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Zinc uptake in fish intestinal epithelial model RTgutGC: Impact of media ion composition and methionine chelation

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ABSTRACT

Apical uptake of zinc as ionic Zn(II) or as Zn-methionine (Zn-Met) was studied in RTgutGC cell line *in vitro* under media compositions mirroring the gut luminal ionic concentration of freshwater (FW) and seawater (SW) acclimated salmonids. Viability of the RTgutGC cells exposed to experimental media preparations showed a time-dependent decrease in SW treated cells, with the effect being significant at 48 h ($P < 0.01$), but not at 12 h or 24 h. Half effective concentration of Zn exposure over 12 h (EC_{50}, in μM) was not differentially affected by media composition (FW, 59.7 ± 12.1 or SW, 83.2 ± 7.2; mean ± SE, $P = 0.43$). Zinc (^{65}Zn) influx in RTgutGC was not different between FW or SW treated cells, but increased significantly in the presence of methionine (2 mM, L-Met or DL-Met). An interaction effect was observed between Zn concentration and media ionic composition on the impact of Met on apical Zn uptake (L-met, $P < 0.001$; DL-met, $P = 0.02$). In the presence of Met, apical Zn uptake in SW medium was significantly lower compared to FW, but only at higher Zn concentrations (12 and 25 μM, $P < 0.01$). Further, Met facilitated Zn uptake was reduced in cells treated with an amino acid transport system blocker with the effect being more significant and stereospecific in SW ionic conditions. The findings of this study showed that (i) Zn speciation in the presence of Met improved apical Zn uptake in RTgutGC cells and Zn-Met species were possibly taken up through Met uptake system. (ii) The effect was differentially affected by the ionic composition of the medium. Implications and limitations of the observations towards practical Zn nutrition of salmonids are discussed.

1. Introduction

While essential as a nutrient, zinc (Zn) is also a potential toxicant and an environmental contaminant of concern [1]. Although fish are able to acquire waterborne Zn via gills, diet is regarded to be the major source of Zn [2]. Knowledge on gastrointestinal (GI) uptake of Zn is therefore significant in fish nutrition and aquatic toxicology [3]. The GI tract is a highly versatile and multi-functional organ in fish [4]. In addition to the primary function of nutrient uptake, the GI tract also serve osmoregulatory functions [5]. The ionic composition of the gut luminal content in seawater (SW) fish varies from that of freshwater (FW) fish due to the fact that marine fish drink and selectively precipitate ions to facilitate water uptake in the hyperosmotic seawater medium [6]. To date, knowledge on the impact of ionic composition of the gut luminal contents on nutrient uptake at the intestinal epithelium is limited. Impact of lumen composition on the GI uptake of Zn was found to be complex and affected by interactions with other ions and ligands at various stages of absorption [3,7].

In aquaculture, formulated fish feeds are supplemented with Zn additives, as the bioavailability of endogenous Zn in feed ingredients is low due to the presence of anti-nutritional factors [8]. The Zn additives used can be categorised into inorganic salts and chelated forms. Inorganic Zn salts of sulphate, chloride or oxide are used, where sulphate is the most studied and relatively more bioavailable form of inorganic Zn to fish [9]. Among chelated forms, Zn chelated with specific amino acids eg. methionine [10,11], glycine [12] or a mix of amino acids have been studied [13,14]. In the aforementioned studies, the chemical form of Zn additive and interactions anti-nutrients have been the focus of investigation towards enhancing bioavailability of dietary Zn [8,9]. In some of these studies, dietary supply of amino acid chelated Zn was found to be more bioavailable than inorganic salts of Zn [11,14]. Nevertheless, results are inconsistent and subject to high variability

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[9,15], such that it remained inconclusive even in a radiotracer study [16]. Physiological studies on Zn uptake in fish using *in vitro* brush border vesicles [17] and *in vivo* perfusion models [3] have revealed that amino acids with high binding affinity for Zn(II) can improve Zn uptake, but the mechanism for which is less understood.

Similar to mammals, GI uptake of Zn in fish is also believed to be orchestrated by more than one transport system involving solute carrier families Slc30 (Znt) and Slc39 (Zip); and potentially L-Type Calcium Channel (LTCC) and divalent metal transporter-1(DMT1) [7,18]. In the intestine, dietary Zn binds to the mucus of the intestinal epithelium, and is transported into the epithelial cells either as the Zn(II) ion or bound to amino acids [1,15]. Uptake of Zn(II) through Zip4 is of vital nutritional significance in mammals; however, the relative efficiency and functional importance of Zip mediated Zn(II) uptake versus amino acid facilitated Zn uptake in GI tract remains to be well understood in mammals, and even more so in fish [1]. The complexity of the environment and multiple dietary interactions have been major constraints in understanding the limiting factors of dietary Zn bioavailability in fish nutritional studies. In this regard, strengthening our knowledge on uptake mechanisms is required to better understand and predict dietary metal bioavailability from feed matrices to fish under varying environmental conditions [19,20].

