Copper transporter 1, metallothionein and glutathione reductase genes are differentially expressed in tissues of sea bream (*Sparus aurata*) after exposure to dietary or waterborne copper.

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Abstract

The high affinity copper transporter 1 (Ctr1), metallothionein (MT) and glutathione reductase (GR) are essential for copper uptake, sequestration and defense respectively. Following rearing on a normal commercial diet (12.6 ± 0.2 mgKg^{-1}Cu), sea bream were fed an experimental control diet lacking mineral mix (7.7 ± 0.3 mgKg^{-1}Cu), an experimental diet enhanced with Cu (135 ± 4 mgKg^{-1} Cu) or an experimental diet (7.7 ± 0.3 mgKg^{-1}Cu) while exposed to Cu in water (0.294 ± 0.013mgL^{-1}). Fish were sampled at 0, 15 and 30 d after exposures. Fish fed the Cu-enhanced experimental diet showed lower levels of expression of Ctr1 in the intestine and liver compared to fish fed control experimental diets, whilst Ctr1 expression in the gill and kidney was unaffected by excess dietary Cu exposure. Waterborne Cu-exposure increased Ctr1 mRNA levels in the intestine and the kidney compared to experimental controls. Excess dietary Cu exposure had no effect on levels of metallothionein (MT) mRNA, and the only effect of dietary excess Cu on glutathione reductase (GR) mRNA was a decrease in the intestine. Both MT mRNA and GR were increased in the liver and gill after waterborne Cu exposure, compared to levels in fish fed experimental control low Cu diets. Thus, Ctr1, MT and GR mRNA expression in response to excess Cu is dependent on the route of exposure. Furthermore, the tissue expression profile of sea bream Ctr1 is consistent with the known physiology of copper exposure in fish and indicates a role both in essential copper uptake and in avoidance of excess dietary and waterborne copper influx.

Footnote: The sea bream Ctr1 cDNA sequence has been deposited with the EMBL/Genbank database under accession number **AJ630205**

Introduction

Copper is an integral part of many enzymes involved in diverse and vital biological processes and is therefore an essential trace element. Copper-dependent enzymes utilize the

ability of copper to readily change its oxidation state from cuprous (Cu^{1+}) to cupric (Cu^{2+}) to catalyze fundamental redox reactions (Linder and Hazeghazam, 1996). However this same characteristic of copper ions can also lead to cytotoxicity through the production of highly damaging reactive oxygen species (ROS) and other free radicals which can cause catastrophic damage to lipids, protein and DNA (Halliwell and Gutteridge, 1984). Thus, all organisms have evolved sophisticated homeostatic mechanisms that regulate uptake, distribution, sequestration and export of copper (Prohaska and Gybina, 2004).

Fish represent a unique model among vertebrates because they can absorb copper through two routes: waterborne copper enters through the gills while dietary copper is absorbed through the intestine (Grosell and Wood, 2002). Diet is the main source of copper for fish under optimal growth conditions (Watanabe et al., 1997;Carpene et al., 1999), whereas waterborne copper can be absorbed through the gills in conditions of dietary copper deficiency (Kamunde et al., 2002). Farmed fish are fed diets supplemented with relatively high levels of Cu and Zn which have been empirically determined to maximise growth rate through the production cycle. Although attractive from a meat production viewpoint, little is known of the effects of such high Cu and Zn levels on fish health or on the environment surrounding farms. The importance of understanding the mechanisms of both essential and excess Cu uptake and effects in fish is highlighted by the observation that alarmingly high sediment Cu and Zn levels have been detected close to farming operations. This has been observed in both sea bream (*Sparus aurata*) aquaculture in the Mediterranean and Atlantic salmon aquaculture (*Salmo salar*) in Canada and Scotland (Chou et al., 2004; Mendiguchia et al., 2006; Dean et al., 2007). Sediment accumulation of Cu and Zn is largely due to the deposition of metal present in uneaten food and faeces and Cu levels of over 800 mgKg^{-1}, which considerably exceed the amount at which ecological effects may be predicted (Long et al., 1998), can be detected in the vicinity of fish cages (Dean et al., 2007). In order to resolve these issues it is necessary to both understand the mechanisms and regulation of essential dietary uptake by fish as well as the toxicological consequences of excess metal and environmental metal contamination.

The high affinity copper transporter 1 (Ctr1) is the principal candidate for mediating essential copper entry in eukaryotes. Ctr1 was first identified in yeast (*Saccharomyces cervisiae*) as the product of a gene essential for copper uptake and CuZn-superoxide dismutase

activity (Dancis et al., 1994). Subsequently homologous genes have been identified in mammals and have been shown to complement Ctr1 deficient yeast mutants (Zhou and Gitschier, 1997). Mammalian Ctr1 is able to mediate uptake of copper with high affinity and specificity in both human and mouse cells (Lee et al., 2001; Lee et al., 2002; Eisses and Kaplan 2005). Furthermore, mice with complete deficiency of Ctr1 die *in utero* following growth and developmental defects, whilst partially deficient heterozygous mice exhibit tissue specific defects in copper accumulation and in the activities of copper-dependent enzymes (Lee et al., 2001). Ctr1 has also been reported in zebrafish (*Danio rerio*) and knockdown of zebrafish Ctr1 mRNA results in embryonic lethality (Mackenzie et al., 2004). However, there have been no studies on the role of Ctr1 in copper uptake in fish. Metallothionein (MT) is a protein inducible by several toxic metals, including copper and zinc. It is also involved in copper and zinc storage and delivery and in free radical scavenging activity (Vasak, 2005; Selvaraj et al., 2005). Thus, MT is a vital component of both storage of essential metals and sequestration of toxic levels of Cu, and has consequently gained popularity as a biomarker for environmental exposure to toxic metals (Hylland et al., 1992). Glutathione reductase (GR) is a ubiquitous enzyme, responsible for regulating homeostatic redox balance through the maintenance of reduced glutathione levels (Carlberg and Mannervik, 1985) and is induced in response to redox perturbation by a variety of agents including Cu (Hansen et al., 2006).

The aim of this study was to investigate the effects of excess Cu exposure to better understand the mechanisms of uptake and toxicity in a farmed marine fish, *S. aurata*. This was achieved by characterising Ctr1 in this species and by measuring the response of Ctr1, MT and GR mRNA expression to excess dietary or waterborne copper.

