# Zinc transporter gene expression in pigs with subclinical zinc deficiency

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# Abstract

This study compared the relative mRNA expression of all mammal zinc (Zn) transporter genes in selected tissues of weaned piglets challenged with short-term subclinical Zn deficiency (SZD). The dietary model involved restrictive feeding (450 g/animal*day$^{-1}$) of a high-phytate, diet (9 g/kg) supplemented with varying amounts of zinc from $ZnSO_4$*$7H_2O$ ranging from deficient to sufficient supply levels (total diet Zn: 28.1, 33.6, 38.8, 42.7, 47.5, 58.2, 67.8, 88.0 mg Zn/kg). Total RNA preparations comprised jejunal and colonic mucosa as well as hepatic and nephric tissue. Statistical modelling involved broken-line regression ($P \leq 0.05$). ZIP10 and ZIP12 mRNAs were not detected in any tissue and ZnT3 mRNA was only identified in the kidney. All other genes were expressed in all tissues but only a few gene expression patterns allowed a significant ($P < 0.0001$) fitting of broken-line regression models indicating homeostatic regulation under the present experimental conditions. Interestingly, these genes could be subcategorized by showing significant turnarounds in their response patterns, either at 40 or 60 mg Zn/kg diet ($P < 0.0001$). In conclusion, the present study showed clear differences in Zn transporter gene expression in response to SZD compared to the present literature on clinical models. We recognized that certain Zn transporter genes were subject to homeostatic regulation under the present experimental conditions by two distinct homeostatic regulation networks. For the best of our knowledge, this represents the first comprehensive screening of Zn transporter gene expression in a highly translational model to human physiology.

Key words: zinc, transporter, ZnT, ZIP, pig

# 1 Introduction

Basic cellular processes are dependent on zinc (Zn) as a structural cofactor of peptides (for example transcription and replication of DNA or maintenance of DNA integrity). In fact, at least 10% of the genes in the human genome encode Zn peptides, highlighting its ubiquitous importance for the mammal organism [1]. In contrast, Zn has a strong toxic potential if the concentration within a biological system exceeds a certain threshold [2]. Therefore, the regulation of Zn uptake, redistribution and excretion within an organism must be tightly controlled.

A complex molecular network that modulates the expression of specific Zn transport peptides to maintain metabolic function under changing dietary and physiological conditions maintains mammalian Zn homeostasis. So far, 24 Zn transporters have been described in mammals mainly based on experiments in rodents and human biopsies. These transporters belong to the solute carrier (SLC) families 30 (ZnT) and 39 (ZIP). Currently, 10 ZnT and 14 ZIP transporters have been described [3].

An increasing body of evidence suggests that ZnT and ZIP transporters differ regarding their transport mechanism as well as the direction of Zn transport. The ZnT transporters seem to remove $Zn^{2+}$ from the cytosol, by either facilitating Zn uptake into subcellular compartments or excretion into the extracellular space. In contrast, ZIP transporters increase cytosolic Zn by promoting $Zn^{2+}$ influx from the extracellular space or subcellular compartments, respectively. Zinc transporters also express differences regarding their tissue specificity, subcellular localization as well as the regulative stimuli to which they respond. Furthermore, differences in response patterns of certain transporters have been reported depending on the biological model used for the investigations [4-7].

Subclinical zinc deficiency is probably the most common form of zinc malnutrition in humans and animals [8]. However, it has not yet been thoroughly investigated. In fact, many of the original experimental *in vivo* studies on the role of zinc in metabolism and nutrition included control groups expressing symptoms of clinical zinc deficiency. Under such conditions, however, the compensation capacities of the metabolism, and in particular the mobilizable Zn pools in the skeleton and the soft tissues, are exhausted [9-12]. This results in

the quite unspecific set of visible symptoms of Zn malnutrition (e.g. growth depression, anorexia, developmental disorders, tissue necrosis etc.) [13] and as a result, an increased background noise from metabolic measurements. In addition, this represents the endpoint in adapting metabolic processes to an ongoing dietary zinc deficiency, which can reduce the informative value of quantitative measurements of Zn-homeostatic mechanisms at the level of the whole organism [14].

To promote translational research on SZD, we have developed an experimental model to promote this phenotype in pigs. It allows high-resolution analyses of the kinetics and dynamics of zinc in the growing pig organism, from the level of the quantitative metabolism to the subcellular level. At the same time, it does not promote any change in the health status of the animals based on continuous veterinary surveillance. The previous findings revealed the adjustment of the quantitative zinc metabolism to a short-term (8d), finely graded reduction in the alimentary zinc intake. Based on this, we were able to derive the gross zinc requirement under the given experimental conditions at 60 mg / kg diet [15]. Already in this early phase of zinc deficiency, various pathophysiological reactions at the metabolic and subclinical level became evident. These included a reduction in pancreatic digestive capacity [16, 17] and cardiac redox capacity [18]. The latter was accompanied by an increased need for the detoxification of reactive oxygen species and the activation of pro-apoptotic signaling pathways when the alimentary zinc supply was ~40 mg / kg diet and below for a period of 8 days [18]. Furthermore, we were able to show that certain tissues that are important for the acute survival of the developing organism (heart, skeletal muscle, thymus, mesenteric lymph nodes, pancreas) replenished their initially depleted zinc concentrations at the expense of other organs or even accumulated Zn above the level of the control group. The critical threshold for these compensatory reactions was also 40 mg Zn / kg diet [19]. Overall, these findings suggested two independent Zn-homeostatic regulatory pathways that act in this early phase of developing zinc deficiency: 1) the regulation of the absorption, redistribution and excretion of zinc in and out of the organism as a result of a decrease in zinc supply below the requirement threshold (~60 mg / kg diet) and 2) the compensation of increased oxidative stress and perhaps inflammatory status as a result of an ongoing nutritional zinc deficiency (critical threshold ≤ 40 mg / kg diet for 8d).

The present study tested this hypothesis on the transcriptomic level of the known ZnT and ZIP genes in important tissues of zinc homeostasis (jejunal and colonic mucosa, liver, kidney). To the best our knowledge, this dataset represents the first comprehensive examination of ZnT and ZIP gene expression in the translational model of the pig. Also, it appears to be the first investigation on zinc transporter gene expression patterns under the terms of such a high-resolution dose-response study *in vivo*.

# 2 Material and Methods

This animal study was evaluated and approved by the animal welfare officer of the faculty TUM School of Life Sciences Weihenstephan, Technical University of Munich, and further approved and registered by the responsible animal welfare authorities (District Government of Upper Bavaria, Federal State of Bavaria: case number 55.2.1.54-2532.3.63-11).

The study design as well as communication of material, methods and results comply to the ARRIVE Guidelines [20]. The dietary model applied for this investigation was carefully developed to allow in-deep physiological research in a large translational animal model with a minimum of animals (n = 6 replicates / treatment group). For further details on the dietary model and the statistical consideration during study preparation see Brugger, Buffler [15] and the subsection on statistical analyses, respectively.

## 2.1 Animals and diets

This study applied the experimental SZD model originally proposed by Brugger, Buffler [15], in which weaned piglets are adapted to a phytate-rich basal diet and subsequently treated with varying dietary Zn supplementation for 8d. Therefore, a total of forty-eight fully weaned piglets from six litters (hybrids of German Large White x Land Race x Piétrain, 8 animals per litter, 50% male-castrated, 50% female, initial average body weight 8.5 ±0.27 kg, 28 d of age, supplier: pig farm of Christian Hilgers (Germany) were individually housed. To ensure full body Zn stores at day one of the experimental period, a basal diet (based on corn and soybean meal, dietary phytate (InsP6) concentration 9 g/kg) with a native Zn concentration of 28.1 mg/kg was supplemented with 60 mg Zn/kg from analytical-grade $ZnSO_4 * 7H_2O$ to adjust the total dietary Zn at a sufficient level of 88.0 mg/kg diet [21]. This diet was provided to all animals ad libitum during an acclimatization phase of 14d prior to the onset of the experiment. Subsequently, all animals were assigned to eight dietary treatment groups in a complete randomized block design [22]. Blocking parameters comprised live weight, litter mates and sex (50% male-castrated, 50% female), thereby yielding a good standardization of body development and genetic background between treatment groups. The treatment groups were fed restrictively the same basal diet as during the acclimatization period (450 g/d representing the average *ad*

*libitum* feed intake at the last day of acclimatization) but with varying dietary Zn concentrations spanning the range from deficient to sufficient dietary supply levels in high resolution. This was achieved by differential supplementation of analytical-grade $ZnSO_4$ * $7H_2O$ (+0, +5, +10, +15, +20, +30, +40, +60 mg added Zn/kg diet; analyzed dietary Zn contents: 28.1, 33.6, 38.8, 42.7, 47.5, 58.2, 67.8, 88.0 mg Zn/kg diet). The group receiving 88.0 mg Zn/kg diet served as control, because it represented the feeding situation during the acclimatization phase from which the dietary Zn contents for all other groups were gradually reduced.

The basal diet was designed to meet all the recommendations of the National Research Council regarding the feeding of weaned piglets except for Zn [21]. Table 1 presents detailed information on the composition and ingredients of the basal diet. Analytical Zn recovery of added Zn from the experimental diets as an indicator of the mixing precision was literally 100% as shown by a highly significant slope of 0.99 mg increase in total analyzed dietary Zn per mg Zn addition to the diets ($P < 0.0001$, $R^2 = 1.00$, data not shown). All diets were pelletized at 70°C with steam to stabilize feed particle size distribution, improve feed hygiene and deactivate native phytase activity originating from plant raw components.

All animals had access to drinking water *ad libitum* during all times of this study and were subject to continuous veterinary surveilance.