Until recently, enterocyte cell models were not available to study nutrient uptake mechanisms for fish nutrition research. However, now an intestinal epithelial cell line (RTgutGC) exhibiting apical and basolateral characteristics has been established [21,22]. RTgutGC cell line has been proposed as a physiologically adequate fish intestinal epithelial model, equivalent to the Caco-2 cell line for human intestinal epithelium [22]. Since then, RTgutGC cells have been well characterised with structural and functional features like forming a monolayer, mucous secretion, tight junction and desmosomes formation between adjacent cells, develop trans-epithelial resistance and polarize over time to exhibit epithelial characteristics [21,23,24]. RTgutGC cells have been used to study metal uptake characteristics for environmental monitoring of potential metal toxicants like silver and its nanoparticles [23,25,26]. However, uptake of nutritionally relevant metals have not been investigated in the RTgutGC model. In this study, we examined the apical uptake of Zn and Zn-Met species as affected by media composition mirroring the luminal ionic concentrations found in freshwater (FW) and seawater (SW) acclimatised salmonids using the RTgutGC cell line.

2. Material and methods

2.1. Cell culture

RTgutGC cells (obtained in kind from Professor Dr. Kristin Schirmer, Dept. of Environmental Toxicology, Eawag, Swiss Federal Institute of Aquatic Science and Technology, Switzerland) were aseptically cultured in Leibovitz' L-15 medium (Invitrogen/Gibco, Switzerland) containing 5% fetal bovine serum (FBS, Eurobio, France) and 1% gentamycin sulfate (BioWhittaker™/Lonza, Belgium) at 19 °C in normal atmosphere as previously described by [22]. The cells were routinely grown in 75 cm^2 cell culture flasks and when confluent after 2 weeks, they were either split to new flasks or harvested to be used in experiments. Cells from the confluent flasks were harvested by washing twice with 1 ml Versene EDTA solution (Invitrogen/Gibco, Switzerland) and detached using 0.7 ml of trypsin (0.25% in phosphate-buffer saline, PBS, Biowest, Nuaille´, France). The trypsin reaction was stopped by adding 10 ml of L15/FBS medium. The resulting cell suspension was centrifuged at 1000 rpm, 19 °C for 3 min. The density of the harvested cells was estimated by manual counting using haemocytometer. The cells were diluted to required volume in L15/FBS and seeded to each well in 24 well plates (Falcon™ Polystyrene Microplates) at a density of 5×10^4 cells $well^{-1}$ and incubated at 19 °C for 48 h prior to experiments.

Table 1
Chemical and ionic composition of the experimental media tested.

Chemical composition (mM)	L15/ex	Freshwater (FW)	Seawater (SW)	Values from literature reports	
Sodium nitrate	155	155	155		
Potassium nitrate	6.2	6.2	6.2		
Magnesium sulfate	3.8	19.5	51.1		
Calcium nitrate	1.5	5.4	5.4		
HEPES	5.0	5.0	5.0		
Magnesium chloride	–	15.0	44.9		
Sodium pyruvate	5.7	5.7	5.7		
Galactose	5.7	5.7	5.7		
pH	7.1	7.4	7.4		
Ionic strength	178.0	258.0	400.0		
Ionic composition (mM)				FW‡	SW§
Calcium, Ca^{2+} *	1.6 ± 0.1	5.3 ± 0.2	5.1 ± 0.2	2 - 4	60 - 185
Magnesium, Mg^{2+} *	3.9 ± 0.3	32.5 ± 0.7	89.4 ± 2.5	15 - 25	50 - 135
Potassium, K^+ *	8.2 ± 1.2	8.6 ± 1.1	7.6 ± 1.3	8 - 10	3 - 14
Sodium, Na^+ *	160 ± 3	157 ± 2	153 ± 3	100 - 150	100 - 225
Nitrate, NO_3^- **	164	172.4	172.4		
Sulfate, SO_4^- **	3.8	18.7	48.6		
Chloride, Cl^- **	1.5	31.5	94.5	25 - 35	60 - 120

*analysed (n = 3); **nominal. L15/ex, adapted from [29]; FW and SW, conceptually formulated to mimic ionic composition of intestinal luminal fluid in freshwater [27,28]‡ and seawater [6] § acclimated salmonids. Ionic strength was calculated using the software Visual MINTEQ.