Materials and Methods

Animals and treatments

Juvenile gilthead sea bream average mass 40 g were obtained from farmed stock. One hundred fish were acclimatised for 15 days in a 1500 l flow through tank at 19°C and at a salinity of 33.6±2.6‰. Before and during acclimatization fish were fed a pre-experimental normal commercial pelleted diet (Diet1; Skretting, Italy) at 2% of body mass per day which contained 12.6±0.2 mgKg^{-1} Cu and 160±2.0 mgKg^{-1} Zn (Table 2). Fish were then divided into

3 tanks of 150l with 20 fish in each. In tank 1 fish were fed an experimental diet lacking mineral mix (Diet2) consisting of a pelleted diet prepared using 60% of fish meal, 20% of wheat middlings, 10% of soya meal, and 10% of fish oil and which contained 7.7 ± 0.3 mgKg^{-1} Cu and 60.1 ± 04.1 mgKg^{-1} Zn (Table 2). Bulk ingredients to make the fish pellets were provided by Skretting and mixed with water. Mixing and extrusion were carried out with a commercial pasta maker and extruded diets were air dried and then chopped into pellets. The experimental diet with high Cu (Diet3) was produced by replacing water with an aqueous solution of $CuSO_4$ to generate a final nominal dietary concentration of 130 mgKg^{-1} Cu (6.17 mM; Diet3) and this was fed to fish in tank 2. This sub-lethal level of dietary Cu was based upon a preliminary experiment where 120 mgKg^{-1} Cu was insufficient to elicit induction of MT (Carpenè unpublished data). Diets were then analyzed by atomic absorption spectrophotometry (AAS) to determine actual copper concentrations. Fish were fed at 1.5% of body mass twice daily. In tank 3 fish were given Diet2 and exposed to copper via water. Copper sulphate ($CuSO_4 \bullet 5H_2O$) was added to the tank to give a nominal Cu concentration of 0.3 mgL^{-1} (1.2μM). In a static renewal protocol 87% of the water was replaced with freshly prepared sea water containing 0.3mgL^{-1} copper sulphate, previously equilibrated in a testing chamber, every 24 hours for 30 days in waterborne treatment and with sea water only in control and diet tanks (tanks 1 and 2). The waterborne concentration of Cu was chosen based on the evidence of previous studies which indicated that this level of Cu was about 25% of the lethal concentration of Cu resulting in 50% mortality after 96 hours for the closely related *Sparus sarba* (96h LC50 between 1.03 and 1.24 mgL^{-1};(Wong et al., 1999). In addition, MT induction was demonstrated in the liver of sea bream at concentrations between 0.1 and 0.5 mgL^{-1} (Isani et al., 2003). Fish in waterborne exposure were also fed the control diet at 1.5% of body mass twice each daily. Cu concentrations were verified by atomic absorption spectrometry (AAS). Fish were weighed before and after sampling and there were no differences in mean masses of animals subjected to copper treatments compared to controls at 15d or at 30d (Table 2).

At 0 days prior to treatment and after 15 days and 30 days seven fish chosen randomly from each tank were removed, sacrificed and tissues immediately sampled for RNA extraction and metal analysis. There were no mortalities in the tanks. Samples for RNA were immediately homogenised in TriReagent RNA extraction buffer (Sigma, UK) using a rotating probe

homogeniser. Tissue samples for metal concentration were stored in acid washed (HCl) Teflon jars at -20˚C.

Metal determination

Copper and zinc were determined in gills, intestine and liver tissues and also in the fish pellet and water. All polyethylene disposables were washed with 1M HCl. The samples were digested with 2 ml 65% HNO_3 and 0.5 ml 30% H_2O_2 in a microwave oven (Model 1200, Milestone, Italy) for 5 min at 250 W, 5 min at 400 W, 5 min at 500W, and one min at 600W. Cooled samples were transferred into 10ml polyethylene volumetric flasks and directly aspired into the flame of an atomic absorption spectrophotometer (Model IL 11, Instrumentation Laboratory USA) equipped with a deuterium lamp background correction. The detection limit for copper was 6 ng/ml; recovery ranged from 94% to 104%. A trace metal standard was run every 20 samples. The accuracy of the method was evaluated by calibration to an international standard (CRM 278). The obtained concentration values fell within the confidence interval given by the Community Bureau of Reference, Brussels.

Sea water copper concentrations in tanks were determined by a Varian Atomic Absorption Spectrophotometer Mod. AA20plus equipped with a graphite furnace Mod. GTA96plus. Water analysis was performed after 50 times dilution of sea water to avoid salt interference. Using this method the detection limit for Cu was $5\mu gL^{-1}$ in seawater.

Synthesis of sea bream Ctr1 cDNA

Total RNA was prepared using TriReagent (Sigma, UK) according to the manufacturers instructions, RNA quality was checked by spectrophotometry and agarose gel electrophoresis. cDNA was synthesised using 1µg of liver total RNA, 25µM of anchored oligo dT_{20} (Invitrogen, UK), 500µM dNTPs (Invitrogen, UK), 200units of SuperScript™ II RT reverse transcriptase with provided buffer (Invitrogen, UK). Reactions were incubated for 10 mins at 40°C followed by 42°C for 50 minutes in a final volume of 20µl.

A partial Ctr1 cDNA was generated using PCR primers, Ctr1F1 and Ctr1R2 (Table 1), which were designed by selecting conserved areas from alignments of protein sequences derived from DNA sequences of human (Genbank Accession number **BC013611**), rat (**BC078745**) and zebrafish (**NM 205717**) Ctr1. Ctr1F1 and Ctr1R2 were included, at a concentration of

0.5µM, in a reaction containing Taq polymerase with supplied buffer IV and 2mM $MgCl_2$ (Taq DNA Polymerase, ABgene, UK). One eighth (2.5µl) of the cDNA synthesis reaction was included in a final volume of 25µl. Annealing temperature was 52 °C.

RACE-PCR (Rapid Amplification of cDNA Ends) was performed using the FirstChoice RLM-RACE kit (Ambion,UK). Primers designed from the sequence of the initial partial cDNA (for 5' RACE 5'Ctr1-R1 and for 3'-RACE, 3'Ctr1F1; Table 1) were used to generate RACE amplicons.

The final full length PCR product was obtained using Ctr1Full-F and Ctr1Full-R (Table 1), designed from the 5' and 3' RACE products. The annealing temperature for all primers was 60 °C.

Sequence analysis

Sequencing was performed using a Beckman 8800 autosequencer. Lasergene SEQman software was used to edit and assemble DNA sequences. The sea bream cDNA sequence was used to search pufferfish (*Tetraodon nigroviridis*), medaka (*Oryzias latipes*), stickleback (*Gasterosteus aculeatus*) and zebrafish genomes (www.ensembl.org) using TBlastX. Regions of chromosomal DNA sequence containing homologous sequences were then processed with GeneWise2 (www.ebi.ac.uk) using the sea bream deduced amino acid sequence as a key to generate predicted polypeptide sequences. ClustalW (Thompson et al., 1994) was used to generate multiple alignments of deduced protein sequences and to infer and bootstrap phylogenetic trees using the neighbour joining method (Saitou and Nei, 1987).

