia, though there is no direct evidence of the mechanism in the gill epithelium at this point (reviewed by Grosell, 2012; Chowdhury et al., 2016). In the DSI cultured epithelium, TJs remain very similar to those found in intact gill (Fletcher et al., 2000) and cultured gill epithelia have been used to show that occludin (Ocln), members of the claudin (Cldn) superfamily and cytosolic proteins such as zonula occludens-1 play important roles in regulating gill epithelium TJ permeability (Chasiotis et al., 2012; Kolosov and Kelly, 2017; Kolosov et al., 2017).

With this background in mind, our study aimed to investigate the toxicological effects of Cu on gill epithelial cultures of rainbow trout (DSI system), emphasizing intracellular and paracellular aspects. We hypothesized that Cu exposure would result in intracellular Cu accumulation and Na^+ depletion, together with disturbances in transepithelial resistance, possibly due to an increase in paracellular permeability (as an indicator of paracellular tightness, Wood et al., 2002b). Because it was reasoned that Cu exposure would impact the cultured model in a manner that may reflect its impact *in vivo*, the activity and transcript abundance of CA were measured as well as mRNA abundance of key Na^+/K^+-ATPase subunits. Finally, to gain molecular insight into possible changes in paracellular permeability, transcript abundance of select TJ proteins were measured following Cu exposure.

2. Material and methods

2.1. Animal husbandry

Rainbow trout (*O. mykiss*; 300–700 g) were obtained from Humber Springs Trout Hatchery (Orangeville, ON, Canada) and maintained in 600-L opaque polyethylene tanks at York University (Toronto, ON). Tanks were supplied with flow-through dechlorinated freshwater (FW, composition in mM: 0.59 Na^+, 0.92 Cl^-, 0.76 Ca^{2+}, 0.043 K^+, pH 7.3), photoperiod 12D: 12 L, and fish were fed *ad libitum* once daily (Martin 133 Profishent, Elmira, ON, Canada). Animal care and experimental procedures were in accordance with an approved York University Animal Care Committee protocol and conformed to the guidelines of the Canadian Council on Animal Care.

2.2. Culture of DSI gill epithelia

Primary culture was performed using a method originally developed by Wood and Pärt (1997) and modified by Fletcher et al. (2000). Methods have been detailed by Kelly et al. (2000) and Schnell et al. (2016). In brief, gill cells are isolated twice over a 2-d period, from two separate fish. For each seed, one fish was sacrificed (by MS-222 overdose and spinal transection), gills were dissected and cells were isolated using trypsin (0.05% Trypsin-EDTA, Canada Inc., Burlington, ON, Canada). Isolated rainbow trout cells were seeded onto polyethylene terephthalate membranes (cell surface area 0.9cm^2, pore size 0.4 μm, Corning® Life Sciences, Durham, NC, USA) at a density of 1.2×10^6 cells per insert, using Leibovitz medium (L-15; Invitrogen®, Thermo Fisher Scientific, Waltham, MA, USA) supplemented on a *v*/v basis with antibiotics (5% foetal bovine serum (FBS, USA origin; Sigma-Aldrich, St. Louis, MO, USA), 2% penicillin and streptomycin (PEST) (Invitrogen®), and 2% gentamicin (Gibco®, Grand Island, NY, USA) on both the upper (apical) and lower (basolateral) surfaces of the inserts. After 24-h incubation at 19 °C in an air atmosphere incubator, the upper surface of the seeded cells were washed with phosphate-buffered saline (PBS) to remove non-attached cells, and a second seeding with freshly isolated cells was performed in the same manner as the first. After a further 24 h, another PBS wash was performed and 2.0 mL of Leibovitz medium (L15) supplemented with FBS and antibiotics was added in the basolateral chamber of the insert. Every 48 h, the medium was completely changed. The transepithelial electrical resistance (TER) of the intact gill epithelium was monitored daily using a custom-modified voltohmmeter (EXOMX, World Precision Instruments, Sarasota, FL, USA) fitted with chopstick electrodes (STX-2). Daily monitoring of resistance allows the development of a sigmoidal TER curve to be visualized so that epithelial preparations can be utilized for experiments when they become electrically stable and tight (*i.e.* upon plateau of TER) (see Kelly et al., 2000). Approximately 7 days after seeding, TER measurements reached a plateau, indicating that preparations were electrically tight and capable of tolerating fresh water on the apical surface. At this point, culture conditions were changed from symmetrical (*i.e.* supplemented L15 media on both sides) to asymmetrical (*i.e.* supplemented L-15 on the basolateral side and fresh water on the apical side).