2.2. Exposure media composition

Two experimental media (i) FW and (ii) SW were conceptually designed from [27,28,6] to closely represent the luminal ionic composition of the freshwater (FW) and seawater (SW) acclimated salmonids, respectively (Table 1). One other medium (L15/ex), adapted from [29], was used as reference to test the viability of cells when treated with experimental media compositions. The composition of the reference exposure medium was based on the ionic concentration of complete L-15 medium used to culture the RTgutGC cells without amino acids or serum and was shown to be able to maintain viability of the cells up to 72 h [21]. The nominal and analysed ionic concentrations of exposure media are presented in Table 1. The concentrations of ions were analysed using a PE NexION 350D ICP-MS instrument following the method as described in [25].

2.3. Cell viability assay

The metabolic activity of the cells, measured with the Alamar blue assay, was used to indicate cell viability. Cell suspension in complete L15/FBS was seeded to 96-well plates at a density of 4×10^4 cells $well^{-1}$ and incubated at 19 °C for 24 h before exposure to different experimental media. After 24 h, the L15/FBS medium was removed from each well, rinsed twice with phosphate buffered saline (PBS) and treated with L15/ex, FW or SW medium with total nominal zinc concentrations of 0, 25, 50, 100 and 150 μM; the respective analysed Zn concentrations were 0.2, 25.2, 52.1, 105.8 and 158.3 μM. The viability of cells was examined at 12 h, 24 h and 48 h post-exposure to experimental media by incubating in dark for 1 h at 19 °C with the Alamar blue reagent (10 μL $well^{-1}$) and absorbance recorded at 570 nm, with 600 nm as a reference wavelength using a spectrophotometric plate reader.

2.4. Zinc (^{65}Zn) influx assays

2.4.1. Effect of ionic composition of the medium

The RTgutGC cells were seeded to 24 well plates at a density of 5×10^4 cells $well^{-1}$ in complete L15/FBS medium, and incubated at 19 °C for 48 h. Subsequently, the medium in the wells were removed, rinsed thoroughly with PBS, treated with respective experimental medium and allowed to acclimatise for 20 min. Later, the cells were treated with the same medium with $^{65}Zn(II)$ (as $ZnCl_2$; approx. 4 kBq/ml; Perkin-Elmer, USA) added at different concentrations (added as $ZnSO_4$ solution, molecular biology grade, Sigma) and incubated at 19 °C for 15 min. The medium was then recovered from the well, rinsed with ice cold medium (with 200 µM Zn, pH 7.4) and quench buffer (with 5 mM EGTA, pH 7.4) for 5 min to remove any adsorbed $^{65}Zn(II)$. The monolayer of cells adhered to the bottom of the wells were digested with 100 µl of 2% hot SDS detergent and the cellular material recovered completely. The cell digests were then counted for radioactivity using 1282 Compugamma Laboratory Gamma Counter, LKB Wallac. The counts per minute (cpm) obtained were corrected for background activity and radioactive decay, and converted using specific activity calculations following the formulae of Glover and Hogstrand [3]. In each treatment group, 6 wells were used; 3 wells were exposed to ^{65}Zn containing medium, whereas 3 others were exposed to equivalent concentrations of Zn from $ZnSO_4$ only. The latter were used for determining the protein concentration of cells (after homogenisation with 500 µl of 0.5 M NaOH) using Bradford assay kit (Bio-Rad) with BSA as the standard. The rate of uptake was expressed as pmoles Zn min^{-1} mg^{-1} protein.

2.4.2. Impact of methionine on Zn uptake

Zn uptake by RTgutGC cells were examined in FW and SW media compositions in the presence of L-methionine (L-Met, Sigma) or DL-methionine (DL-Met, Alfa Aesar). The cells were exposed to nominal concentrations of 3.07, 6.14, 12.27 and 24.55 µM $^{65}Zn(II)$ in FW and SW media (i) without amino acids (control) or with 2 mM of (ii) L-Met or (iii) DL-Met. The pH of all experimental media preparations were adjusted to 7.4 using 0.5 M NaOH. The preparation of cells prior to experimental exposure and the assay conditions were as described in 2.4.1. The exposure period was 15 min and experiments were performed in triplicate (n = 3) with three technical (well) replicates per experiment. The influx of $^{65}Zn(II)$ was calculated as described in 2.4.1.