Quantitative PCR

All cDNA for quantitative PCR (QPCR) was synthesised using Superscript reverse transcriptase and supplied buffer components (Clontech, UK) and an oligo-dT containing primer (Table 1). Reactions contained 3µg of total RNA, 500ng oligo-dT primer in a volume of 20µl and were incubated at 42°C for 1.5 hours followed by 70°C for 15min. QPCR primers for target genes Ctr1, qCtr1F and qCtr1R, MT (U58774), qMT-F and qMT-R and GR (**AJ937873**), qGR-F and qGR-R and reference genes sea bream β-actin (**X89920**), qActinF and qActinR, sea bream glyceraldehydes-6-phosphate dehydrogenase (GAPDH; (**DQ641630**) qGapdhF and qGapdhR and elongation factor1α (EF1α ; **AF184170**) qEF1αF

and qEF1α (Table 1) were used at 0.5μM with one fortieth of the cDNA synthesis reaction and SYBR-green QPCR mix (ABgene, UK) in a total volume of 20μl. Reactions were run in a Techne Quantica thermocycler at an anneal temperature of 58°C for CTR1 and MT, 55°C for β-actin and GR and 60°C for GAPDH and EF1α to give a PCR product of 186, 171, 171, 204, 250 and 174bp respectively. Each QPCR product was than sequenced to confirm its identity and in each case was found to be identical to the predicted sequence. Gene copy number in each reaction was automatically calculated by the Quantica software by comparison to a standard curve constructed from the results of a parallel set of reactions containing serially diluted linearised plasmids containing sea bream CTR1, MT, GR, β-actin, GAPDH and EF1α cDNA sequences. Concentrations of standards were determined spectrophotometrically and converted to copy number by consideration of plasmid size and DNA absorption coefficient. Normalisation of copy number across biological samples was achieved by using a normalization factor (NF) based on the geometric mean expression of reference genes (β-actin, GAPDH or EF1α) determined using geNorm software (Vandesompele et al., 2002). Within tissues, the stability of individual reference genes varied and geNorm was used to select the two most stable genes for a particular tissue, which were then used to normalize target gene expression level. Since the expression of all reference genes varied widely between tissues, the comparison of expression levels of Ctr1 in different tissues was achieved by normalising Ctr1 copy number to mass of total input RNA.

Statistical analyses

Statistical analysis was performed using the Minitab v.15.1 statistical software package (Minitab Inc., USA). Data was first assessed for normality with the Kolmogorov-Smirnov test and for homogeneity of variances by Bartlett's test and examination of residual plots. Where necessary sample data was transformed to improve normality. Sample data was compared by analysis of variance (ANOVA) using a General Linear Model. Post hoc multiple comparisons were applied using Tukey's test (Zar 1999). A significance of $p<0.05$ was applied to all statistical tests performed. All data are presented as mean ± SD.

Results

Sea Bream Ctr1 cDNA

Ctr1 has not been previously reported from sea bream and in this study it was important to isolate a full length cDNA to confirm its identity and to determine the expression profile of Ctr1 mRNA in different tissues. Following the first round of RT-PCR a DNA fragment of 376 base pairs was generated which, on sequencing, showed 88% identity to the zebrafish Ctr1 and 76% identity to human Ctr1. To amplify the 5' end of the sea bream Ctr1 cDNA, two primers were synthesised based on the sequence of this fragment and used in a nested PCR procedure whereby a portion of the reaction mix after use of the 5'Ctr1-R1 was subjected to a further round of 5'-RACE using the 5'Ctr1-R2 primer. This yielded a fragment of 728 base pairs which overlapped with 100% identity with the original sequence. The 3'RACE using 3`Ctr1-F1 yielded a fragment of 398 bp which also showed 100% identity with the original PCR product in the region of overlap. This allowed the inference of a full length cDNA for sea bream Ctr1 (saCtr1) which consisted of an open reading frame of 222 amino acids and 5' end 3' untranslated regions of 197 bp and 243 bp respectively. To confirm the saCtr1 sequence primers (Ctr1Full-F and Ctr1Full-R) were designed to amplify the entire sea bream Ctr1 ORF. The sequence obtained (saCtr1) was 100% identical to the assembled sea bream sequence and showed 78% identity with the human sequence and 89% with the zebrafish sequence.

Alignment of the deduced amino acid sequence of saCtr1 with Ctr1 proteins from other fish species, frog, lizard, chick and human show a strong sequence conservation, notably the MXM copper-binding motif, three trans-membrane domains and the C-terminal HCH motif (Puig et al., 2002; Guo et al., 2004; Guo and Petris, 2006; Fig. 1). Moreover, amino acids G34, N53, M69, K178 and D84 (of human Ctr1) which were shown by mutational analysis to affect V_{max} and K_m of copper uptake (Eisses and Kaplan, 2005) are completely conserved in sea bream and in all of the other vertebrate Ctr1 sequences, with the exception of lizard where a histidine residue in transmembrane domain 2 is replaced with aspartate (Fig 1).

In view of the possibility that fish species may contain multiple genes for which only single mammalian examples exist, the sea bream cDNA sequence was used to search for similar sequences in other fish genome sequences. In each species a single Ctr1 gene was identified and the Ctr1 derived from the zebrafish genome was identical to that previously described (Mackenzie et al., 2004). Furthermore phylogenetic comparison of all of the fish

sequences with Ctr1 from other vertebrates indicated that the fish genes were monophyletic and that the sea bream Ctr1 was most closely related to the stickleback protein (Fig. 2).

The tissue expression profile of saCtr1 was determined by QPCR (Fig. 3). Of the eight tissues tested levels of Ctr1 mRNA were significantly higher in small intestine and gill than in kidney, liver, brain, heart, white and red muscle in which levels of Ctr1 mRNA were 6 to 10-fold lower than in intestine.

Growth and Metal levels in tissues

Growth was not significantly affected by inclusion of Cu in the diet or in water after 15 days or after 30 days (Table 2).

Cu and Zn levels in fish tissues and diets are provided in Table 2. Cu levels in waterborne exposure tanks were 0.283 ± 0.004 mgL^{-1} at the beginning of the experiment and 0.294 ± 0.013 mgL^{-1} at the end of the experiment, whereas in control and dietary treatment tanks copper was < 0.005 mgL^{-1} throughout the experiment.