2.3. Copper exposure

The Cu concentrations used in the present study (200 and 1000 μg/L) were selected based on reports of environmentally relevant Cu levels found in contaminated FW (USEPA, 2007) and the observations of Smith et al. (2001), who reported on the effect of Cu on a primary cultured trout gill epithelium preparation. When TER reached about ~25 KΩ, the medium in the apical chamber was replaced (using three rinses) with sterile freshwater in the absence (control) or presence of Cu (200 and 1000 μg/L). Cu was added as $CuCl_2$ (Merck, St. Louis, MO, USA). The exposures were maintained for 24 h. TER was monitored every hour until 7 h, followed by measurements at 12 h, 18 h and 24 h of exposure. After the 24-h measurement, cells were washed with an EDTA solution to remove the loosely bound Cu on the cell surface. The EDTA was prepared using 12 mM EDTA (with no Na^+) in a sucrose solution with the same osmolality as PBS. After removal of the EDTA solution, trypsin was added for 2 min (0.05% Trypsin-EDTA; Invitrogen®) to detach the cells. Detached cells were transferred into a 1.5-mL vial and centrifuged for 3 min at 360*g*. The supernatant was discarded and the pellet formed was analyzed for intracellular and paracellular properties as described below.

2.4. Gene of interest transcript abundance by quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cultured gill epithelia using Trizol Reagent® (Invitrogen®) according to the manufacturer's instructions. RNA concentration was determined using a Multiskan Spectrum UV/Vis microplate spectrophotometer (Thermo Fisher Scientific, Nepean, ON, Canada). A fixed quantity of RNA (2 μg) was treated with DNase I (Amplifications Grade, Invitrogen®) and used for cDNA synthesis. First-strand cDNA was synthesized using SuperScriptTM III reverse transcriptase and Oligo (dT)12–18 primers (Invitrogen®). SYBR Green I

Table 1
Primer sets, PCR annealing temperatures and amplicon size, and gene accession numbers for genes encoding carbonic anhydrase, Na^+-K^+-ATPase, tight junction proteins and elongation factor-1 of rainbow trout.

Intracellular proteins	Gene	Primer sequence (5′ – 3′)	Annealing temperature (°C)	Amplicon size (bp)	Accession number
Carbonic anhydrase	*ca-II*	F: AGGAGTCAATCAGCGTCAGC R: TCCTGCTTGGTGCTCCCATA	60	207	NM_001172549
Na^+-K^+-ATPase, subunit α1a	*nka* α1a	F: AGAAAGCCAAGGAGAAGATG R: AGCCCGAACCGAGGATAGAC	56	133	NM_001124461.1
Na^+-K^+-ATPase, subunit α1b	*nka* α1b	F: AGCAAGGGAGAAGAAGGACA R: GAGGAGGGGTCAGGGTG	59	178	NM_001124460.1
Zonula occludens-1	zo-1	F: AAGGAAGGTCTGGAGGAAGG R: CAGCTTGCCGTTGTAGAGG	60	291	HQ656020
Occludin	Ocln	F: CAGCCCAGTTCCTCCAGTAG R: GCTCATCCAGCTCTCTGTCC	58	341	GQ476574
Cingulin	Cgn	F: CTGGAGGAGAGGCTACACAG R: CTTCACACGCAGGGACAG	56	156	K008767
Claudin-8d	cldn-8d	F: GCAGTGTAAAGTGTACGACTCTCTG R: CACGAGGAACAGGCATCC	60	200	BK007966
Claudin-28b	cldn-28b	F: CTTTCATCGGAGCCAACATC R: CAGACAGGGACCAGAACCAG	60	310	EU921670
β-actin	*actb*	F: GGACTTTGAGCAGGAGATGG R: GACGGAGTATTTACGCTCTGG	58	354	AF157514
Elongation fator-1 alpha	*ef1α*	F: GGCAAGTCAACCACCACAG R: GATACCACGCTCCCTCTCAG	60	159	AF498320

F, forward.
R, reverse.