2.4.3. Impact of amino acid transport inhibitor on zinc uptake

Cells were seeded in 24 well plates as described in 2.4.1 and exposed to FW or SW medium with a nominal concentration of 10 µM $^{65}Zn(II)$ either without amino acids (i) or with 2 mM of (ii) L-Met, (iii) D-methionine (D-Met, Sigma) or (iv) DL-Met. The exposure to the above media preparations were made in the presence or absence of 10 mM of an amino acid transport inhibitor (2-Aminobicyclo [2.2.1] heptane-2-carboxylic acid, BCH, Sigma). The exposure period was 15 min and assay conditions were as described in 2.4.1. The experiment was performed in triplicate (n = 3) with three technical (well) replicates each time.

2.4.4. Data analyses

The data presented in this manuscript are mean of three repeated observations (n = 3), analysed using GraphPad Prism version 7 for Windows, GraphPad Software, California, USA. Data on EC_{50} for Zn exposure in FW and SW medium were estimated using a four parameter, variable slope model. The test of significance between EC50 in FW and SW media was obtained through fit-comparison option available in GraphPad Prism. Rest of the data were analysed by two-way ANOVA followed by Tukey's multiple comparison test; whenever the interaction effect was significant, one-way ANOVA was employed to make group-wise comparisons using Tukey's multiple comparison test.

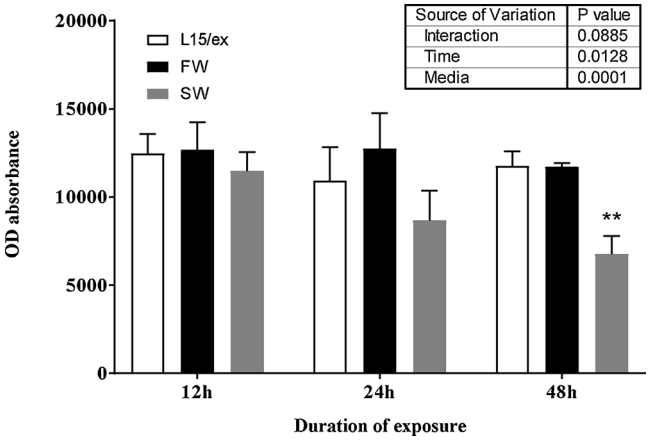

Fig. 1. Viability of RTgutGC cells (measured with Alamar blue assay) exposed to FW (black bar) and SW (grey bar) experimental media relative to L15/ex (white bar) as reference medium. Data are represented as mean ± SD (n = 3) of 12, 24 and 48 h exposure periods. Two-way ANOVA showed significant effect of both media composition ($P < 0.01$) and time ($P < 0.05$). Asterisk (*) indicates significant difference at each time point as obtained through Tukey's multiple comparison test (**, $P < 0.01$) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3. Results

3.1. Cell viability assay

The metabolic activity of the cells exposed to the FW and SW media compositions were on par with the cells exposed to the reference medium (L15/ex) at 12 h, and declined thereafter in SW treated cells in a time-dependent manner (Fig. 1). The exposure to increasing concentrations of Zn in the medium reduced the metabolic activity of the cells in a dose dependent manner (Fig. 2). Analysis of the cell viability data using a four parameter, variable slope model indicated that the EC_{50} for Zn (as µM, mean ± SE) was not significantly different between RTgutGC cells exposed to FW (59.7 ± 12.1) or SW (83.2 ± 7.2) media (P = 0.43).

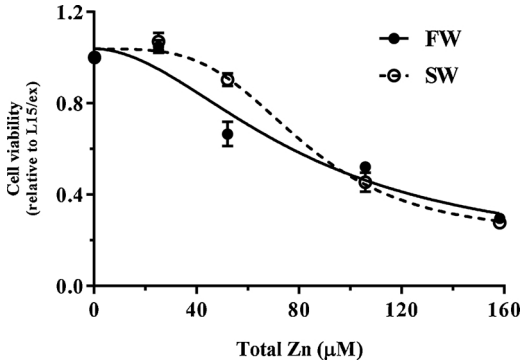

Fig. 2. Concentration dependent decrease in metabolic activity of RTgutGC cells exposed to total zinc concentrations from 0 to 160 µM in FW (filled circle, solid line) or SW (open circle, dashed line) after 12 h exposure, normalised to L15/ex. Four parameter, variable slope model used to calculate EC_{50} for Zn (mean ± SE, in µM) in RTgutGC cells exposed to FW (59.7 ± 12.1) and SW (83.2 ± 7.2) media, however the difference was not statistically significant (P = 0.43). The test of significance was performed in GraphPad Prisim by the option to compare best-fit values of specific parameter (EC_{50}) between two data sets.