## 2.2 Sampling conditions

Diet samples were collected and processed as described previously [15]. At experimental day 8, all animals were killed by exsanguination under anesthesia (azaperone and ketamine) without fasting and tissue samples were taken from jejunal and colonic mucosa as well as liver and kidney. Tissue samples for gene expression analyses were immediately incubated in RNAlater (Life Technologies GmbH) overnight and subsequently stored at -80°C according to the manufacturer´s instructions.

## 2.3 Analyses of dry matter and total zinc concentration in diets

Analyses of dry matter and Zn in diets occurred as described earlier [15]. Zn concentrations were measured by atomic absorption spectrometry (NovAA 350; Analytik Jena AG) applying certified AAS Zn standard material (Merck 109953, Merck Millipore) after microwave wet digestion (Ethos 1, MLS GmbH).

## 2.4 Gene expression analysis

Primer design, assay quality control and chemical procedures (total RNA extraction, reverse transcription, quantitative PCR (qPCR)) were performed as described earlier[15, 18]. Purity (measured with the NanoDrop 2000 system, Thermo Scientific) and integrity (measured with the Experion system, Biorad) of all total RNA extracts from all tissues met or exceeded the minimum thresholds necessary for gene expression profiling applying qPCR methodology [23, 24]. Primer pairs (Eurofins Scientific) were designed with Primer Blast [25] for the potential reference transcripts glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-glucuronidase (GUSB), histone H3 (H3), ubiquitin C (UBC), β-actin (ACTB) and divalent metal transporter 1 (DMT1) as well as the target transcripts SLC30 (ZnT) 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and SLC39 (ZIP) 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, based on published porcine sequence information [3] (Supplementary Tables 1 and 2). All oligonucleotides bind to homologous regions of respective transcripts to amplify the pool of potential transcript variants within one reaction. We identified *ZnT5, ZnT6* and *ZIP9* in jejunum, *DMT1, ZIP1* and *ZIP7* in colon, *DMT1, ZIP7* and *ZIP13* in liver as well as *ZnT4, ZnT5* and *ZnT6* within the kidney as suitable reference genes by applying earlier published statistical approaches [26]. The $2^{-\Delta\Delta Ct}$ method [27] was used to normalize the gene expression data because the determination of the amplification efficiency revealed comparable values between 95% and 100% of applied RT-qPCR assays. A detailed description of the procedure for the determination of amplification efficiency has been provided earlier[15].

*ZIP10* and *ZIP12* transcripts were not detected in any of the porcine tissues examined within the present study. These assays amplify sequences which appear to be highly conserved between mammal species. Therefore, murine brain and liver cDNA preparations were used for testing.

## 2.5 Statistical analyses

Data analysis was performed with SAS 9.4 (SAS Institute Inc.) applying the procedure NLMIXED to estimate linear broken-line regression models ($y = a + bx + cx$) based on independent group means relative to dietary Zn concentration ($n = 8$). The decision for linear broken-line models over non-linear models was made by following the approach proposed by McDonald [28] by which the goodness-of-fit of linear vs. polynomial models is statistically compared using F-statistics. In case of our dataset we found that applying non-linear models over the linear broken-line models yielded no significant increase in the quality of the curve-fitting. Furthermore, using single datapoints from individual animals instead of the group mean values was not advisable in light of the present experimental design because the imbalance in the ratio of X (eight dietary treatment groups) to Y (six response values per treatment group) coordinates would have caused a severe overestimation of the degrees of freedom and, therefore, false results. Broken-line regression is an iterative procedure to estimate a potential statistical threshold (breakpoint) within non-linear data sets above and below which a significant difference in the response behavior of a certain parameter to the dietary treatment is evident [29]. If no significant breakpoint in parameter response could be estimated from a certain data set, a linear regression model was tested instead (y= a + bx) (procedure REG). It is noteworthy, that none of the observed gene expression patterns fitted a significant linear model. Only significant regression models were applied for data presentation and interpretation in the present manuscript. A threshold of $P \leq 0.05$ was considered to indicate statistical significance. All $2^{-\Delta\Delta Ct}$ gene expression values [27] were presented as x-fold differences compared to a relative mRNA abundance of 1.0 (not regulated) within the control group (88.0 mg Zn/kg diet).

The justification of the total sample size for the present experiment was done prior to the study by power analysis with the software package G*Power 3.1.9.6 [30] assuming a two-factor model (8 treatment groups, 6 experimental blocks) including interactions (treatment*block) and a strong biological effect. Based on this analysis it has been concluded that forty-eight animals (n = 6 replicates/treatment group) in a completely randomized block design are sufficient to meet the generally accepted minimum statistical power of $1 - \beta = 0.8$ [31]. The correctness of these assumptions were confirmed by estimating the power of the applied

regression models and associate T-statistics on regression parameters, which met in any case the necessary minimum of $1-\beta = 0.8$.

# 3 Results

All animals remained in good health throughout the whole trial. There were no signs of clinical Zn deficiency (for example growth retardation, anorexia [13]) evident at any time [15].

## 3.1 Tissue specificity of *ZnT* and *ZIP* transcripts in weaned piglets challenged with finely graded differences in zinc supply status

Table 2 highlights the qualitative expression pattern of analysed transcripts within respective tissues. Most of the analysed *ZnT* and *ZIP* transcripts were abundant within the tissues examined in the present study. This excludes *ZIP10* and *ZIP12*, which were not expressed in any of the tissues as well as *ZnT3*, which was only recognised within the kidney. Testing *ZIP10* and *ZIP12* assays in murine liver and brain cDNA preparations yielded positive results and excluded technical problems to be the cause of negative results derived in porcine cDNA from jejunum, colon, liver and kidney, respectively. Some transcripts (*ZnT5*, *ZnT6* and *ZIP9* in jejunum, *ZIP1* and *ZIP7* in colon, *ZIP7* and *ZIP13* in liver as well as *ZnT4*, *ZnT5* and *ZnT6* in kidney) were expressed in such a highly stable manner over treatment groups that they served as reference genes for data normalisation (based on data analyses using earlier published statistical approaches [26]).

## 3.2 Effects of varying dietary zinc supply on the relative *ZnT* and *ZIP* transcript abundance in examined porcine tissues of weaned piglets challenged with finely graded differences in zinc supply status

Many transcripts recognized within the jejunum, colon, liver and kidney of growing piglets showed significant dietary thresholds in response to changes in dietary Zn supply. This was evident by significant breakpoint parameter estimates ($P \leq 0.05$ for X and Y intercepts of respective breakpoints). The only exceptions were *ZIP2* and *ZIP3* in colonic tissue as well as the candidate genes that served as reference genes for data normalisation within respective tissues. Significant dietary thresholds either lay at ~40 or ~60 mg Zn/kg diet, respectively. However, the slopes of the respective segments within many broken-line regression models were

not significant and coefficients of determination of respective models were low ($R^2$). Subsequently, only models expressing at least one significant slope over changes in dietary Zn supply are described within figures and tables.

Figure 1 presents the broken-line response of jejunal Zn transporter gene expression as affected by varying dietary Zn supply. Table 3 presents the corresponding statistical measures of the respective regression curves. Above significant dietary thresholds of 57.1, 62.3, 38.8, 41.6, 62.6 and 52.3 mg Zn/kg diet ($P < 0.0001$, respectively) jejunal *ZIP5* and *ZIP11* significantly increased or decreased, respectively, in response to changes in dietary Zn ($P \leq 0.05$, respectively) whereas *ZIP1* and *ZIP13* did not change in a significant manner. Below these thresholds, the relative mRNA abundance of *ZIP1* and *ZIP13* significantly increased whereas *ZIP11* significantly decreased with further reduction in dietary Zn supply ($P \leq 0.001$, $\leq 0.05$ and $\leq 0.0001$, respectively). The *ZIP5* gene expression did not change significantly with stepwise decrease in dietary Zn concentration below its respective breakpoint.

Figure 2 presents the broken-line response of colonic Zn transporter gene expression as affected by varying dietary Zn supply. Table 4 presents the corresponding statistical measures of the respective regression curves. Relative mRNA abundance of *ZnT4, ZnT9, ZIP4, ZIP5, ZIP7, ZIP11* and *ZIP13* showed significant breakpoints in response to a finely graded reduction in dietary Zn concentration at 60.6, 63.9, 59.6, 39.0, 42.7, 44.8 and 68.3 mg Zn/kg diet, respectively ($P \leq 0.0001$, respectively). Above the respective dietary thresholds, *ZIP4, ZIP5* and *ZIP7* plateaued in response to changes in dietary Zn. These genes significantly increased their relative expression levels in response to further reduction in dietary Zn below these breakpoints ($P \leq 0.001$, $\leq 0.001$ and $\leq 0.05$ for *ZIP4, ZIP5* and *ZIP7*, respectively). On the contrary, colonic *ZnT4* and *ZIP11* significantly increased ($P \leq 0.05$ and $\leq 0.001$, respectively) whereas *ZnT9* and *ZIP13* significantly decreased ($P \leq 0.05$, respectively) with reduction in dietary Zn concentration from 88.0 mg Zn/kg to their respective breakpoints. Below these dietary thresholds, *ZnT4* and *ZIP11* significantly decreased ($P \leq 0.001$ and $\leq 0.05$, respectively) whereas *ZnT9* and *ZIP13* did not change significantly.

Figure 3 presents the broken-line response of hepatic Zn transporter gene expression as affected by varying dietary Zn supply. Table 5 presents the corresponding statistical measures of the respective regression curves.