Cu levels in all tissues examined from day 0 (Diet 1), day 15 and day 30 control fish (Diet2) were not significantly different. Copper levels in gill, intestine and liver of dietary exposed animals (Diet3; 135.5 ± 4.4 mgKg^{-1} Cu dry diet) were not different from control animals at 15 days but after 30 days Cu accumulated in the liver (1.6 fold greater) and intestine (1.5 fold greater). After waterborne Cu exposure, gill Cu levels were higher at both 15 and 30 d timepoints, whilst hepatic Cu was higher at day 15 and had returned to control levels at day 30. Zn levels in liver and intestine from control fish sampled at 0, 15 and 30 d did not differ. Zn levels were reduced in the gills of control fish sampled at 30 d, as well as those fish exposed to enhanced dietary and waterborne Cu for 30 d, as compared to fish fed a commercial diet. In the liver of fish exposed to waterborne Cu for 30 days, Zn levels were lower compared to those in all other treatments. Due to a lack of tissue, metal levels were not determined in kidney.

Effect of excess dietary and waterborne copper exposure on Ctr1, MT and GR gene expression

Results are presented in Figure 4. Changing the control diets from Diet1 (pre-experimental normal commercial diet) to Diet2 (reduced mineral mix) had no effect on intestinal, gill or kidney Ctr1 mRNA levels after 15 or 30 d. Switching from Diet1 to Diet2 resulted in an increase of Ctr1 mRNA in the liver of control fish. After adding copper to Diet2

(Diet3; 135±4.4 mgkg^{-1} Cu in diet) Ctr1 mRNA levels were affected differently in different tissues. Intestinal Ctr1 mRNA was 3.9 fold lower at 15 d, however, at 30 d, no significant decrease was detected (P-Value 0.0593) whilst Ctr1 levels were not affected in gill or kidney. In the liver of fish fed Cu-enhanced diets (Diet3), Ctr1 mRNA decreased relative to Diet2 fed controls and returned to the levels measured in pre-experimental control fish at day 0, which had been maintained on Diet1. After exposing fish on Diet2 to waterborne Cu for 30 days, intestinal and renal Ctr1 mRNA was higher than in controls on commercial Diet1 or on experimental control Diet2 alone. Hepatic Ctr1 mRNA did not change after waterborne Cu exposure relative to experimental controls on Diet 2.

Intestinal, gill and liver MT mRNA levels were lower in Diet2 fed control fish (15 and 30d) and in dietary and waterborne Cu-exposed fish compared to Diet1 pre-experimental controls. There was no change in renal MT mRNA after switching control diets. Dietary Cu had no effect on MT mRNA relative to levels in control Diet2 in any of the tissues tested. In contrast waterborne exposure to Cu dramatically increased MT mRNA in liver relative to levels in Diet 1, Diet2 or Diet3 fish. Smaller but significant increases in MT mRNA were also measured in kidney and in gill after exposure to waterborne Cu relative to Diet2 controls. Waterborne Cu had no effect on intestinal MT mRNA relative to Diet2 fed control fish.

Changing from Diet 1 to Diet2 had no effect on GR in gill, kidney or liver. Intestinal GR mRNA levels were decreased in experimental control fish after switching from Diet1 to Diet2, similar to effects observed on MT. Inclusion of Cu in either diet or water led to further reductions in intestinal GR mRNA. Dietary Cu had no effect on GR in gill or liver, but did lead to increases in kidney of diet 2 fed experimental control fish after 30 days. Waterborne Cu increased GR mRNA in gill and liver but had no apparent effect in kidney.

Discussion

In attempting to understand the mechanisms of Cu uptake in fish, whether under conditions of deficiency, sufficiency or excess, it is essential to consider known Cu-specific transport proteins. Consequently we characterised the cDNA for the sea bream homologue of Ctr1, saCtr1. The deduced amino acid sequence of this cDNA possesses all of the domains that characterize Ctr1 in other species (Fig 2): the metal binding motif, the three

transmembrane domains and the HCH motif. Moreover, the saCtr1 protein sequence possesses all of the amino acid residues shown to be essential for optimal Ctr1 activity (Eisses and Kaplan, 2005). The metal binding motif in the sea bream sequence differs from mammalian sequences in consisting solely of MXM (where X represents any amino acid residue) rather than the more extensive MXXMXM. However mutant mammalian Ctr1 proteins lacking the first methionine but containing either of the second or third methionines in the MXXMXM motif retain near maximal copper uptake activity (Puig et al., 2002). Based on phylogenetic and comparative genome analysis it is very unlikely that sea bream contain more than one Ctr1 gene. Therefore, considering the conserved structural features, comparative genome and phylogenetic analyses, it is most likely that the cDNA described here encodes a functional high affinity copper transporter, and has no other homologues in sea bream.

Ctr1 is essential for normal cellular Cu uptake and its expression is widespread among mammalian tissues (Kuo et al., 2001; Holzer et al., 2006) which would infer a primary role in systemic rather than dietary uptake. Ctr1 expression during zebrafish embryogenesis is mainly in the intestinal region after 24 hours and it continues to be strongly expressed in intestine until adulthood, when expression is more widespread (Mackenzie et al., 2004). In the present study we found that adult sea bream Ctr1 mRNA was also widely expressed, but most highly in intestine with levels some 4 to 10 fold lower in other tissues, consistent with results in other species. The role of Ctr1 in dietary uptake of Cu is unclear. Ctr1 is essential for absorption of Cu from the diet in neonates (Nose et al., 2006), but its expression has in some studies been localised to the basolateral membrane of intestinal cells, implying a role in delivering bloodborne Cu specifically for incorporation into intracellular intestinal proteins (Zimnicka et al., 2007) whilst in other studies Ctr1 is present on the apical membrane of intestinal cells (Kuo et al., 2006) consistent with a direct role in dietary Cu uptake. Since intestinal uptake of copper predominates in fish (Kamunde et al., 2002; Kamunde et al., 2001) this suggests a role for Ctr1 in uptake of dietary copper in fish, as in mammals. Indeed previous studies on Cu uptake in fish intestine provides evidence for the presence of a transporter with similar kinetics (high affinity/low capacity) to that of Ctr1 (Grosell and Wood, 2002; Burke and Handy, 2005) and our finding that Ctr1 mRNA is most highly expressed in intestine supports this contention.