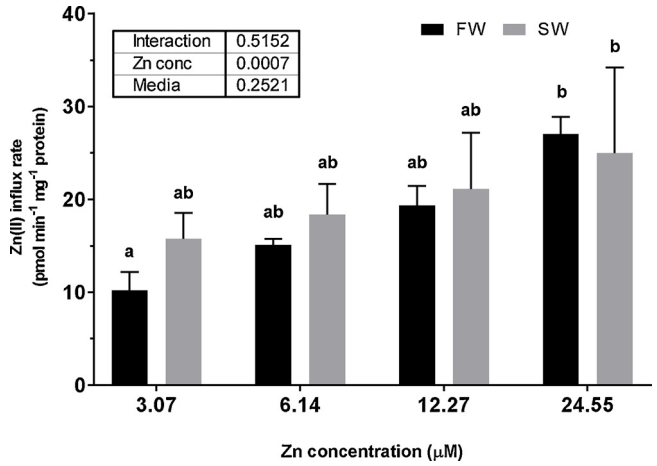

Fig. 3. Impact of ion composition in the medium on Zn influx in RTgutGC cells (mean ± SD; n = 3): Zn influx under FW (black bars) and SW (grey bars) media. Data were analysed through two-way ANOVA, followed by Tukey's multiple comparison test. The P-values of ANOVA are presented in insets and the post-hoc differences among groups are represented as superscript letter above the bars. Bars with different are statistically different ($P < 0.05$).

3.2. Effect of media composition and methionine chelation on apical Zn uptake

The influx of ^{65}Zn increased with increasing Zn concentration in the media ($P < 0.001$). The difference in the ion composition between FW or SW media did not have a significant impact the influx of ^{65}Zn in RTgutGC cell line (Fig. 3). Methionine (L- or DL-) inclusion in the media at 2 mM concentration significantly influenced the apical influx of Zn in RTgutGC cells (Fig. 4). Two-way ANOVA showed significant interaction effect (L-met, $P < 0.001$; DL-met, $P = 0.02$) between Zn concentration and media ionic composition on the impact of methionine on Zn uptake. Post-hoc comparison following one-way ANOVA showed that Zn uptake in the presence of methionine (L-Met or DL-Met) was significantly lower in SW treated cells at higher Zn concentrations (12 and 25 μM) ($P < 0.05$). Cells exposed to amino acids along with the amino acid transporter blocker (BCH) showed a significant ($P < 0.001$) reduction in ^{65}Zn uptake when compared to cells untreated with BCH. The effect was more pronounced and stereospecific in SW than in FW conditions (Fig. 5).