Supermix (BioRad Laboratories Canada Ltd., Mississauga, ON, Canada) and a Chromo4TM Detection System (CFB-3240; Bio-Rad Laboratories) were used for qRT-PCR analysis. The following reaction conditions were used: 1 cycle for denaturation (95 °C, 4 min), followed by 40 cycles of: denaturation (95 °C, 30 s), annealing (30 s) and extension (72 °C, 30 s). To ensure that a single PCR product was synthesized during reactions, a dissociation curve analysis was carried out after each qRT-PCR run. Transcript abundance of each gene of interest was normalized using rainbow trout β-actin (*actb*; see Table 1). The use of *actb* for gene of interest normalization was validated by statistically comparing *actb* threshold cycle values between control and Cu-exposed preparations to confirm that no statistically significant changes occurred ($p = 0.176$). Genes of interest examined were *ocln*, *cldn-8d* and *-28b*, *zo-1*, cingulin (*cgn*), *nka α1a* and *nka α1b* as well as *ca-II*. Primer sequences used were those previously published (see Chen et al., 2015; Kelly and Chasiotis, 2011; Kolosov et al., 2014), except for those used to amplify *ca-II* which was designed from identified coding sequence (accession number NM_001172549) using Primer3 software (v. 0.4.0). In this regard, a PCR reaction was carried out with stock rainbow trout gill cDNA, and amplicon size was verified using agarose gel electrophoresis. The putative *ca-II* amplicon was then isolated and purified using a QIAquick gel extraction kit (Qiagen Inc., Mississauga, ON, Canada) and subsequently sequenced (Bio Basic, Markham, ON, Canada). Sequence identity for the amplicon was confirmed using a BLAST search. All primer sequences, annealing temperatures, amplicon sizes and gene accession numbers are detailed in Table 1.

2.5. Model epithelium Cu accumulation and intracellular Na^+ content

Analysis of intracellular Cu content was performed as described by Nogueira et al. (2013). Pellets of detached exposed cells were counted using trypan blue assay and the number of cells used to normalize the data at the end of the analyses. Following that, cells were dried in an incubator (65 °C) overnight, digested with 50 μL of HNO_3 (Suprapur, Merck, St. Louis, MO, USA), and diluted with Milli-Q water as necessary. Measurements were performed by graphite furnace atomic absorption spectrometry (GFAAS, Varian SpectraAA-20 equipped with graphite tube atomizer [GTA-110], Mulgrave, Australia). Na^+ intracellular contents were measured in another round of experiments using the pellets of detached exposed cells. Pellets were sonicated to lyse the cells (Sonozap, Ultrasonic Processor, New York, NY, USA) and diluted with Milli-Q water as necessary for measurements by flame atomic absorption spectrometry (Varian SpectraAA-20, Mulgrave, Australia).

2.6. Model epithelium CA activity

Analysis of CA activity was performed by the method described by Henry (1991). This method is based on the catalysis of the hydration of the carbon dioxide present in a saturated CO_2 solution by the enzyme, with subsequent release of H^+ and consequent reduction of the solution pH. Pellets of detached cells were homogenized (50 μL) and added (10 μL) into the reaction solution (500 μL; composition in mM: 225 mannitol; 75 sucrose; 10 Tris Base; 10 Na_2HPO_4) with an aliquot of a CO_2-saturated solution (100 μL). The pH was measured every 15 s up to 1 min. The enzyme activity was calculated based on the slope of the linear regression for the pH change over time and the protein content in the cell homogenate. Protein concentration in the cell homogenate was determined using a commercial reagent based on the method of Bradford (1976). The specific enzyme activity was expressed as arbitrary unit of CA (enzyme units/mg protein).