In this study, we focussed on the effect of excess Cu on gene expression during two possible routes of intake, diet and water. In order to generate control diets containing low copper, the mineral mix containing Cu, Zn and other trace minerals was omitted resulting in an experimental control diet (Diet2) with Cu and Zn levels lower than the pre-experimental commercial diet (Diet1) which was used until the start of the experiment (day 0). Thus it is possible that effects on gene expression during exposure to high Cu may have been the result of lower levels of minerals other than Cu. However, the low mineral experimental control diet (Diet2) did not cause reductions in the concentration of Cu and Zn in the tissues of control fish at 15d or on growth rates, despite clear effects on gene expression. There were reductions in zinc in gill in all treatments after 30 days, although these reductions did not change the pattern of gene expression compared to 15 days, indicating that the reduction in dietary zinc was not a major factor in causing the differences in gene expression between fish fed the experimental diets. Furthermore, even though the level of Zn in the experimental control (60 mg Zn kg^{-1}) diet was lower than that of the pre-experimental diet (160 mg Zn kg^{-1}), this level would not be considered deficient since the dietary Zn requirement for various fish species is known to range between 15 and 30 mg kg^{-1}(NRC 1993). Nevertheless, it is clear from the results that omission of mineral premix did affect gene expression in sea bream tissues, based on the differences seen between fish at day 0, reared on the commercial diet, and fish at days 15 and 30 fed the experimental diets. The most pronounced effects were reductions in MT expression in intestine, gill and liver and reductions in GR in the intestine. These reductions were not subsequently reversed by inclusion of high Cu in the diet, in fact intestinal GR mRNA was further reduced by inclusion of high dietary Cu. Therefore these effects on GR and MT are most likely to be caused by reductions in constituents of the mineral premix, other than Cu. The most likely candidate constituent is Zn, as it was reduced from 160 to 60 mg kg^{-1} in the control diets, whilst Cu was reduced to a much lesser extent, and MT is known to mainly respond to Zn status (Coyle et al., 2002). Thus, the relatively high level of MT and GR mRNA observed under normal commercial dietary conditions indicates that Zn levels may be excessive in this diet.

The effects of switching control diets on Ctr1 mRNA levels were distinct from effects on MT and GR. No effects on Ctr1 were observed in any control diets in intestine, gill and kidney, indicating that omission of the mineral premix is not a factor in considering the effects

of excess Cu on this gene in these tissues. However, after dietary exposure to excess Cu, sea bream exhibited up to a 4-fold decrease in intestinal Ctr1 mRNA with no associated increase in tissue Cu levels. This decrease may be a protective response to excess dietary Cu, resulting in decreased Cu-uptake. Previous studies on Cu uptake in fish have concentrated on the physiology of Cu distribution and have mainly considered the uptake and effects of copper in freshwater. Similar studies on marine species are few in number and there may be large differences in physiology between salt and freshwater fish. For example, in freshwater (low Na^+) it is well established that copper uptake can occur through the ENaC sodium transporters of gill, although in conditions of high Na^+ concentrations, such as the seawater used here, these transporters are thought unlikely to play a significant part in either gills or gut (Handy et al., 2002). Even so, making use of the available data, the results presented here are consistent with a role for a high affinity carrier such as Ctr1 in regulating excess and normal copper uptake. Thus, Cu-uptake exhibits kinetics in isolated rainbow trout (*Oncorhynchus mykiss*), consistent with the presence of Ctr1-like activity (Burke and Handy, 2005) and rainbow trout acclimated to Cu in the diet show increased Cu tolerance to both waterborne and subsequent dietary exposure (Miller et al., 1993). In another freshwater species, the catfish, *Clarias gariepinus*, Cu uptake across the gut has also been shown to be negatively regulated in isolated, perfused intestine, Cu uptake efficiency declining with increasing luminal Cu concentration (Handy et al., 2000). Additionally Cu absorption in rainbow trout reportedly declined with increasing dose of Cu infused into the intestine (Clearwater et al., 2000). Taken together these results strongly suggest that a specific high affinity Cu-uptake system exists in freshwater fish intestine and that some component of this system may be down-regulated by exposure to excess dietary Cu. Although sea bream is a marine species and not strictly comparable to freshwater models, the high expression levels of saCtr1 mRNA in intestine and the decreases induced by excess copper suggest that the Cu-uptake component which is downregulated by excess Cu is Ctr1.

Waterborne Cu exposure showed very different effects on gene expression to dietary exposure. Cu levels were raised in the liver of fish after 15d of waterborne exposure and returned to control levels at 30 days, despite dramatic induction of GR and MT at both timepoints. Large increases of GR were also observed in gill of fish exposed to waterborne Cu. These effects are most likely a response to excess Cu exposure since the observed

increases were not observed in low mineral mix control diets and were much higher than the levels observed on pre-experimental high Zn diets. Remarkably, and quite distinct from dietary exposure, waterborne Cu exposure also resulted in an increase in Ctr1 mRNA in sea bream intestine and in kidney after 30d. In freshwater rainbow trout, renal excretion of excess Cu in fish is negligible compared to hepatobiliary routes (Grosell et al., 1998) and although we did not measure copper levels in the kidney, marine fish, in contrast to freshwater species, can accumulate Cu in the kidney following brachial exposure, although at considerably lower levels than liver (Grosell et al., 2003; Grosell et al., 1997). Furthermore rainbow trout exhibit a rapid turnover of Cu in the kidney following brachial exposure (Grosell et al., 1997) indicating that, although accumulation may be low, the kidney is an important site of Cu transport and distribution in freshwater fish. Therefore the increase in Ctr1 mRNA in kidney is consistent with the observed low renal excretion of copper and with re-absorption by kidney. In conditions of copper scarcity renal Ctr1-dependent re-absorption may be an essential mechanism for maintaining optimal whole body copper concentrations. In conditions of excess copper it might be thought that renal excretion of copper in plasma filtrate would be an efficient way to remove Cu. This is not the case and it may be that excess Cu is reabsorbed in the kidney, via increased Ctr1, as protection from renal toxicity which would otherwise be induced by an increased concentration of Cu in the tubules, an effect which would be particularly acute in marine fish which discharge a highly concentrated urine (Beyenbach, 2004). The increase in Ctr1 in intestine after waterborne-Cu exposure after 30 d is difficult to explain, particularly in the light of decreases in GR.

Considering the results in total it is clear that Cu has different effects on Ctr1, MT and GR mRNA expression depending on its route of uptake. Although we did not observe any effects, either of control diet or of waterborne Cu exposure on gill Ctr1, intake of Cu across the gill had very distinct effects on sea bream gene expression in other tissues compared to uptake across the intestine. Intestinal Cu uptake, although resulting in similar increases in tissue Cu levels, does not induce markers of toxicity such as MT or GR expression in liver or other tissues. This suggests that Cu from dietary sources is associated with different hepatic components or compartments than Cu from gill uptake, in turn inferring that the form in which Cu is delivered to the liver and other tissues must differ in some way between intestinal- and gill-derived Cu. In mammals Cu uptake is entirely from the diet via the intestine, after which it