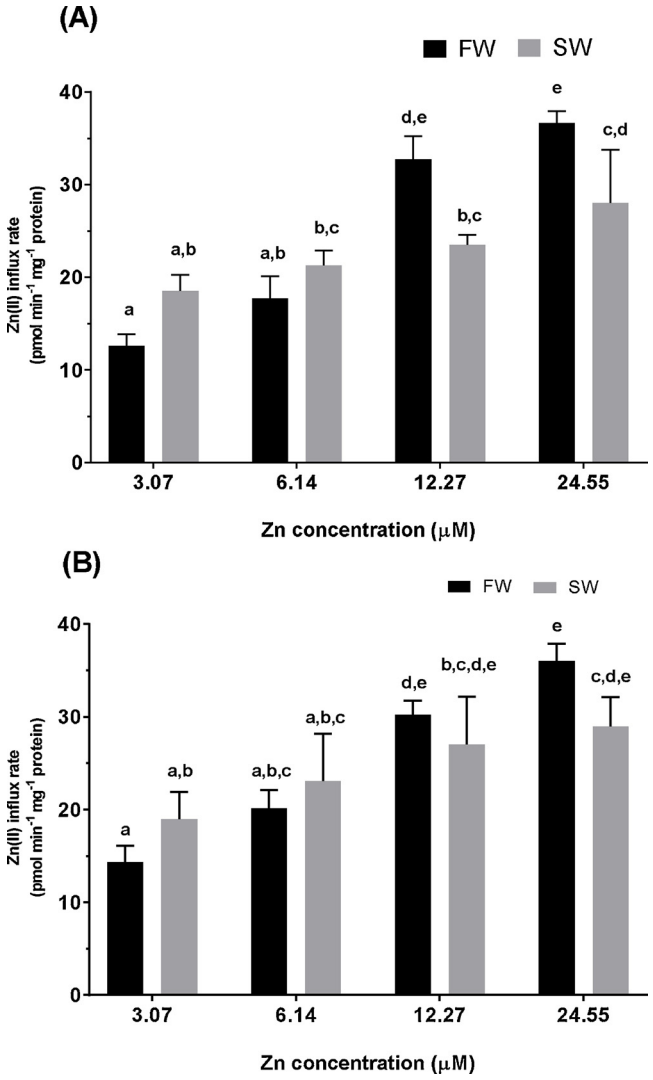

Fig. 4. Impact of methionine chelation on Zn influx in RTgutGC cells (mean ± SD; n = 3): Zn influx under FW (black bars) and SW (grey bars) medium (A) with 2 mM L-methionine; (B) 2 mM DL-methionine. Data were initially analysed by two-way ANOVA, which showed a significant interaction effect. Therefore, one-way ANOVA was performed followed by Tukey's multiple comparison test. The post-hoc differences among groups are represented by superscript letter above the bars. Bars with different letters differ significantly ($P < 0.05$).

4. Discussion

Ionic composition in the gastrointestinal (GI) lumen of fish can vary depending on the environment (salinity induced hypo- or hyper-osmoregulation), feeding status (time after a meal) and diet composition (ion concentration of the diet). In rainbow trout, *in vitro* metal uptake studies have used artificial saline preparations to closely mimic the ionic composition found in the GI lumen [30–33]. However, large variations seem to exist in the Ca^{2+} and Na^{+} concentrations between the artificial saline and actual measurements in rainbow trout. The Na^{+} concentration in the intestinal luminal content of rainbow trout 10–20 h post-meal and 24 h after sudden change to seawater varies from about 100–225 mM [6]; whereas, in the mucosal saline used for the *in vitro* studies the Na^{+} concentration ranges between 0 and 60 mM [30–33]. Similarly, the Ca^{2+} concentration in the mucosal saline used in studies in rainbow trout [33] and other marine/seawater adapted fish was about 5 mM [30–32]; whereas, in intact marine fish, the luminal content was reported to vary from 60 to 185 mM [6]. Higher Ca^{2+} levels tend to precipitate chloride and sulfate, and hence it might be a methodological consideration with *in vitro* studies to use less Ca^{2+}. Moreover, in marine fish, the high Ca^{2+} entering the GI lumen through drinking of seawater is precipitated as calcium carbonate aided by Ca^{2+} induced bicarbonate secretions from the intestinal epithelium [34]. In this context, it is not known if bicarbonate secretion is active in RTgutGC cell line and this merits further investigation. In the present study, although the Na^{+} concentrations were closer to the range of those found in seawater living rainbow trout during digestion of a meal [6], the Ca^{2+} concentrations were considerably lower (see Table 1). The effect of luminal Ca^{2+} on intestinal zinc uptake is complex; it stimulated epithelial Zn uptake in rainbow trout, but inhibited post-intestinal accumulation of Zn [7]. It is therefore possible that the difference in Ca^{2+} concentrations used in the present study and that in the seawater rainbow trout intestine influenced our results but it is difficult to predict the directionality of this uncertainty.

Zinc transport across cellular and intracellular membranes takes place through Zn transport proteins (Zip and Znt) [18]. Transport of Zn into the cytosol is mediated by members of the slc39 (Zip) transporter family, while movement of Zn away from the cytosol, either into organelles or out of the cell, is achieved through members of the slc30 (Znt) transporter family [35]. In mammals, Zip1–6 and Zip14 are