Gene expression patterns of *ZnT4, ZnT6, ZnT8, ZIP1* and *ZIP14* exhibited significant changes in their response to varying dietary Zn supply at breakpoints of 48.4, 38.8, 57.3, 47.5 and 42.7 mg Zn/kg diet, respectively ($P \leq 0.0001$). Above the respective dietary thresholds, *ZnT4, ZnT8, ZIP1* and *ZIP14* did not change significantly in response to changes in dietary Zn supply whereas *ZnT6* significantly decreased directly to a reduction in dietary Zn from 88.0 mg Zn/kg diet to the respective breakpoint ($P \leq 0.001$). On the contrary, *ZnT4* and *ZnT6* significantly decreased ($P \leq 0.001$ and $\leq 0.05$, respectively) whereas *ZnT8, ZIP1* and *ZIP14* significantly increased ($P \leq 0.05$, $\leq 0.05$ and $\leq 0.0001$, respectively) with reduction in dietary Zn concentration below the respective dietary thresholds.

Figure 4 presents the broken-line response of nephric Zn transporter gene expression as affected by varying dietary Zn supply. Table 6 presents the corresponding statistical measures of the respective regression curves. Gene expression of *ZnT1, ZnT3, ZnT7* and *ZIP4* changed significantly around dietary thresholds of 70.4, 42.6, 35.2 and 41.9 mg Zn/kg diet ($P < 0.0001$, respectively). All these genes plateaued in response to a reduction of dietary Zn concentration from 88.0 mg/kg to the respective breakpoints. Further reduction in dietary Zn below these thresholds promoted a significant increase of *ZnT3, ZnT7* and *ZIP4* ($P \leq 0.05$, $\leq 0.0001$ and $\leq 0.05$, respectively) as well as a significant decrease of *ZnT1* gene expression ($P \leq 0.05$).

# 4 Discussion

We investigated the gene expression response of the currently known members of the ZnT (SLC30) and ZIP (SLC39) family of Zn transporter genes in weaned piglets, challenged with finely graded differences in dietary Zn concentration (ranging from deficient to sufficient dietary concentrations: 28.1 to 88.0 mg Zn/kg). We chose the jejunal and colonic mucosa as well as liver and kidney as target tissues for the analyses because they represent important hubs in the complex network of body Zn acquisition, (re)distribution and excretion [32]. Pancreatic gene expression could not be investigated in the present study due to technical issues.

## 4.1 Specificity of ZnT and ZIP family member gene expression for selected porcine tissues

Most of the transcripts were present in all tissues investigated. The only exceptions included *ZIP10* and *ZIP12*, which transcripts were completely absent within any of our porcine cDNA preparations. Furthermore, *ZnT3* mRNA was only detected in nephric tissue.

According to RNA sequencing experiments in humans and drosophila, *ZIP12* gene expression is limited to the brain [33, 34]. Furthermore, studies on its functional genomics and proteomics highlighted its role in neuronal development and function [35, 36] as well as the schizophrenic brain [37]. Hence, the absence of *ZIP12* cDNA in any of our porcine tissue preparations is in good agreement with the present state of knowledge. Otherwise, the absence of *ZIP10* cDNA strongly contradicts earlier studies. In fact, ZIP10 transcription has been identified in a variety of tissues including such examined in the present study [33, 34]. Apart from its role in cancer progression, specifically its function as a zinc transporter within the renal proximal tubule and collecting duct system [38-40] has been studied in various species including humans, which potentially highlights its involvement in reabsorption of $Zn^{2+}$ from primary urine. Hence, the question arises why we were not able to identify its mRNA signature in any of our samples including those of the kidney. Our *ZIP10* qPCR assay was designed to amplify an area on the porcine mRNA sequence that is conserved also in other mammals including rodents and humans [41] and it successfully worked with murine brain and liver preparations (data not shown). Furthermore, we did not sample from specific sections of the kidney but tried to achieve a representative cross-section of this organ for the total RNA preparation.

Therefore, we conclude that the absence of *ZIP10* transcripts in the porcine tissues may not be due to technical bias and that the growing pig does not express this gene in the tissues sampled for the present study, at least under the given experimental conditions. However, this should be confirmed in future studies comprising microdissection of different parts of the kidney and associated analysis of *ZIP10* mRNA expression.

*ZnT3* expression has been recognized earlier in several organs including brain, adipose tissue, pancreatic beta-cells, epithelial cells, testis, prostate and retina [42]. Until now, the majority of studies focused on its role in transporting cytosolic Zn into synaptic vesicles [43]. Interestingly, this also involved enteric neurons within the gastrointestinal tract of pigs [44, 45]. The absence of *ZnT3* gene expression in any cDNA obtained from porcine jejunum and colon during the present study may have been because these samples represented the mucosal layer. To the best of our knowledge, no study has yet examined *ZnT3* gene expression within liver and kidney of pigs. Therefore, this may be the first report on the absence of hepatic *ZnT3* gene expression and its detection within nephric tissue of growing pigs, respectively.

The detection of gene expression signatures of all the remaining zinc transporter genes within the tissues investigated appeared to be in good agreement with the available literature (summarized in [4-7])

## 4.2 Response of *ZnT* and *ZIP* family member gene expression to changes in the dietary zinc concentration

Certain mammalian Zn transporter mRNAs have been earlier demonstrated to be directly affected by deficient dietary Zn supply, including *ZnT1, ZnT2, ZnT4, ZnT5, ZnT6, ZIP4* and *ZIP10* [39, 46-48]). In the present study, we could confirm this for *ZnT1* (kidney), *ZnT4* (colon, liver), *ZnT6* (liver) and *ZIP4* (colon, kidney) but not *ZnT2, ZnT5* and *ZIP10*. Furthermore, we identified several other transcripts to respond to deficient dietary Zn supply that have to our knowledge not been reported so far, including *ZnT3* (kidney), *ZnT7* (kidney), *ZnT8* (liver), *ZnT9* (colon), *ZIP1* (jejunum, liver), *ZIP5* (jejunum, colon), *ZIP7* (colon), *ZIP11* (jejunum, colon), *ZIP13* (jejunum, colon) and *ZIP14* (liver). Most of the earlier studies used models of clinical Zn deficiency. Therefore, our data seem to highlight differences in the physiological adaption of Zn transporter gene expression to short-term SZD. Indeed, clinical Zn deficiency is associated with a multitude of secondary

metabolic events during which the integrity of tissues may be impaired [13]. This could hamper the resolution of measurements due to increased background noise. Finally, clinical Zn deficiency represents an endpoint in physiological adaption to body Zn depletion. Hence, early response patterns may have already been changed, which also explains why some results from the present study (with a total experimental period of only 8d) have not been described earlier.

Several of the analyzed *ZnT* and *ZIP* gene expression patterns revealed highly significant breakpoints in response to changes in dietary Zn concentration. However, only the response of genes presented in the present figures and tables allowed a significant curve-fitting. We hypothesize that only these genes were regulated directly by changes in the organisms Zn supply status in response to the dietary treatment. On the contrary, all other Zn transporter genes with only significant breakpoints may have just indirectly adapted to the changes in Zn fluxes initiated by these key transporters and are therefore not further demonstrated or discussed within this manuscript.

Interestingly, all genes which expressed a significant non-linear dose-response behavior to the dietary treatment could be subcategorized into two groups, which showed statistical breakpoints at either around 60 mg Zn/kg diet or 40 mg Zn/kg diet, respectively. The gross Zn requirement (net zinc requirement plus necessary minimum supplementation to compensate for negative phytate effects) under given experimental conditions was earlier estimated to be ~60 mg Zn/kg diet [15]. Therefore, it may be hypothesized that the presented *ZnT* and *ZIP* genes which expressed a statistical breakpoint of ~60 mg Zn/kg diet, are involved in the regulative network that ensures the satiation of the temporary whole-body demand for Zn. A prominent example was colonic *ZIP4* gene expression, which plateaued in groups receiving dietary Zn concentrations >60 mg/kg. However, a decrease in dietary Zn concentration below this threshold induced a linear upregulation of *ZIP4* gene expression with further decline in dietary Zn supply. In fact, this response pattern correlates positively to earlier data on the response of apparently digested feed Zn under the present experimental conditions ($r = -0{,}91$; $P = 0.002$; data not shown) [15]. This nicely reflects the classical concept of Zn homeostasis according to which the organism is increasing its Zn absorption capacity at the gut barrier during periods of Zn malnutrition [49]. It is also in agreement with the earlier published work that identified

the intestinal ZIP4 as the major active transport route for luminal Zn into the enterocytes of mammals [48, 50, 51]. This again confirms this gene´s role within the homeostatic network that controls the body Zn level and especially the Zn absorption in swine and other mammals. Interestingly, we did not identify any significant reaction of the *ZIP4* mRNA abundance in jejunal tissue relative to changes in dietary Zn supply. The jejunum is generally considered to be the main side of Zn absorption [52]. Hence, we expected a significant up-regulation of *ZIP4* mRNA abundance in jejunal before colonic mucosal RNA preparations as has been shown earlier in mice fed low Zn diets [51, 53-55]. This was not the case, which seem to highlight again clear differences between models of subclinical compared to clinical Zn deficiency. Considering the already mentioned broken-line response of colonic *ZIP4* gene expression to changes in dietary Zn supply together with its strong positive correlation to the net absorption of feed Zn, we hypothesize that under the conditions of a short-term subclinical Zn deficiency the colon is the major side of Zn acquisition from the intestinal lumen. This has already been proposed by other authors, which recognized peaks in the expression of Zn responsive genes as affected by increased cytosolic $Zn^{2+}$, under the terms of mild Zn deficiency in adult rats [56, 57]. Furthermore, there are reports of a significant contribution of caecal and colonic Zn absorption in times of reduced Zn acquisition from the small intestine [58, 59]. We demonstrated earlier a decrease in pancreatic digestive capacity under the present experimental conditions [16, 17]. Therefore, we conclude that a shift of the main site of Zn absorption to the large intestine may have happened in favor of Zn-dependent digestive enzymes like carboxypeptidases A and B [60], thereby stabilizing the already impaired protein digestion within the small intestine under the present experimental conditions. However, this must be further investigated in appropriate follow-up studies involving e.g. Ussing chambers and patch-clamp techniques.