2.7. Statistical analyses

Each endpoint (Na^+ content, Cu accumulation, CA activity and gene expression) was evaluated in a separate set of experiments ($N = 8$ for each of 0, 200 and 1000 μg/L Cu treatments). Data were expressed as mean ± standard error. One-way analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$) was performed for comparisons among treatments. ANOVA assumptions (data normality and homogeneity of variances) were previously verified and no data transformations were necessary.

3. Results

3.1. Transepithelial resistance (TER) measurements

TER had reached stability by 7 days (175 h; Fig. 1) when the apical medium was replaced with sterile, temperature-equilibrated freshwater of the same composition as in the husbandry conditions. The TER values increased immediately in control and 200 μg/L Cu treatments but briefly dropped significantly at 1000 μg/L Cu (176 h). After the first

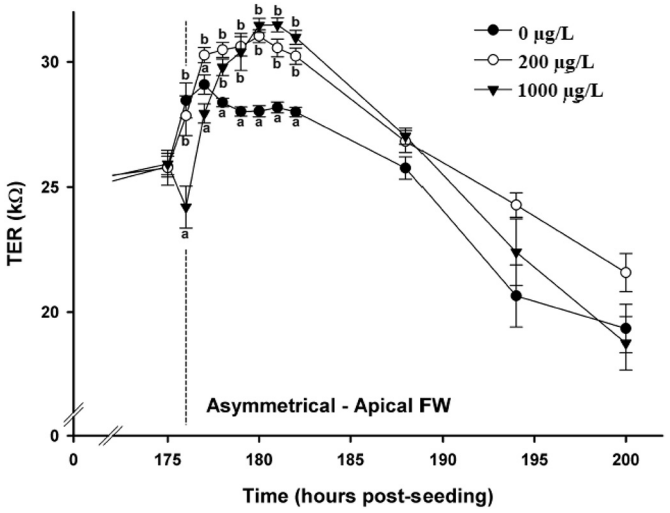

Fig. 1. Measurements of the transepithelial resistance (TER) of DSI cultured gill epithelia of rainbow trout at time 0 h (175 h) until the end of 24 h of exposure (200 h) to Cu. Treatments were 0 µg/L, 200 µg/L, and 1000 µg/L. Different letters indicate significantly different mean values among treatments ($p < 0.05$).

hour (177 h), the 1000 µg/L Cu treatment TER rebounded so that it was similar to that of the control, but still significantly different from the 200 µg/L Cu TER. Over the course of the next 5 h, the TER values in the control group stabilized, whereas those in the two Cu treatments continued to increase to maximum values, and were significantly different from the control. After 12 h of exposure, TER values were similar in all three treatments and remained without significant difference until the end of the experiment.

3.2. Transcript abundance of genes encoding TJ proteins

There were no significant differences in the mRNA abundance of *zo-1* (Fig. 2a), *ocln* (Fig. 2b), *cldn-8d*d (Fig. 2c), *cldn-28b* (Fig. 2d) or *cgn* (Fig. 2e) among the three treatment groups, measured at 24 h of exposure (Fig. 2).

3.3. Cu accumulation, intracellular Na^+ and transcript abundance of nka α1a, nka α1b and ca-II

The cultured DSI gill epithelia exposed to 200 and 1000 µg Cu/L did not exhibit differences in their Na^+ content compared to control, measured at 24 h of exposure (Fig. 3a). However, a significant Cu accumulation (5.5-fold; Fig. 3b) in concert with a significant decrease of carbonic anhydrase activity (76%; Fig. 3c) was observed at 1000 µg Cu/L. No significant difference in *ca-II* (Fig. 3d), *nka α1a* (Fig. 3e) or *nka α1b* (Fig. 3f) mRNA abundance was observed between control and Cu-treated preparations.