enters the hepatic portal vein and progresses to the liver where it mixes with arterial blood and is taken up by hepatocytes. Within the hepatocyte a complex system of Cu chaperone proteins deliver Cu to various intracellular compartments and cupro-enzymes. In conditions of excess, Cu is excreted into the bile and at the same time intestinal uptake is reduced (Linder et al., 1998). Our results suggest that Cu in the hepatic portal vein derived from the intestine is likely to be complexed with a specific carrier enabling normal physiological uptake by liver cells. In contrast, Cu entering the arterial blood via the gill may be more likely to exist in a form which bypasses normal uptake and distribution pathways and thus causes induction of GR and MT, markers of redox and metal stress. To date three candidates for circulatory Cu transport have been suggested. Ceruloplasmin, a protein essential for iron homeostasis, contains up to 95 % of Cu in the blood, but deficiency of the protein has no effect on Cu transport (Meyer et al., 2001). Similarly, deficiency of serum albumin, to which Cu^{2+} binds with high affinity, has no Cu-transport or distribution effects (Vargas et al., 1994). Furthermore serum albumin binds Cu in a state which is not available for cellular uptake, although cells will take up Cu^{2+} complexed with L-histidine which in blood may couple albumin-bound Cu with cellular uptake systems (Deschamps et al., 2005). The third candidate is transcuprein (also known as α2-macroglubulin and α1-inhibitorIII) and the bulk of copper derived from intestine and thence absorbed by liver is bound to this protein and to serum albumin with which it readily exchanges Cu^{2+} (Liu et al., 2007), although conclusive evidence for a central role in Cu transport is still lacking.

Although the decreases in sea bream intestinal Ctr1 mRNA and increases in kidney Ctr1 mRNA induced by dietary and waterborne Cu respectively may reflect an important protective adaptation to conditions of excess copper, in mammals the only known mechanism of Ctr1 regulation is post-translational (Kuo et al., 2006; Nose et al., 2006). However mammalian studies have concentrated on models of Cu deficiency and little attention has been paid to gene expression in models of Cu excess. In non-vertebrates more information exists. Yeast Ctr1p expression is controlled by Mac1p a transcription factor which both up-regulates Ctr1p gene expression in conditions of Cu-deficiency and represses Ctr1p expression under conditions of Cu excess (Labbe et al., 1997). A Drosophila Ctr1 homologue is up-regulated by copper depletion and this is dependent on MTF-1, a transcription factor which paradoxically also functions to up-regulate MT in conditions of excess Cu from species as diverse as insects

and mammals (Selvaraj et al., 2005). The differential effects of Cu on sea bream liver following gill or intestinal uptake may provide a useful model in which to study the diverse transcriptional effects of Cu in vertebrates.

In summary, the tissue distribution profile and Cu-dependent intestinal expression of sea bream Ctr1 mRNA is consistent with the physiological evidence from various fish species for the involvement of a functional high affinity transporter in copper homeostasis under conditions of both normal and excess dietary copper exposure. Furthermore, Cu derived from uptake via gill was associated with the induction of markers of metal toxicity, whilst uptake from intestine, using the synthetic diets used in this study, was not. In view of the potential for toxic metal contamination below aquaculture sites it is also notable that the concentration of Zn in a normal commercial diet may also result in increased MT in intestine and liver, indicating that dietary Zn levels might be excessive, from both fish health and environmental health viewpoints.

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Tables

Table 1. Primers used for cDNA isolation and QPCR.

ID	Sequence 5`→3`
Ctr1F1	gccgcaaatgaccttctactt
Ctr1F2	ctacaactccatgcc
Ctr1R1	ggcatggagttgtag
Ctr1R2	actgccttcttccagctgaa
5'Ctr1-R1	agcgatgcagaggtaagcgttgta
3'Ctr1-F1	gctgatggagacgcacaagact
Ctr1Full-F	ctcgcgactttgtgagtttcgtgt
Ctr1Full-R	caaggtgttggttaccacggttac
qCtr1F	cgggtctgctcatcaacaccc
qCtr1R	tgtgcgtctccatcagcaccg
qMT-F	gctcctgcacctcctgcaag
qMT-R	gggtcacacacaggcgccat
qGR-F	caaagcgcagtgtgattgtgg
qGR-R	ccactccggagttttgcatttc
qActinF	gaccaactgggatgacatgg
qActinR	gcatacagggacagcacagc
qGapdhF	tgcccagtacgttgttgagtccac
qGapdhR	cagaccctcaatgatgccgaagtt
qEF1αF	catggttgtggagcccttct
qEF1αR	tcctgcacgaccattcatttc
Oligo dT	aagcagtggtatcaacgcagagtactttttttttttttttttttttttttttttttnv

Table 2. Fish weights**,** copper and zinc tissue and diet concentrations at 0, 15 and 30 d after exposure to dietary (mgKg^{-1} dry mass) or waterborne (mgL^{-1}) copper.

|	Day0 Commercial Diet	Day15 diet2	Day15 diet3	Day15 diet2+Cu in water	Day30 diet2	Day30 diet3	Day30 diet2+Cu in water
Fish weights	46.7 ± 5.9	51.1 ± 8.2	48.9 ± 9.1	51.6 ± 6.6	55.1 ± 6.6	52.3 ± 9.8	55.1 ± 7.5
Water [Cu]	<0.005	<0.005	<0.005	**0.294±0.013***	<0.005	<0.005	**0.294±0.013***
Diet [Cu]	12.6 ± 0.2	7.7 ± 0.3	**135.5 ± 4.5***	7.7 ± 0.3	7.7 ± 0.3	**135.5 ± 4.5***	7.7 ± 0.3
Diet [Zn]	160.0 ± 2.0	60.1 ± 4.1	67.6 ± 2.3	60.1 ± 4.1	60.1 ± 4.1	67.6 ± 2.3	60.1 ± 4.1
Gills [Cu]	1.5 ± 0.1	1.5 ± 0.2	1.4 ± 0.3	**2.5 ± 0.3***	1.7 ± 0.1	1.4 ± 0.2	**2.2 ± 0.2***
Gills [Zn]	27.9 ± 1.7	33.5 ± 3.2	30.7 ± 10.7	33.3 ± 4.3	**19.4 ± 2.4***	**19.1 ± 7.3***	**20.1 ± 1.1***
Liver [Cu]	5.4 ± 2.0	8.7 ± 1.8	8.9 ± 1.4	**10.4 ± 1.7***	8.5 ± 2.3	**13.3 ± 4.2***	8.5 ± 2.6
Liver [Zn]	38.3 ± 11.2	61.1 ± 29.1	54.8 ± 18.3	44.3 ± 18.6	52.1 ± 13.8	40.0 ± 17.0	**20.4 ± 7.8***
Intestine [Cu]	2.8 ± 0.6	3.2 ± 0.4	3.3 ± 0.8	3.1 ± 1.1	2.6 ±0.9	**3.9 ± 0.8***	2.9 ± 0.5
Intestine [Zn]	19.5 ± 5.2	22.5 ± 0.5	31.4 ± 9.8	26.4 ± 15.3	24.4 ± 4.2	31.3 ± 13.6	21.8 ± 6.1

Values are means ± S.D. n=5, asterisks indicate a significant (P<0.05; ANOVA, Tukeys test) difference from control experimental diet at day 15 or day 30. Copper and zinc content in the tissues diet and water was determined by AAS.