With regard to those genes with a statistical breakpoint close to 40 mg Zn/kg diet, we postulate these may play a key role in the regulation of tissue Zn in context to redox and immune functions. Indeed, it has been demonstrated earlier that under the present experimental conditions the heart muscle of Zn deficient piglets restored its initially depleted total Zn concentration. This was probably due to an up-regulation of transporter proteins importing Zn from the circulation into the tissue. It seemed to occur in response to increased cardiac oxidative stress during SZD, in groups fed <~43 mg Zn/kg diet [18]. Therefore, breakpoints of Zn transporter

genes around ~40 mg Zn/kg diet may indicate similar events in other tissues. A prominent example for a stress/inflammation responsive Zn transporter is the hepatic ZIP14. This parameter has been earlier identified as an acute-phase protein that transports circulating Zn as well as non-transferrin associated iron into the liver in times of systemic inflammatory activity [61, 62]. In the present study, its gene expression significantly increased in piglets fed ≤42.7 mg Zn/kg diet, which suggests these animals developed a systemic inflammatory status in response to SZD. Earlier published data supports this hypothesis, by demonstrating an inverse correlation between systemic inflammatory activity and the Zn status in the elderly [63] the functional background of which has been recently been comprehensively reviewed [64].

It is noteworthy that the threshold of ~40 mg Zn/kg diet does seem to not represent a minimum dietary Zn concentration above which no adverse effects in terms of redox metabolism occur. It rather seems to be related to the maximum tolerable bone Zn depletion during our experiment. In fact, animals receiving ≤43 mg Zn/kg diet showed a reduction in bone Zn concentration between ~20-25% [15]. This represents depletion of the mobilizable skeletal Zn fraction, which has been demonstrated earlier in $^{65}$Zn-labelled rats [65-67]. A continuation of the study >8 d until final emptying of mobilizable body Zn stores of deficiently supplied animals, most likely would have increased this dietary threshold over time until it equals the gross Zn requirement threshold defined earlier [15].

Further Zn transporter genes other than the ones discussed above expressed significant dose-response behavior to changes in the dietary Zn concentration and have therefore been presented in the present tables and figures. However, their precise role in the regulative hierarchy of Zn homeostasis or stress/inflammatory response, respectively, is currently unclear and needs further scientific investigations. In contrast, certain members of the *ZnT* and *ZIP* genes exhibited a very stable expression level compared between dietary treatment groups under the present experimental conditions. These included jejunal *ZnT5* and *ZnT6*, colonic *ZIP1* and *ZIP7*, hepatic *ZIP7* and *ZIP13* as well as nephric *ZnT4*, *ZnT5* and *ZnT6*. Therefore, they served as reference genes for data normalization in the present study. Future studies should further investigate under which conditions the transcription of these genes changes in the pig. Furthermore, their regulation on the proteomic level must

be studied, which is currently hampered due to the limited availability of suitable antibodies for pigs. This is crucial in order to understand their role in whole-body Zn homeostasis and associated metabolic pathways.

In conclusion, we identified significant differences in the homeostatic adaption to SZD compared to earlier studies on clinical Zn deficiency. This was evident by the identification of gene expression patterns that contradict the present state of knowledge. Many of the investigated Zn transporter transcripts expressed significant breakpoints in response to a reduction in dietary Zn. These thresholds lay close to either ~ 40 or ~ 60 mg Zn/kg diet. This indicates clear differences in the respective stimuli to which these genes respond. A breakpoint close to ~60 mg Zn/kg diet equals the gross Zn requirement threshold under the present experimental conditions. This may highlight a role of certain genes in the regulation of Zn fluxes, to meet the basal requirements and/or compensate for body Zn depletion, respectively. In addition to these genes, a subset of Zn transporters seemed to be involved in the regulation of Zn fluxes for the compensation of stress and inflammatory processes. This was evident by a breakpoint close to ~40 mg Zn/kg diet, which has been earlier related to the response of oxidative stress-associated measures under the present experimental conditions. Taken together, this manuscript presents the first comparative study of the effects of a finely-graded reduction in dietary Zn concentration on the gene expression patterns of all known ZnT and ZIP genes in jejunum, colon, liver and kidney of weaned piglets under condition of SZD. Future studies must include their regulation on the proteomic level to better understand the complex interactions within the whole-body Zn homeostasis. This data may be translated from pigs to humans in respect to the high similarity between these species regarding their nutrition physiology.

# CRediT author statement

**Brugger:** Conceptualization, Methodology, Validation, Formal Analysis, Visualization, Project administration, Writing – Original Draft Preparation **Hanauer:** Investigation **Ortner:** Investigation **Windisch:** Resources, Writing – Reviewing and Editing, Supervision, Funding Acquisition.

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Table 1. Composition as well as concentrations of metabolizable energy and crude nutrients of the basal diet[15]

| Dietary composition | | Chemical composition | |
|---|---|---|---|
| Corn, % | 46.0 | Analyzed values | |
| Soy bean meal (40% crude protein), % | 26.0 | Dry matter, g/kg diet | 902 |
| Potato protein, % | 10.0 | Crude protein, g/kg diet | 238 |
| Wheat bran, % | 5.00 | Total lipids, g/kg diet | 45.7 |
| Sugar beet pulp, % | 3.00 | Crude fiber, g/kg diet | 51.2 |
| Premix[1], % | 2.70 | Crude ash, g/kg diet | 61.0 |
| Feeding sugar, % | 2.00 | Estimated values[2] | |
| Soybean oil, % | 1.50 | Metabolizable Energy, MJ/kg diet | 13.3 |
| $Ca(H_2PO_4)_2$, % | 1.60 | Lysine, g/kg diet | 13.8 |
| $CaCO_3$, % | 1.40 | Methionine, g/kg diet | 4.10 |
| NaCl, % | 0.50 | Threonine, g/kg diet | 10.3 |
| $TiO_2$, % | 0.30 | Tryptophan, g/kg diet | 2.90 |

[1]Premix composition: 2.80% MgO; 0.08% $CuSO_4 \cdot 5H_2O$; 2.00% $FeSO_4 \cdot 7H_2O$; 0.20% $MnSO_4 \cdot H_2O$; 0.002% $Na_2SeO_3 \cdot 5 \cdot H_2O$; 0.002% KI; 0.05% retinyl propionate; 0.007% cholecalciferol; 0.20% all-*rac*-α-tocopherol; 0.002% menadione; 0.01% thiamin; 0.03% riboflavin; 0.10% nicotinic acid; 0.02% pantothenic acid; 0.02% pyridoxine; 0.15% hydroxocobalamin; 0.03% biotin; 0.002% folic acid; 6.70% choline; 77.6% corn meal. [2]The concentrations of metabolizable energy and essential precaecal digestible (synonymous with “ileal digestible”) amino acids were estimated according to feed table information (http://datenbank.futtermittel.net/). Vitamin and trace element concentrations (except zinc) met the requirements according to NRC (10).

Table 2: Qualitative expression pattern of ZnT and ZIP genes within the jejunum, colon, liver and kidney of weaned piglets fed diets with different Zn concentrations for 8d[1].

| Transcript | Jejunum | Colon | Liver | Kidney |
|---|---|---|---|---|
| ZnT1 | ✓ | ✓ | ✓ | ✓ |
| ZnT2 | ✓ | ✓ | ✓ | ✓ |
| ZnT3 | N/A | N/A | N/A | ✓ |
| ZnT4 | ✓ | ✓ | ✓ | ✓[R] |
| ZnT5 | ✓[R] | ✓ | ✓ | ✓[R] |
| ZnT6 | ✓[R] | ✓ | ✓ | ✓[R] |
| ZnT7 | ✓ | ✓ | ✓ | ✓ |
| ZnT8 | ✓ | ✓ | ✓ | ✓ |
| ZnT9 | ✓ | ✓ | ✓ | ✓ |
| ZnT10 | ✓ | ✓ | ✓ | ✓ |
| ZIP1 | ✓ | ✓[R] | ✓ | ✓ |
| ZIP2 | ✓ | ✓ | ✓ | ✓ |
| ZIP3 | ✓ | ✓ | ✓ | ✓ |
| ZIP4 | ✓ | ✓ | ✓ | ✓ |
| ZIP5 | ✓ | ✓ | ✓ | ✓ |
| ZIP6 | ✓ | ✓ | ✓ | ✓ |
| ZIP7 | ✓ | ✓[R] | ✓[R] | ✓ |
| ZIP8 | ✓ | ✓ | ✓ | ✓ |
| ZIP9 | ✓[R] | ✓ | ✓ | ✓ |
| ZIP10 | N/A | N/A | N/A | N/A |
| ZIP11 | ✓ | ✓ | ✓ | ✓ |
| ZIP12 | N/A | N/A | N/A | N/A |
| ZIP13 | ✓ | ✓ | ✓[R] | ✓ |
| ZIP14 | ✓ | ✓ | ✓ | ✓ |

Notes: [1]The applied dietary Zn concentrations were 28.1, 33.6, 38.8, 42.7, 47.5, 58.2, 67.8 and 88.0 mg Zn/kg. [R]evaluated as a suitable reference gene using published mathematical procedures [26]. N/A, transcript not detected in respective tissue sample; ZnT1 to 10, solute carrier (SLC) family 30 members 1 to 10; ZIP1 to 14, SLC family 39 members 1 to 14.