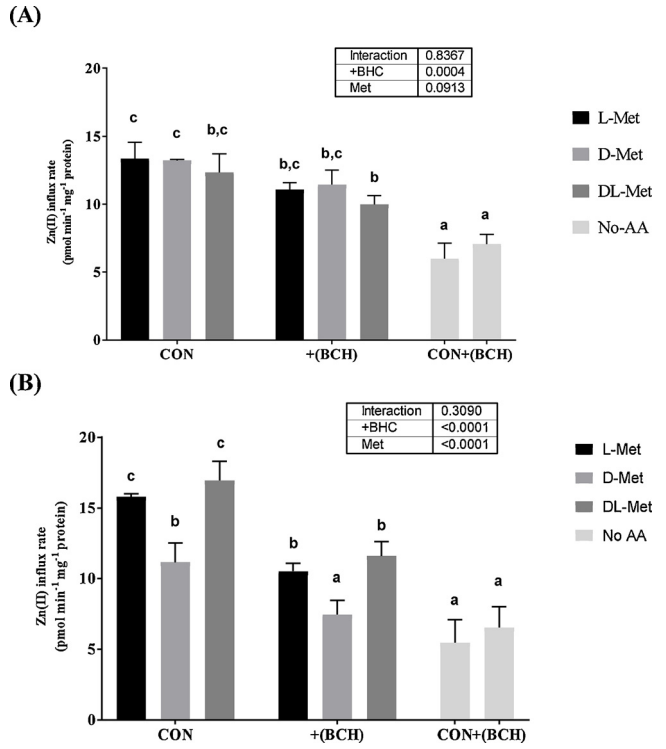

Fig. 5. Impact of amino acid transport inhibitor (2-Aminobicyclo [2.2.1] heptane-2-carboxylic acid, BCH, 10 mM) on zinc influx in RTgutGC cells exposed to FW (A) and SW (B) medium. L-methionine (L-Met), D-methionine (D-Met) or DL-methionine (DL-Met) added at 2 mM concentrations. No-AA (without methionine, negative control); CON, control with methionine; + BCH, with BCH. Data presented as mean ± SD (n = 3) analysed using two-way ANOVA, with Met and BCH as main effects (No-AA treatments were not included in the two-way ANOVA model). P-values of the main effects and their interaction are provided as insets. The differences between groups as obtained by Tukey's multiple comparison test are shown as superscript letter over the bars. Bars with different letters are significantly different ($P < 0.05$).

involved in Zn uptake from extracellular fluid; Zip4 located at the apical surface and Znt1 at the basolateral membrane of intestinal epithelia are of vital importance for uptake of dietary Zn [36,37]. The mRNA and protein levels of key Zn transporters were differentially expressed in the duodenum of pigs fed $ZnSO_4$ or Zn-chitosan chelate as the dietary Zn source; the $ZnSO_4$ group, with more ionic Zn(II) had significantly higher Zip4 and Zip5 protein abundance [38]. Although Zip transporters are involved in the uptake of ionic Zn(II), other divalent metal transport systems (DMT1, CTR1, TRPV6) [39,40] and amino acid mediated pathways may also contribute to Zn uptake in mammals [41,42]. However, in fish Trpv6 is not expressed in the intestine and the importance of Dmt1 and Ctr1 for intestinal Zn uptake are not known. It has been suggested that amino acid-linked Zn transport occurs in fish intestine [3,17]. Zn uptake in brush-border membrane vesicles was correlated to mono-histidine species [17] and the bis-histidine Zn complex was bioavailable to rainbow trout *in vivo* [3]. The effects of histidine and cysteine on the uptake of Zn by mammalian erythrocytes have also been suggested to be mediated by the bis-complex of metal-amino acid species [42]. The donor ligand hypothesis and/or the transported chelate hypothesis have been suggested as the two plausible means of amino acid aided metal transport in fish [3,17,20,43]. The donor-ligand hypothesis assumes that amino acid ligand aids is shuttling metals from inhibitory ligands in the luminal chyme to dedicated metal transporters; whereas, in the transported-chelate hypothesis an alternative transport pathway which accepts the metal-amino acid chelate as a substrate is proposed. In rainbow trout brush border membrane vesicles, lack of stereospecific action of histidine (L- or D-) upon apical Zn(II) uptake suggested of the donor-ligand exchange, but was not in support of the transported chelate hypothesis [17]. However, the possibility of fish amino acid transport system being less stereospecific was also suggested, contrary to mammals [44]. Nevertheless, the impact of different stereoisomers is important to be studied as the efficiency of organic Zn additive used in fish feeds can be related to the stereoisomer used to chelate. Moreover, it is of relevance in understanding the possibility of methionine supplements (L- or DL-) used in fish feeds improving Zn uptake. By studying the uptake of copper (Cu) from Cu-histidine in the presence of an array of potential histidine transport system inhibitors, Glover et al. [20] suggested a distinct transport system for Cu-histidine chelates in rainbow trout brush border membrane vesicles, *in vitro*. Recently, manganese (Mn) from Mn-lysine complex has been suggested to be transported by amino-acid uptake pathways (y^+ and $b^{0,+}$), different from the ionic Mn^{2+} uptake pathway in primary rat intestinal epithelial cells [45]. In the present study, Zn uptake increased in the presence of methionine and it was reduced upon simultaneous exposure to BCH, a potent blocker of Na^+-dependent methionine transport systems in intestinal epithelial cells [46,47]. These data suggest that the Zn-methionine chelate is transported through an amino acid mediated uptake pathway, similar to that suggested for Cu-histidine and Mn-lysine [20,45]. Therefore, the transported chelate hypothesis merits further investigation.