4. Discussion

We are able to accept our hypothesis that Cu exposure would result in intracellular Cu accumulation in DSI preparations and that Cu exposure would disturb the electroresistive properties of the cultured model. However, after a brief initial drop in TER in the 1000 µg Cu/L treatment, there were increases in TER (at least in the short term for a number of hours), in the Cu-exposed preparations, which is counter to what would be the expected observation if paracellular permeability had been permanently compromised. Furthermore, intracellular Na^+ content did not differ between control and treated epithelia and no transcriptional changes were observed in genes encoding TJ proteins or those associated with transcellular ion transport, all of which oppose our original hypothesis. Nevertheless, a decrease in CA activity at the end of 24-h exposure was observed, so the cultured model does appear to reflect several conditions reported to be associated with Cu exposure of the intact gill.

TER is an indicator of overall tightness of the model gill epithelium, a function of both transcellular and paracellular resistance (Wood et al., 2002b). In our study, the DSI epithelium of rainbow trout presented TER values around 25 kΩ.cm^2 in the final measurements under symmetrical conditions (around 6–8 days of culture), and exhibited a modest increase when the apical L-15 medium was replaced by fresh water (absence of Cu). These high TER values in symmetrical conditions, with a modest increase, followed by a gradual decline upon apical exposure to fresh water, are typical of DSI epithelia and, at least in part, are thought to reflect the presence of mitochondria-rich cells (Fletcher et al., 2000). Both levels of Cu exposure resulted in larger increases in TER following FW exposure than in the control treatment during the first 6 h of fresh water presence. The higher concentration (1000 µg Cu/L) actually caused a biphasic effect – an initial immediate drop, followed by the greater increase at 2 h to 6 h. However, there were no significant differences between the three treatments from 12 h onwards. These observations suggest some disruptive effects of Cu on permeability, quickly followed by compensation or repair. By way of comparison, Smith et al. (2001) using SSI preparations (*i.e.* epithelia comprising only pavement cells) reported that TER was unaffected by 1600 µg/L Cu exposure, but their single measurement occurred after 24 h of Cu exposure which is similar to our finding at this time.

Although alterations in TER values in the first 6 h of exposure may be related to the modification of paracellular integrity, no changes in transcript abundance of select TJ proteins were observed in samples taken at 24 h. This may not be surprising given that permeability (as measured by TER) of control and Cu-treated preparations was not significantly different at this time. However, the absence of a transcriptional response does not necessarily imply a lack of change in abundance of these proteins, a point reinforced by our results with CA discussed below. Although we observed an absence of Cu effects on mRNA abundance of TJ proteins in the DSI cultured gill epithelium of rainbow trout, Wang et al. (2015) observed a decrease in mRNA abundance of gill *ocln*, *zo-1*, and some other TJ proteins in grass carp exposed *in vivo* to a comparable concentration of Cu (700 µg/L), but for a much longer time (96 h). The authors associated the decrease of TJ protein mRNA abundance with oxidative stress. Therefore, future studies should examine temporal changes in both transcript and protein abundance of model gill epithelia exposed to Cu.

At the end of 24-h exposure of the DSI cultured epithelium to 1000 µg Cu/L, a significant intracellular Cu accumulation occurred. Cu may have entered through an apical Na^+ channel, a Cu-specific channel called CTR1, or *via* the divalent metal transporter DMT1 (Handy et al., 2002; Grosell and Wood, 2002; Grosell, 2012). However, the classic pattern of Na^+ depletion associated with Cu accumulation *in vivo* was not observed. Cu is considered an ionoregulatory toxicant in aquatic organisms, out-competing Na^+ for sites of ion transport on the apical and basolateral surfaces of gill ionocytes, in addition to increasing paracellular permeability (Grosell et al., 2002; Grosell, 2012). However, it must be remembered that Na^+ loss in fish exposed to Cu *in vivo* occurs from the extracellular compartment, whereas we measured Na^+ content in the intracellular compartment of the cultured epithelium. We are aware of no information of Cu effects on gill intracellular ion concentrations *in vivo*. However, Smith et al. (2001), using SSI cultured gill epithelia, similarly reported no depletion of intracellular Na^+ content by Cu levels greater than those used here. Smith et al. (2001) did note a relationship between depressed O_2 consumption of the preparation and depressed intracellular Na^+ content, but again the Cu threshold (1600 µg/L) for effects on O_2 consumption was higher than that those tested in the present study.