Table 3. Broken-line regression analysis of relative jejunal gene expression (xfold) of ZIP1, 5, 11 and 13 in weaned piglets fed diets with different zinc concentrations for 8d[1].

| | Regression model | Breakpoint | Slopes | $R^2$ |
|---|---|---|---|---|
| ZIP1 | $y = 1.40 + b_1x$ for $x \leq X_B$ | $X_B$ 62.6*** ± 5.07 | $b_1$ -0.01** ± 0.002 | *0.75* |
| | $y = 0.26 + b_2x$ for $x \geq X_B$ | $Y_B$ 0.79*** ± 0.05 | $b_2$ 0.008 ± 0.004 | |
| ZIP5 | $y = 0.93 + b_1x$ for $x \leq X_B$ | $X_B$ 62.3*** ± 5.38 | $b_1$ -0.007 ± 0.003 | *0.70* |
| | $y = -0.64 + b_2x$ for $x \geq X_B$ | $Y_B$ 0.52*** ± 0.07 | $b_2$ 0.02* ± 0.006 | |
| ZIP11 | $y = -0.52 + b_1x$ for $x \leq X_B$ | $X_B$ 38.8*** ± 0.02 | $b_1$ 0.05*** ± 0.009 | *0.79* |
| | $y = 1.66 + b_2x$ for $x \geq X_B$ | $Y_B$ 1.40*** ± 0.05 | $b_2$ -0.007* ± 0.002 | |
| ZIP13 | $y = 2.63 + b_1x$ for $x \leq X_B$ | $X_B$ 52.3*** ± 6.05 | $b_1$ -0.03* ± 0.01 | *0.75* |
| | $y = 0.94$ for $x \geq X_B$ | $Y_B$ 0.94*** ± 0.09 | N/A | |

Notes: [1]The applied dietary zinc concentrations were 28.1, 33.6, 38.8, 42.7, 47.5, 58.2, 67.8, and 88.0 mg Zn/kg. Broken-line regression models were estimated based on independent arithmetic group means relative to dietary zinc concentration (*n = 8*). Parameter estimates are presented as means ± SEs to indicate the precision of estimation. $P \leq 0.05$ was considered to be significant. *, **, *** indicate $P \leq 0.05, 0.001, 0.0001$, respectively. $b_1$, slope of the broken-line regression curves over dietary zinc doses ≤XB; $b_2$, slope of the broken-line regression curves over dietary zinc doses >$X_B$; xfold, difference in the gene expression value according to Livak and Schmittgen [27] compared to a relative mRNA abundance of 1.0 in the control group (88.0 mg Zn/kg diet); $X_B$, X intercept of the breakpoint in the parameter response; $Y_B$, Y intercept of the breakpoint in the parameter response; ZIP1, 5, 11, 13, solute carrier (SLC) family 39 members 1, 5, 11, 13.

Table 4. Broken-line regression analysis of relative colonic gene expression (xfold) of ZnT4 and 9 as well as ZIP4, 5, 7, 11 and 13 in weaned piglets fed diets with different zinc concentrations for 8 d[1].

| | Regression model | Breakpoint | Slopes | $R^2$ |
|---|---|---|---|---|
| ZnT4 | $y = 0.50 + b_1x$ for $x \leq X_B$ | $X_B$ 60.6*** ± 4.01 | $b_1$ 0.01** ± 0.002 | *0.85* |
| | $y = 1.69 + b_2x$ for $x \geq X_B$ | $Y_B$ 1.21*** ± 0.04 | $b_2$ -0.008* ± 0.003 | |
| ZnT9 | $y = 0.67 + b_1x$ for $x \leq X_B$ | $X_B$ 63.9*** ± 4.82 | $b_1$ -0.002 ± 0.003 | *0.82* |
| | $y = -0.75 + b_2x$ for $x \geq X_B$ | $Y_B$ 0.52*** ± 0.06 | $b_2$ 0.02* ± 0.005 | |
| ZIP4 | $y = 4.85 + b_1x$ for $x \leq X_B$ | $X_B$ 59.6*** ± 7.14 | $b_1$ -0.06** ± 0.02 | *0.76* |
| | $y = 0.98$ for $x \geq X_B$ | $Y_B$ 0.98* ± 0.29 | N/A | |
| ZIP5 | $y = 3.51 + b_1x$ for $x \leq X_B$ | $X_B$ 39.0*** ± 1.32 | $b_1$ -0.06** ± 0.01 | *0.90* |
| | $y = 1.04$ for $x \geq X_B$ | $Y_B$ 1.04*** ± 0.04 | N/A | |
| ZIP7 | $y = 2.48 + b_1x$ for $x \leq X_B$ | $X_B$ 42.7*** ± 2.50 | $b_1$ -0.04* ± 0.008 | *0.81* |
| | $y = 0.96$ for $x \geq X_B$ | $Y_B$ 0.96*** ± 0.05 | N/A | |
| ZIP11 | $y = 0.34 + b_1x$ for $x \leq X_B$ | $X_B$ 44.8*** ± 2.43 | $b_1$ 0.04* ± 0.01 | *0.87* |
| | $y = 3.67 + b_2x$ for $x \geq X_B$ | $Y_B$ 2.38*** ± 0.09 | $b_2$ -0.03** ± 0.005 | |
| ZIP13 | $y = 1.18 + b_1x$ for $x \leq X_B$ | $X_B$ 68.3*** ± 5.3 | $b_1$ -0.007*** ± 0.001 | *0.86* |
| | $y = -0.26 + b_2x$ for $x \geq X_B$ | $Y_B$ 0.71*** ± 0.04 | $b_2$ -0.01* ± 0.004 | |

Notes: [1]The applied dietary zinc concentrations were 28.1, 33.6, 38.8, 42.7, 47.5, 58.2, 67.8, and 88.0 mg Zn/kg. Broken-line regression models were estimated based on independent arithmetic group means relative to dietary zinc concentration (*n = 8*). Parameter estimates are presented as means ± SEs to indicate the precision of estimation. $P \leq 0.05$ was considered to be significant. *, **, *** indicate $P \leq 0.05, 0.001, 0.0001$, respectively. $b_1$, slope of the broken-line regression curves over dietary zinc doses ≤XB; $b_2$, slope of the broken-line regression curves over dietary zinc doses >$X_B$; xfold, difference in the gene expression value according to Livak and Schmittgen [27] compared to a relative mRNA abundance of 1.0 in the control group (88.0 mg Zn/kg diet); $X_B$, X intercept of the breakpoint in the parameter response; $Y_B$, Y intercept of the breakpoint in the parameter response; ZnT4, 9, solute carrier (SLC) family 30 members 4, 9; ZIP4, 5, 7, 11, 13, SLC family 39 members 4, 5, 7, 11, 13.

Table 5. Broken-line regression analysis of relative hepatic gene expression (xfold) of ZnT4, 6 and 8 as well as ZIP1 and 14 in weaned piglets fed diets with different zinc concentrations for 8 d[1].

| | Regression model | Breakpoint | Slopes | $R^2$ |
|---|---|---|---|---|
| ZnT4 | $y = -0.001 + b_1x$ for $x \leq X_B$ | $X_B$ 48.4*** ± 2.36 | $b_1$ 0.02** ± 0.003 | *0.92* |
| | $y = 0.95$ for $x \geq X_B$ | $Y_B$ 0.95*** ± 0.03 | N/A | |
| ZnT6 | $y = 0.34 + b_1x$ for $x \leq X_B$ | $X_B$ 38.8*** ± 0.04 | $b_1$ 0.01* ± 0.003 | *0.91* |
| | $y = 0.64 + b_2x$ for $x \geq X_B$ | $Y_B$ 0.81*** ± 0.02 | $b_2$ 0.004** ± 0.0007 | |
| ZnT8 | $y = 3.30 + b_1x$ for $x \leq X_B$ | $X_B$ 57.3*** ± 7.95 | $b_1$ -0.04* ± 0.01 | *0.80* |
| | $y = 0.92$ for $x \geq X_B$ | $Y_B$ 0.92*** ± 0.13 | N/A | |
| ZIP1 | $y = 1.81 + b_1x$ for $x \leq X_B$ | $X_B$ 47.5*** ± 3.84 | $b_1$ -0.02* ± 0.004 | *0.80* |
| | $y = 0.62 + b_2x$ for $x \geq X_B$ | $Y_B$ 0.84*** ± 0.06 | $b_2$ 0.005 ± 0.003 | |
| ZIP14 | $y = 1.53 + b_1x$ for $x \leq X_B$ | $X_B$ 42.7*** ± 0.02 | $b_1$ -0.01*** ± 0.001 | *0.92* |
| | $y = 0.99$ for $x \geq X_B$ | $Y_B$ 0.99*** ± 0.008 | N/A | |

Notes: [1]The applied dietary zinc concentrations were 28.1, 33.6, 38.8, 42.7, 47.5, 58.2, 67.8, and 88.0 mg Zn/kg. Broken-line regression models were estimated based on independent arithmetic group means relative to dietary zinc concentration (*n = 8*). Parameter estimates are presented as means ± SEs to indicate the precision of estimation. $P \leq 0.05$ was considered to be significant. *, **, *** indicate $P \leq 0.05, 0.001, 0.0001$, respectively. $b_1$, slope of the broken-line regression curves over dietary zinc doses ≤XB; $b_2$, slope of the broken-line regression curves over dietary zinc doses >$X_B$; xfold, difference in the gene expression value according to Livak and Schmittgen [27] compared to a relative mRNA abundance of 1.0 in the control group (88.0 mg Zn/kg diet); $X_B$, X intercept of the breakpoint in the parameter response; $Y_B$, Y intercept of the breakpoint in the parameter response; ZnT4, 6, 8 solute carrier (SLC) family 30 members 4, 6, 8; ZIP1, 14, SLC family 39 members 1, 14.