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Human       MDHS.HHMGMSYMDSNSTMQP.....SHHHPTTSASHSHGGGDSSMMMMPMTF YFGFKNVELLFSG
Chick       ..MS.HHMMNSSMLP..TTHP.....EHHHSTASSGGGHGSG...MMMMAMTF HFSYENVPLLFSG
Lizard      ..MSNHHMAHMGHDMNSSTTPTPDPHAHHHAMTTAGHIHDGG...MMMMHMTF YFSYNNVPLLFSG
Frog        .......MDHSHHTVT.TSDPHG...GHHHPTTSGNHGDHGG..SMHMMQMTF YFGYENVEVLFTG
Pufferfish  .......MDHSHHLSHPMATPTSGGHDHGG..DGSSGGHGGHMG....MAMTF YVGYHNVELLFSG
Seabream    .......MDHSHHHHPTMAPPTTSGHDHGGGHGGNQGGHEGHGG....MVMTF YFGYNNVELLFTG
Zebrafish   .....MDSSHSHHHVEETTMPSPASGDHGGHLTTTGNGHGDH...MMMMQMTF YFGYKNVELLFAG
                                                               ******
                                                             Met motif

Human       LVINTAGEMAGAFVAVFLLAMFYEGLKIARESLLRKSQVSIRYNSMPVPGPNGTILMETHKTVGQQ
Chick       LTINTPGEMAGAFVAVFFLAMFYEGLKIARECLLRKSQVSIRYNSMPVPGPNGTILMETHKTVGQQ
Lizard      LVINTAGEMAGRFVAIFLLAMFYEGLKIARESLLRKSQVSIRYNSMPVPGPNGTVLMETHKTVGQQ
Frog        LVINSAGEMAGAFVAVFLLALLYEGLKISREALLRKSQVSIRYNSMPVPGPNGTILMETHKTVGQR
Pufferfish  LVINSAGEMVAACIGVFLLAILYEGLKIGREVLLRRSQVNVRYNSMPLPGTDGTVLMETHKTVGQR
Seabream    LLINTPGEMVGACIGVFLLAVLYEGLKMGRETLLRRSQVNVRYNSMPLPGADGTVLMETHKTVGQR
Zebrafish   LVINTPGEMAGACIGVFLLAVLYEGLKIGREVLLRRNQVNVRYNSMPVPGSDGTVLMETHKTVGQR
                *************************
                                    TMD1