Cu exposure (1000 µg Cu/L, but not 200 µg Cu/L) did cause a marked inhibition of carbonic anhydrase activity, a known sensitive

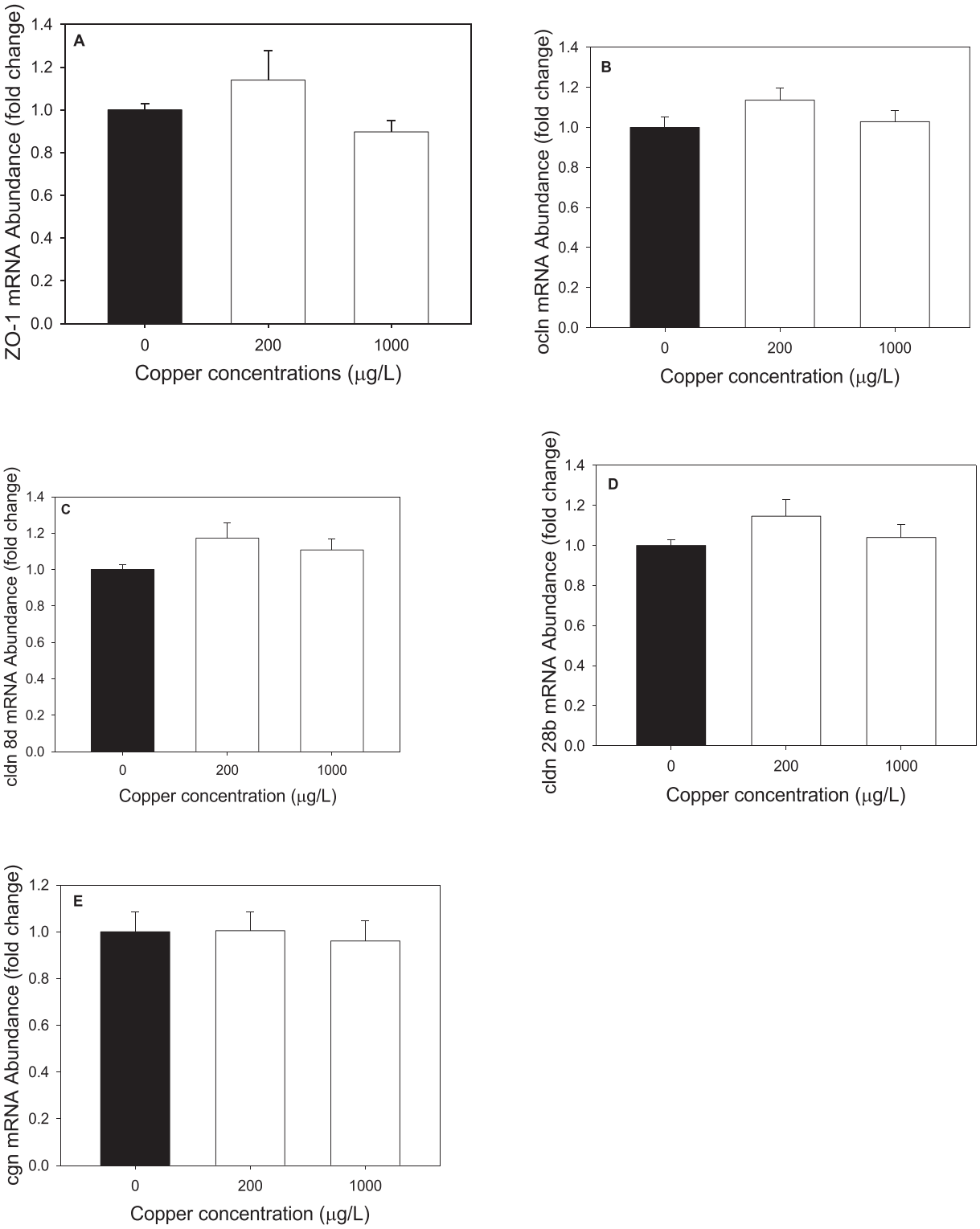

Fig. 2. Extracellular responses of DSI cultured gill epithelia of rainbow trout to 24 h of Cu exposure. Treatments were 0 μg/L, 200 μg/L, and 1000 μg/L. Responses were analyzed through gene expression (mRNA abundance) of (A) zonula occludens-1 (*zo-1*), (B) occluding (*Ocln*), (C) claudin-8d (*cldn-8d*), (D) claudin-28b (*cldn-28b*), and (E) cingulin (*Cgn*). Different letters indicate significantly different mean values among treatments ($p < 0.05$).