Table 6. Broken-line regression analysis of relative nephric gene expression (xfold) of ZnT1, 3 and 7 as well as ZIP4 in weaned piglets fed diets with different zinc concentrations for 8 d[1].

| | Regression model | Breakpoint | Slopes | $R^2$ |
|---|---|---|---|---|
| ZnT1 | $y = 0.48 + b_1x$ for $x \leq X_B$ | $X_B$ 70.4*** ± 9.84 | $b_1$ 0.007* ± 0.002 | *0.75* |
| | $y = 1.00$ for $x \geq X_B$ | $Y_B$ 1.00*** ± 0.05 | N/A | |
| ZnT3 | $y = 4.40 + b_1x$ for $x \leq X_B$ | $X_B$ 42.6*** ± 3.40 | $b_1$ -0.08* ± 0.02 | *0.82* |
| | $y = 1.18$ for $x \geq X_B$ | $Y_B$ 1.18*** ± 0.08 | N/A | |
| ZnT7 | $y = 4.65 + b_1x$ for $x \leq X_B$ | $X_B$ 35.2*** ± 0.76 | $b_1$ -0.10*** ± 0.01 | *0.95* |
| | $y = 1.08$ for $x \geq X_B$ | $Y_B$ 1.08*** ± 0.02 | N/A | |
| ZIP4 | $y = 8.78 + b_1x$ for $x \leq X_B$ | $X_B$ 41.9*** ± 2.26 | $b_1$ -0.18* ± 0.04 | *0.89* |
| | $y = 1.41$ for $x \geq X_B$ | $Y_B$ 1.41*** ± 0.13 | N/A | |

Notes: [1]The applied dietary zinc concentrations were 28.1, 33.6, 38.8, 42.7, 47.5, 58.2, 67.8, and 88.0 mg Zn/kg. Broken-line regression models were estimated based on independent arithmetic group means relative to dietary zinc concentration (*n = 8*). Parameter estimates are presented as means ± SEs to indicate the precision of estimation. $P \leq 0.05$ was considered to be significant. *, *** indicate $P \leq 0.05, 0.0001$, respectively. $b_1$, slope of the broken-line regression curves over dietary zinc doses $\leq X_B$; $b_2$, slope of the broken-line regression curves over dietary zinc doses $> X_B$; xfold, difference in the gene expression value according to Livak and Schmittgen [27] compared to a relative mRNA abundance of 1.0 in the control group (88.0 mg Zn/kg diet); $X_B$, X intercept of the breakpoint in the parameter response; $Y_B$, Y intercept of the breakpoint in the parameter response; ZnT1, 3, 7, solute carrier (SLC) family 30 members 1, 3, 7, ZIP4, SLC family 39 member 4.

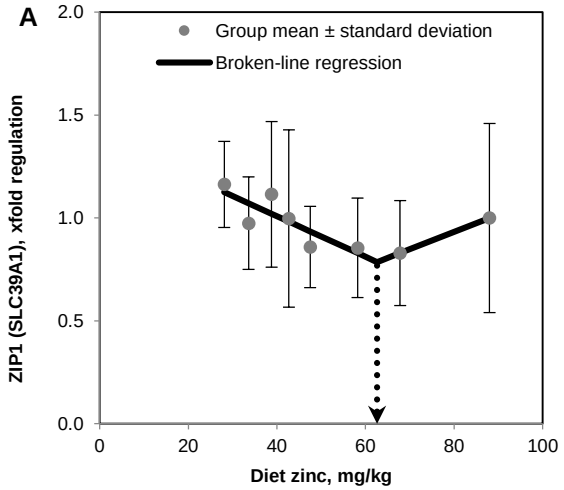
Figure 1. Response of relative jejunal gene expression of ZIP1 (A), ZIP5 (B), ZIP11 (C) and ZIP13 (D) in weaned piglets fed diets with different zinc concentrations for 8 d (see Table 3 for detailed information on the statistical measures of the respective regression models). Values are arithmetic means ± SDs, *n = 6*. Diet zinc, dietary zinc; xfold, difference in the gene expression value according to Livak and Schmittgen [27] compared to a relative mRNA abundance of 1.0 in the control group (88.0 mg Zn/kg diet); ZIP1, 5, 11, 13, solute carrier (SLC) family 39 members 1, 5, 11, 13.

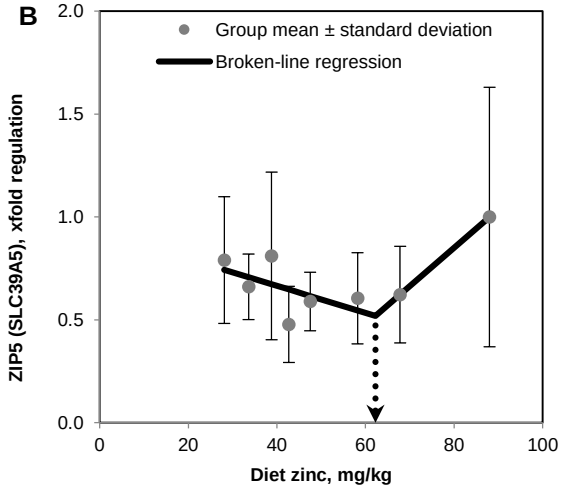
Figure 2. Response of relative colonic gene expression of ZnT4 (A), ZnT9 (B), ZIP4 (C), ZIP5 (D), ZIP7 (E), ZIP11 (F) and ZIP13 (G) in weaned piglets fed diets with different zinc concentrations for 8 d (see Table 4 for detailed information on the statistical measures of the respective regression models). Values are arithmetic means ± SDs, n = 6. Diet zinc, dietary zinc; xfold, difference in the gene expression value according to Livak and Schmittgen [27] compared to a relative mRNA abundance of 1.0 in the control group (88.0 mg Zn/kg diet); ZnT4, 9, solute carrier (SLC) family 30 members 4, 9; ZIP4, 5, 7, 11, 13, SLC family 39 members 4, 5, 7, 11, 13.

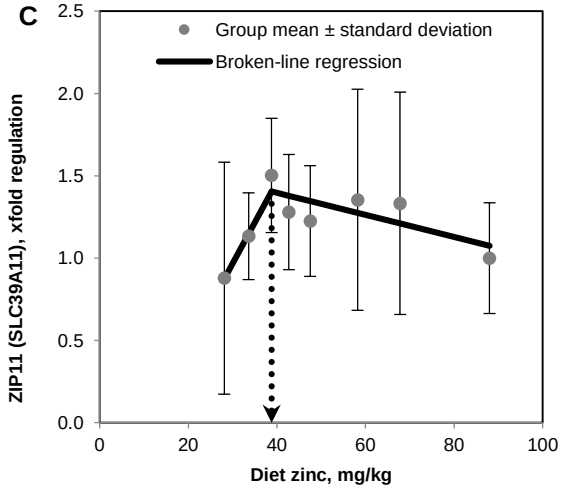
Figure 3. Response of relative hepatic gene expression of ZnT4 (A), ZnT6 (B), ZnT8 (C), ZIP1 (D) and ZIP14 (E) in weaned piglets fed diets with different zinc concentrations for 8 d (see Table 5 for detailed information on the statistical measures of the respective regression models). Values are arithmetic means ± SDs, n = 6. Diet zinc, dietary zinc; xfold, difference in the gene expression value according to Livak and Schmittgen [27] compared to a relative mRNA abundance of 1.0 in the control group (88.0 mg Zn/kg diet); ZnT4, 6, 8 solute carrier (SLC) family 30 members 4, 6, 8; ZIP1, 14, SLC family 39 members 1, 14.

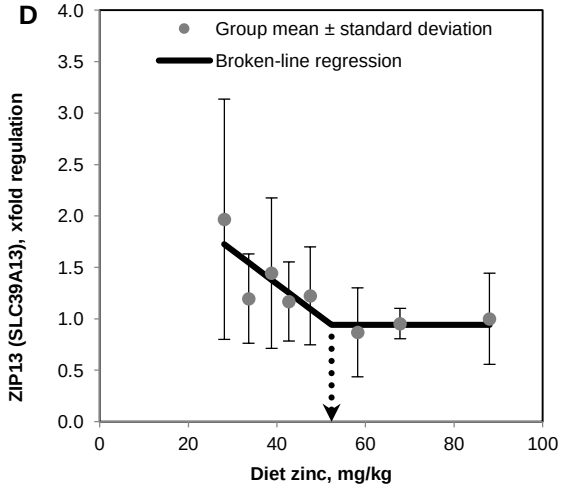
Figure 4. Response of relative nephric gene expression of ZnT1 (A), ZnT3 (B), ZnT7 (C) and ZIP4 (D) in weaned piglets fed diets with different zinc concentrations for 8 d (see Table 6 for detailed