Human       MLSFPHLLQTVLHIIQVVISYFLMLIFMTYNGYLCIAVAAGAGTGYFLFSWKKAVVVDITEHCH
Chick       MLSFPHLLQTVLHIIQVVVSYFLMLIFMTYNGYLCIAVAAGAGMGYFFFSWKKAVVVDITEHCH
Lizard      MLSFPHLFQTALDIVQVVVSYFLMLIFMTYNGYLCIAVAAGAGTGYFLFSWKKAVVVDITEHCH
Frog        MLSVPHLLQTLLHIIQVVVSYFLMLIFMTYNAYLCIAVAAGAGTGYFLFSWKKAVVVDITEHCH
Pufferfish  MLSPAHFLQTFLHIIQVAVSYILMLVFMTYNVYLCIAVALGAGMGYFLFSWRKAVVVDITEHCH
Seabream    MLSPAHFLQTVLHIVQVVVSYFLMLVFMTYNAYLCIAVAAGAGMGYFLFSWRKAVVVDITEHCH
Zebrafish   MLSMAHFLQTLLHIIQVVVSYFLMLVFMTYNGYLCIAVAAGAGLGYFLFSWKKAVVVDITEHCH
                *******************  ****************       ***
                    TMD2                      TMD3            His/Cys motif
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Figure 1. Alignment of vertebrate Ctr1 polypeptide sequences.
Residues identical in all proteins are shaded. The positions of the conserved methionine-rich copper binding motif (Met motif), transmembrane domains (TMD1-3) and the C-terminal HCH motif referred to in the text are indicated. The residues shown to be required for the activity of human Ctr1 are boxed
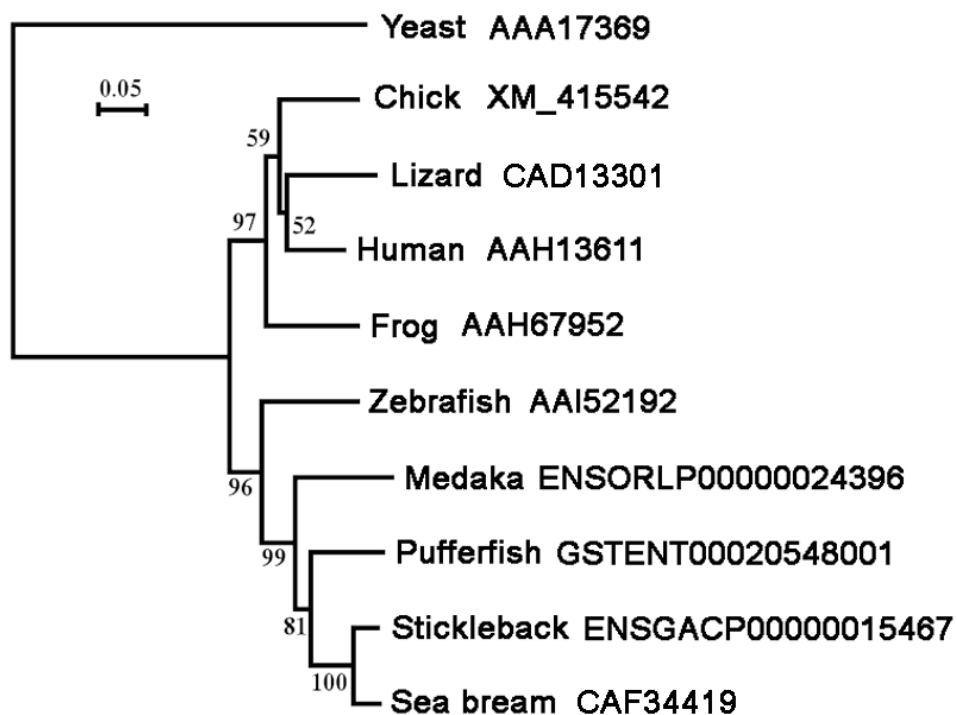
Figure 2. Phylogenetic relationships of vertebrate Ctr1 polypeptide sequences.
Sea bream (*Sparus aurata*), medaka (*Oryzias latipes*), stickleback (*Gasterosteus aculeatus*), zebrafish, (*Danio rerio*), pufferfish, (*Tetraodon nigroviridis*) along with human, frog, (*Xenopus tropicalis*) lizard, (*Podarcis sicula*), chick (*Gallus gallus*) and yeast (*Saccaromyces cerevisae*) Ctr1 were used to generate the phylogenetic tree using ClustalW. Numbers (bootstrap values) represent the percentage of times the associated branch topology was returned after 1000 iterations of tree generation. The Genbank accession numbers or the ENSEMBL peptide ID for the protein sequence of each species is provided.
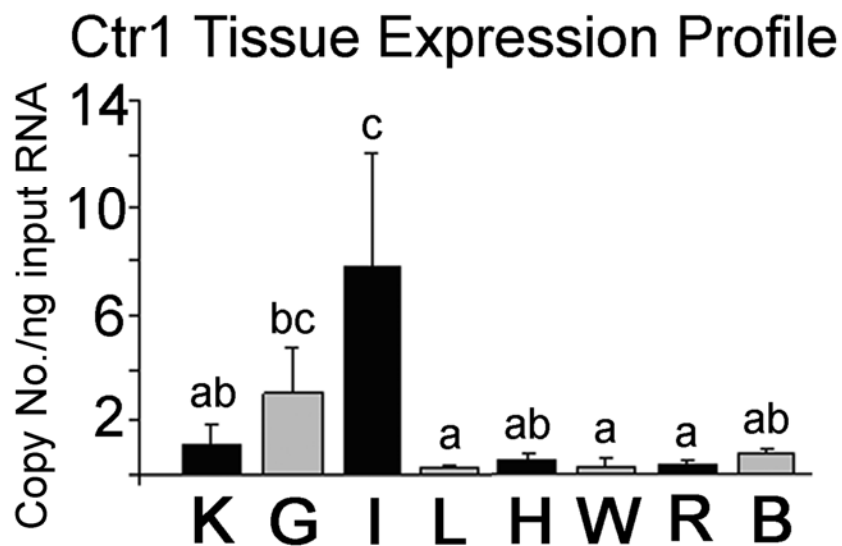
Figure 3. Tissue expression profile of saCtr1 mRNA in sea bream fed a commercial diet.
Data are presented as mean ± standard deviation (n=5 fish/replicate).Means bearing different
lettering are significantly different between tissues (p<0.05, ANOVA, Tukeys test). Data
shown are day 15 Diet2 fish. L, liver; K, kidney; G, gill; I, intestine; B, brain; W, white
muscle; R, red muscle; H, heart. "The data shown represent the fish fed Diet 2 (7.7±0.3 mgKg[-1] Cu) for 15 d.
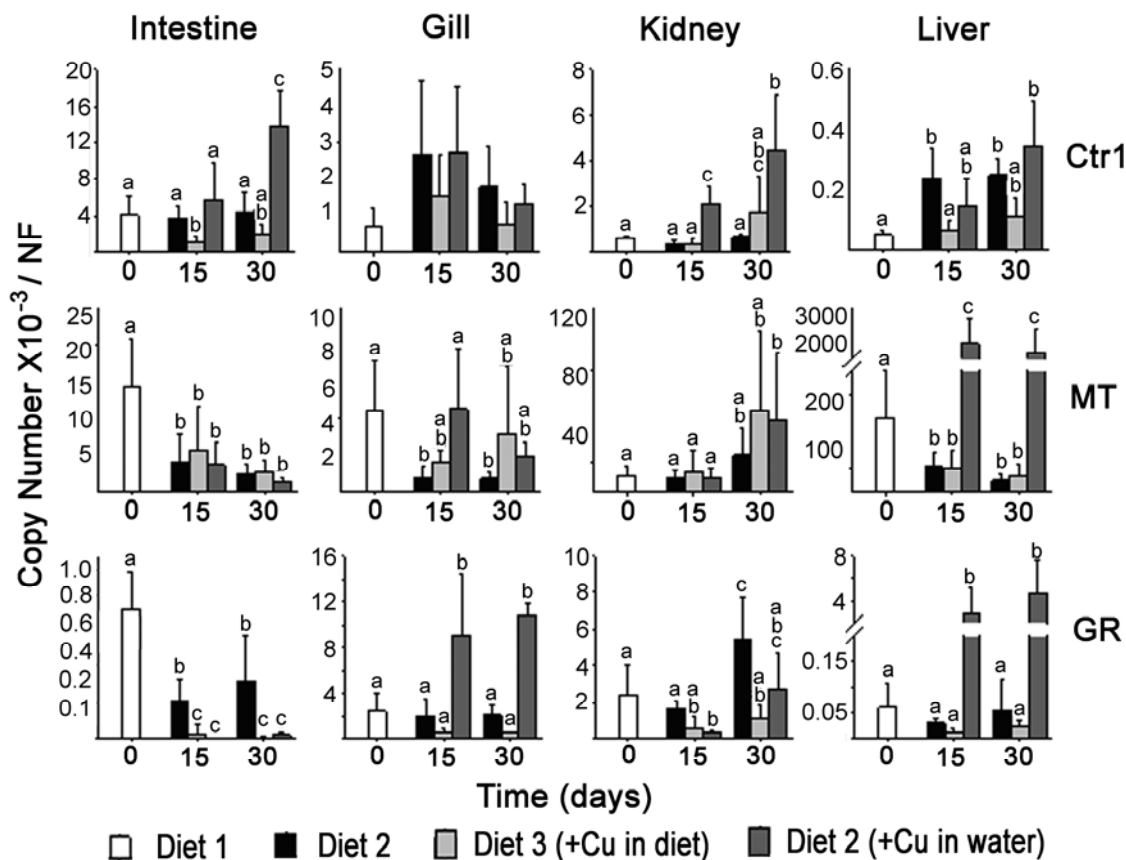
Figure 4. Tissue- and copper-specific regulation of sea bream Ctr1, MT and GR mRNA. Ctr1, MT and GR mRNA levels were measured in fish after 15 and 30d fed an experimental diet (7.7±0.3 mgKg^{-1} Cu, Diet2), or a diet containing excess copper (130mgKg-1, Diet3), or after exposure to excess waterborne copper (0.3mgL^{-1}, Diet2+Cu in water). Fish at day 0 had been maintained on a normal commercial diet (12.6 ± 0.2 mgKg^{-1} Cu, Diet1). Ctr1, MT and GR copy numbers were normalized by geNorm using a normalization factor (NF) based on the geometric mean of β-actin, EF1α or GAPDH reference genes. Values are means ± S.D. N=5. Bars bearing different lettering are significantly different (P<0.05; ANOVA, Tukeys test).