marker of trace metal exposure (Lionetto et al., 2012; Lionetto et al., 2016; Ali et al., 2017). This is the first time that this has been seen in cultured fish gill epithelia, but CA inhibition by Cu has been seen previously in a number of studies on invertebrates (see Introduction), and in one *in vivo* study on fish (guppies; Zimmer et al., 2012). As CA is intimately involved in Na^+ and Cl^- transport (by providing the counter ions H^+ and HCO_3^-), ammonia excretion (in providing H^+ for NH_3 trapping) and of course in acid-base regulation, its inhibition by Cu is thought to play an important role in Cu toxicity *in vivo* (Grosell, 2012). However, the absence of an effect of Cu on CA at 200 μg Cu/L will require further study. One possible explanation may relate to the presence and Cu-induced actions of metallothionein (MT), which at this

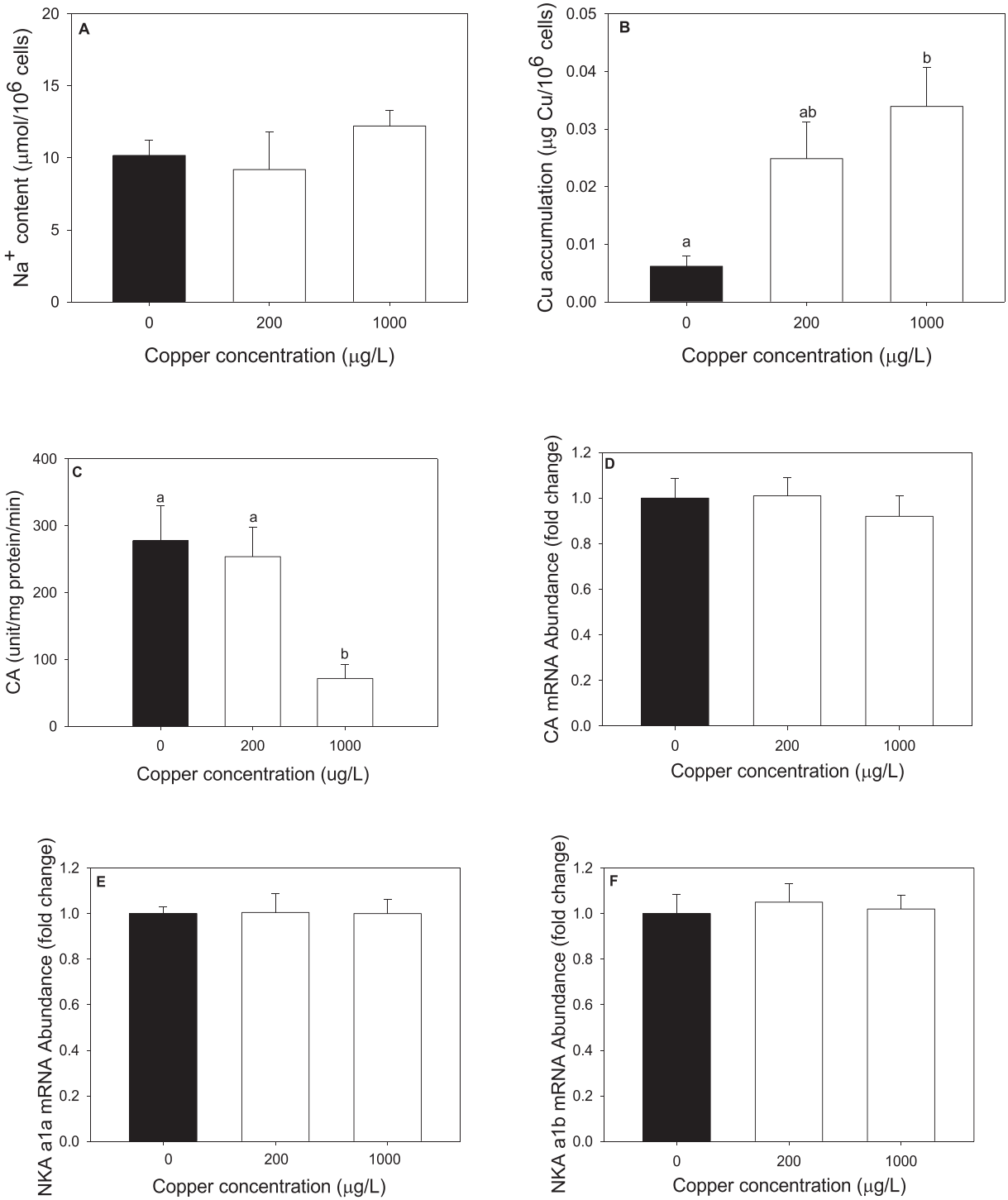

Fig. 3. Intracellular responses of DSI cultured gill epithelia of rainbow trout to 24 h of Cu exposure. Treatments were 0 μg/L, 200 μg/L, and 1000 μg/L. The parameters analyzed were (A) Na^+ content, (B) Cu accumulation, (C) carbonic anhydrase activity, and gene expression (mRNA abundance) of (D) carbonic anhydrase (*ca-II*) (E) Na^+-K^+-ATPase, subunit α1a (*nka α1a*), and (F) Na^+-K^+-ATPase, subunit α1b (*nka α1b*). Different letters indicate significantly different mean values among treatments ($p < 0.05$).

lower dose may be capable of Cu detoxification through a metal-binding pathway. Notably, transcript abundance of MT has been reported to increase in a cultured trout gill model following exposure to Cu (Walker et al., 2007).

Despite the decrease in CA activity at 24-h exposure to 1000 μg Cu/L, we observed no change in the *ca-II* mRNA abundance at this time. The gene encoding *ca-II* is metal-, tissue- and species-specific (Ali et al., 2017). The up- and down-regulation of *ca-II* mRNA abundance in the freshwater-acclimated sheepshead minnow exposed *in vivo* to a range of Cu and salinities depended on the metal concentration, time of exposure, and the interactive effects of two stressors (Cu and salinity) (de Polo et al., 2014). We also saw no changes in the mRNA abundance of *nka α1a* and *nka α1b*, even though the Na^+/K^+ATPase enzyme is a well-known target of Cu (*e.g.