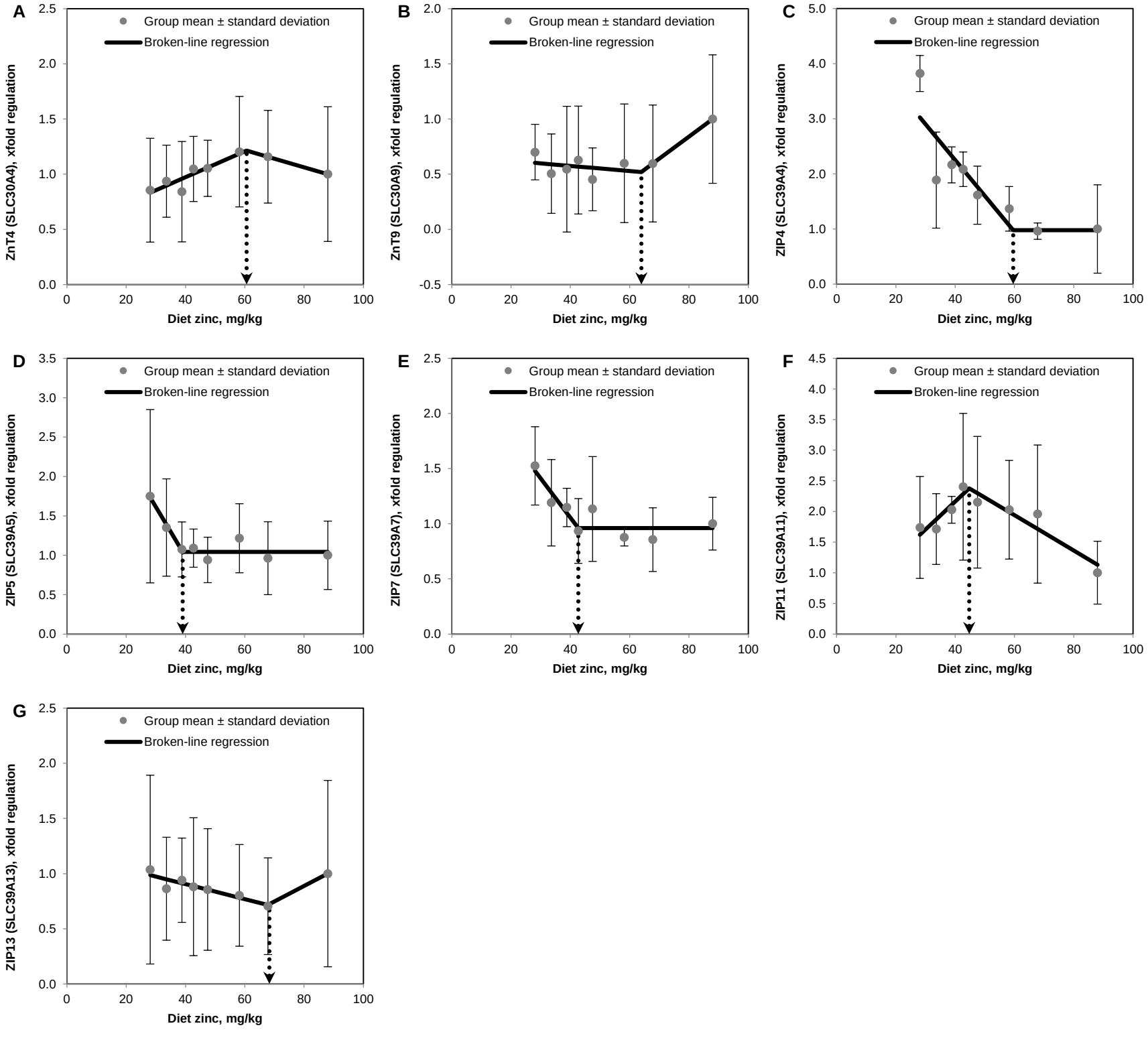
information on the statistical measures of the respective regression models). Values are arithmetic means ± SDs, n = 6. Diet zinc, dietary zinc; xfold, difference in the gene expression value according to Livak and Schmittgen [27] compared to a relative mRNA abundance of 1.0 in the control group (88.0 mg Zn/kg diet); ZnT1, 3, 7, solute carrier (SLC) family 30 members 1, 3, 7, ZIP4, SLC family 39 member 4.

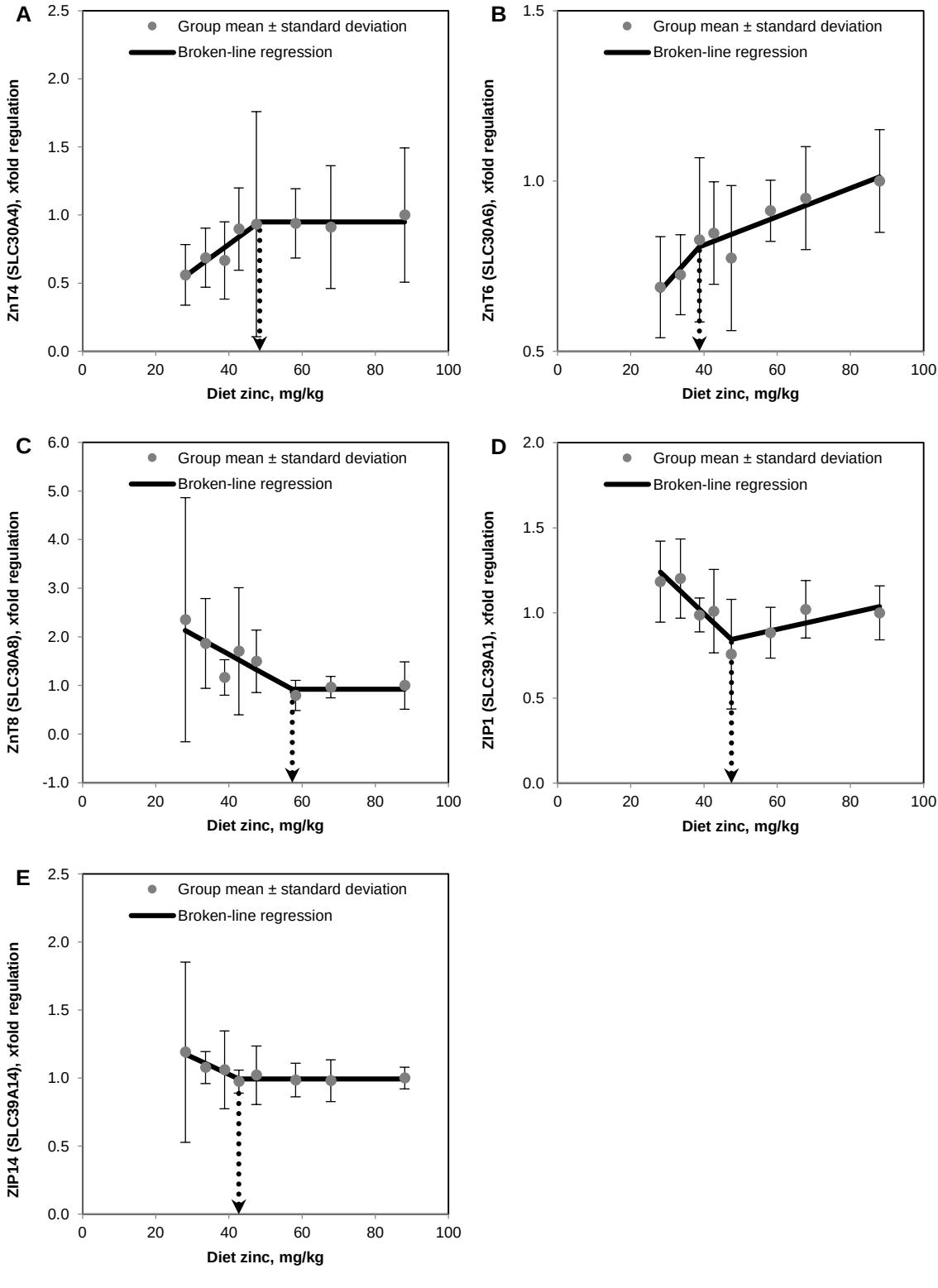

A
Group mean ± standard deviation
Broken-line regression
ZIP1 (SLC39A1), xfold regulation
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
Diet zinc, mg/kg

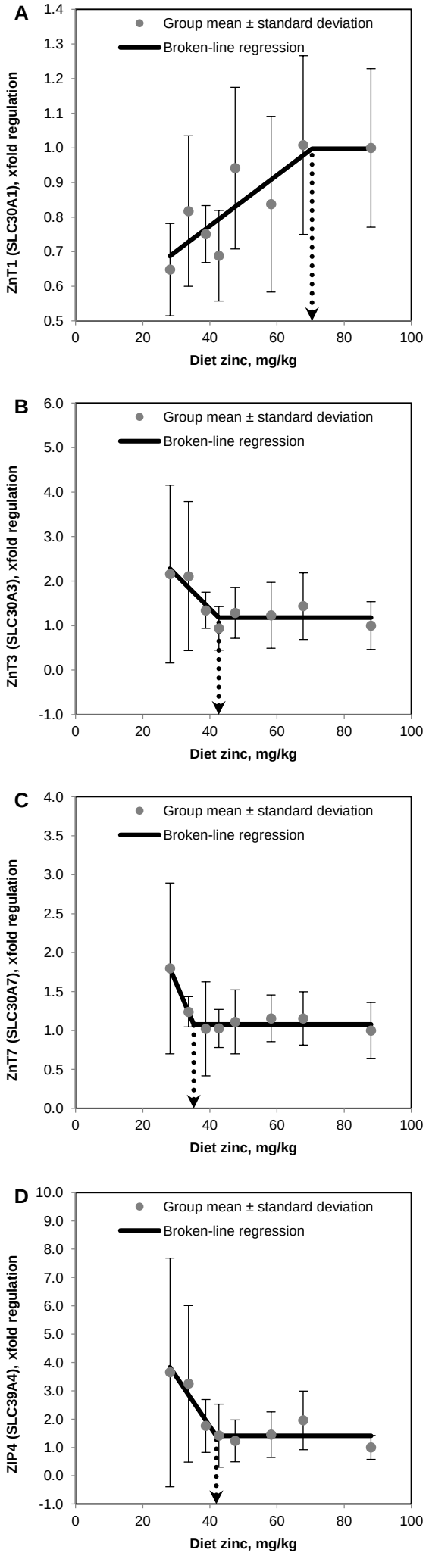

B
Group mean ± standard deviation
Broken-line regression
ZIP5 (SLC39A5), xfold regulation
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
Diet zinc, mg/kg