In mammals, amino acid uptake systems are pH sensitive and are either Na^+-dependent (B° and $B^{0,+}$), or Na^+-independent ($b^{0,+}$, L, and y^+); whereas, systems $B^{0,+}$, $b^{0,+}$, and y^+ are used by cationic amino acids, systems B° and L are specific for neutral amino acids [46,48]. Understanding of the different amino acid uptake pathways and the mode of action of the amino acid transport blocker used (BCH) is required to comprehend the differential effects of BCH on Zn uptake in RTgutGC cells exposed to FW and SW medium. BCH competes with methionine as a substrate of transport systems, which includes both the Na^+-dependent system (B° and $B^{0,+}$) and a part of the Na^+-independent (L-type) amino acid transport systems [46]. While the usefulness of BCH to study amino acid uptake systems is documented, no reports are available to refer if BCH has the ability to chelate Zn. We hereby show that BCH alone was neither able to significantly increase or decrease the uptake of Zn(II), which implies that Zn chelation with BCH was not favored under the test conditions. In fish, the Na^+-mediated components of amino acid transport in the intestine are dependent on luminal Na^+ concentration, whereas only the non-mediated components are functional in the absence of luminal Na^+ [49,50]. Although the Na^+ concentration and pH of the medium was not different between FW and SW, the effect of BCH in reducing Zn uptake in the presence of methionine was more potent in cells exposed to SW medium. This observation could possibly be due to a differential contribution of the Na^+-independent system in amino acid uptake under FW and SW luminal conditions. Indeed, in the European seabass (*Dicentrarchus labrax*), the contribution of the saturable Na^+-independent component was much higher for epithelial transport of methionine, compared to other amino acids namely glycine or alanine [51]. Further studies targeting specific Zn(II) and putative Zn-amino acid chelate transport systems are required to better understand the underlying mechanisms.

With increasing inclusion of plant derived protein sources used in salmonid feeds, availability of dietary Zn is reduced and requires higher supplementation levels than those recommended by NRC [52] to meet the Zn requirement of salmonids [53]. Due to environmental concerns, the European Food Safety Authority (EFSA) opinion suggested reduction in the maximum permissible Zn levels in salmonid feeds from 200 to 150 mg kg^{-1} complete feed and also laid emphasis on improving the availability of dietary Zn to limit Zn emissions [54]. In this context, our present study provides basic data in support of Zn-Met chelation to improve apical Zn uptake in the enterocytes, but also the possibility of a physiological limitation under luminal conditions of high ionic

concentration. The lack of functional adaptability in RTgutGC cells to SW ionic conditions due to short exposure and lack of convincing data to discern if the cells exhibited uptake at initial rate or saturation kinetics are potential limitations of this study. RTgutGC exhibited a transient induction in mRNA expression of Na^+/K^+-ATPase (α-subunit) only up to 24 h upon exposure to a high ionic SW medium [21]. Therefore, the uptake characteristics displayed by the RTgutGC cells herein can be more comparable to rainbow trout enterocytes exposed to seawater challenge and not that displayed by a seawater adapted fish. Our previous research on zebrafish suggests that there is no or limited systemic control of zinc uptake across the intestine, in the sense of humoral regulation, and the intestinal epithelium is responding directly to the Zn availability in the gut [55]. This improves the relevance of an *in vitro* system for studies on Zn uptake; nevertheless, a cell culture will always be a model of reality. Hence, translating the results from this *in vitro* uptake study in an enterocyte cell line to intestinal Zn uptake *in vivo* should be viewed with these constraints in mind.

The findings of this study towards understanding intestinal Zn uptake under different ionic conditions and dietary Zn forms will be of high practical interest in fish nutrition. To conclude, using RTgutGC we found no evidence for a difference in Zn uptake in media representing the intestine of FW and SW salmonids. However, Zn uptake in the presence of methionine was influenced by the ionic concentration in the media.

Declarations of interest

None.

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