* Laurén and McDonald, 1987; Li et al., 1998). Again, the lack of a transcriptional response does not necessarily mean the absence of change in activity and abundance of the enzyme;

indeed many investigations have shown that Cu inhibits Na^+/K^+ATPase activity, but there are few transcriptional studies on Cu effects on this enzyme (Grosell et al., 2012). Again, the study of de Polo et al. (2014) on sheepshead minnow is relevant, where there were no changes in *nka* mRNA abundance in a variety of Cu exposure scenarios, similar to the present results.

5. Conclusion

Results from the present study on a reconstructed trout gill epithelium in primary culture allowed us to consider paracellular and intracellular responses to Cu exposure. It is important to note that this is the only *in vitro* cell culture preparation which allows apical exposure to metals under conditions that are close to those *in vivo* – *i.e.* a robust response in the presence of apical fresh water. Importantly, the DSI system methodology was able to demonstrate the sensitivity of CA activity to inhibition by Cu, despite a lack of change in the mRNA abundance of *ca-II* after 24 h of exposure. Although Cu also had no effect on mRNA abundance of genes encoding select TJ proteins at 24 h, the significant differences in TER values between control and Cu treatments in the first 6 h of exposure, followed by return to control values thereafter suggest that some changes in paracellular permeability occurred. These deserve further attention in the future studies. Both the DSI methodology as an *in vitro* replacement for live fish tests and the use of CA inhibition as an endpoint may be useful for future mechanistic investigations and environmental monitoring.

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Declaration of competing interest

None.

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