C
Group mean ± standard deviation
Broken-line regression
ZIP11 (SLC39A11), xfold regulation
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
Diet zinc, mg/kg


D
Group mean ± standard deviation
Broken-line regression
ZIP13 (SLC39A13), xfold regulation
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
Diet zinc, mg/kg


A
Group mean ± standard deviation
Broken-line regression
ZnT4 (SLC30A4), xfold regulation
Diet zinc, mg/kg
B
Group mean ± standard deviation
Broken-line regression
ZnT9 (SLC30A9), xfold regulation
Diet zinc, mg/kg
C
Group mean ± standard deviation
Broken-line regression
ZIP4 (SLC39A4), xfold regulation
Diet zinc, mg/kg
D
Group mean ± standard deviation
Broken-line regression
ZIP5 (SLC39A5), xfold regulation
Diet zinc, mg/kg
E
Group mean ± standard deviation
Broken-line regression
ZIP7 (SLC39A7), xfold regulation
Diet zinc, mg/kg
F
Group mean ± standard deviation
Broken-line regression
ZIP11 (SLC39A11), xfold regulation
Diet zinc, mg/kg
G
Group mean ± standard deviation
Broken-line regression
ZIP13 (SLC39A13), xfold regulation
Diet zinc, mg/kg


A
Group mean ± standard deviation
Broken-line regression
ZnT4 (SLC30A4), xfold regulation
Diet zinc, mg/kg
B
Group mean ± standard deviation
Broken-line regression
ZnT6 (SLC30A6), xfold regulation
Diet zinc, mg/kg
C
Group mean ± standard deviation
Broken-line regression
ZnT8 (SLC30A8), xfold regulation
Diet zinc, mg/kg
D
Group mean ± standard deviation
Broken-line regression
ZIP1 (SLC39A1), xfold regulation
Diet zinc, mg/kg
E
Group mean ± standard deviation
Broken-line regression
ZIP14 (SLC39A14), xfold regulation
Diet zinc, mg/kg


A
Group mean ± standard deviation
Broken-line regression
ZnT1 (SLC30A1), xfold regulation
Diet zinc, mg/kg
B
Group mean ± standard deviation
Broken-line regression
ZnT3 (SLC30A3), xfold regulation
Diet zinc, mg/kg
C
Group mean ± standard deviation
Broken-line regression
ZnT7 (SLC30A7), xfold regulation
Diet zinc, mg/kg
D
Group mean ± standard deviation
Broken-line regression
ZIP4 (SLC39A4), xfold regulation
Diet zinc, mg/kg

Supplementary Table 1. PCR primer and PCR product specifications – Part I

| Gene | Accession number[1] | Forward sequence | Reverse sequence | Position on template[2] 5'→3' | Product Length[3] bp | Annealing temperature Jejunum/Colon/Liver/Kidney °C |
|---|---|---|---|---|---|---|
| GAPDH | NM_001206359.1 | CACATGGCCTCCAAGGAGTAA | GGAGATGCTCGGTGTGTTGG | 1082 → 1210 | 129 | 58.6 / 58.6 / 58.6 / 58.6 |
| GUSB | NM_001123121.1 | TCACGAGGATCCACCTCTCAT | CCTATGGCCCTCTGAGGTGA | 1647 → 1808 | 162 | 60.0 / 60.0 / 58.6 / 60.0 |
| H3 | NM_213930.1 | CTTTGCAGGAGGCAAGTGAG | GCGTGCTAGCTGGATGTCT | 333 → 445 | 113 | 60.0 / 60.0 / 58.6 / 60.0 |
| UBC | XM_003483411.1 | AGTGATGGCCAGTGAAGCAA | GCAGGCCACTGAGAGCTAAT | 2306 → 2442 | 137 | 64.1 / 64.1 / 64.1 / 64.1 |
| ACTB | XM_003357928.1 | GACTCAGATCATGTTCGAGACCTT | CATGACAATGCCAGTGGTGC | 449 → 551 | 103 | 60.0 / 60.0 / 60.0 / 60.0 |
| DMT1 | NM_001128440.1 | TAGCAGCAGTCCCCATAGTG | GCCCGAAGTAACACCCTAGC | 1011 → 1108 | 98 | 60.0 / 60.0 / 63.4 / 58.6 |
| ZnT1 | NM_001139470.1 | CAGGAGGAGACCAACATCCT | TCTGGACTTTTCTGGATCTGTC | 563 → 646 | 84 | 60.0 / 60.0 / 62.5 / 62.5 |
| ZnT2 | NM_001139475.1 | TGCCTTTATCCACGTGATTGGA | TCTATATACTTGTACTCGGGCTTG | 773 → 868 | 96 | 57.2 / 60.0 / 62.5 / 61.3 |
| ZnT3 | NM_001139474.1 | ACAGAGATGTCCTTCCAGCAC- | CATGAAGACGCAGCACACAG- | 214 → 333 | 120 | N/A / N/A / N/A / 63.4 |
| ZnT4 | NM_001130972.1 | AGTTGCAGTTAATGTGATAATGGG | ACATCTAGAACCTGTGGTGGGA | 722 → 827 | 106 | 60.0 / 60.0 / 63.4 / 64.1 |
| ZnT5 | NM_001137624.1 | CGGATCACAAGGGTGGAGTA | CGTTTGGTTCCACCAACATCT | 612 → 717 | 106 | 60.0 / 62.5 / 64.1 / 64.1 |
| ZnT6 | NM_001137623.1 | ACTGCCTCAGCCATAGCCATC | CAATAACGTGAGGTGGTGTGGT | 693 → 798 | 106 | 57.2 / 58.6 / 64.0 / 64.0 |
| ZnT7 | NM_ 001136211.1 | ACACATAATATTTTTACTCAGGCCG | CTTTTCATTTGTTACAGGGCTGC | 1132 → 1211 | 80 | 60.0 / 60.0 / 55.1 / 63.4 |
| ZnT8 | XM_001925124.5 | CCGAGCAGAGATCCTTGGTG | CGGTGGCTTGGATCTGGTAA | 534 → 652 | 119 | 57.2 / 60 / 56.0 / 58.6 |
| ZnT9 | NM_001137632.1 | CGCCAGGAATGGCAGAATTTAG | AACTTTACTTGATTCAGCGTGC | 181 → 160 | 80 | 58.6 / 58.6 / 63.4 / 57.2 |
| ZnT10 | XM_003357627.1 | GGAGCTGATGAGTAAACTGTCTG | CTGTCCTGCTGACACTTGATG | 1039 → 1158 | 120 | 60.0 / 60.0 / 64.0 / 60.0 |

Notes: [1]Sequence information was provided by the National Center for Biotechnology Information (NCBI) RefSeq database; O´Leary NA, Wright MW, Brister JR, Ciufo S, Haddad D, McVeigh R, Rajput B, Robbertse B, Smith-White B, Ako-Adjei D et al. 2016. Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion and functional annotation. Nucleic Acids Research. 44(D1):D733-D745. [2]Position on template indicates first base at 5' and last base at 3' side (5'→3') at which the respective primer pair binds. [3]Product length presented as base pairs. bp, base pairs; N/A, not determined; PCR, polymerase chain reaction; ZnT1-10, solute carrier (SLC) family 30 member 1-10.

Supplementary Table 2. PCR primer and PCR product specifications – Part II

| Gene | Accession number[1] | Forward sequence | Reverse sequence | Position on template[2] 5'→3' | Product Length[3] bp | Annealing temperature Jejunum/Colon/Liver/Kidney °C |
|---|---|---|---|---|---|---|
| Zip1 | XM_001929505.1 | CATGTGACGCTCCAGTTCCC | CCCGACTGCTCCTTGTAAGC | 414 → 517 | 104 | 60.0 /60.0 /57.2 / 57.2 |
| Zip2 | NM_001244460.1 | CCCTGCTGGTTCACACTCTA | GTGACGACCTGTGGCTCTAT | 221 → 303 | 83 | 61.3 / 61.3 / 63.4 / 63.4 |
| Zip3 | XM_003123026.1 | CACTCACAGTGGCTAGGCTGA | CCAAACCATGTGTGGGCGTG | 16 → 111 | 96 | 60.0 / 60.0 / 64.1 / 58.6 |
| Zip4 | XM_001925360.2 | GCCAGTCAGAGAGGTACCTG | CGTAGTGGGTAGCAGCAT | 1041 → 1154 | 114 | 60.0 / 60.0 / 64.0 / 64.1 |
| Zip5 | XM_003481622.1 | CACTGACGGACTGGCGATAG | CGAAGTCACCCAGTTCGTGG | 243 → 352 | 110 | 60.0 / 58.6 / 56.0 / 60.0 |
| Zip6 | XM_003356412.1 | AGATCATGCCTGATTCATACGACA | GCCACCAACCCAGGCTATTTG | 106 → 192 | 87 | 60.0 / 58.6 / 56.0 / 58.6 |
| Zip7 | NM_001131045.1 | GAGTCCAACTCACCTCGGCA | TGAGAATGGGGTTCCAGAGCA | 895 → 1013 | 119 | 60.0 / 58.6 / 64.0 / 58.6 |
| Zip8 | XM_003129295.3 | AGCTGCACTTCAACCAGTGTT | TTGAGCTGGTTATCTGCGTCG | 203 → 286 | 84 | 61.3 / 61.3 / 54.4 / 58.6 |
| Zip9 | XM_001926183.3 | TGACCACACACAGCTACAG | GCTGTTTCTGGATCGTCAGTAGA | 219 → 338 | 120 | 57.2 / 58.6 / 63.4 / 60.0 |
| Zip10 | BC.101516.1 | CCACGGCGAGAACAAAACTG | CGGATCCAGAATGACAGGGG | 1958 → 2052 | 95 | N/A / N/A / N/A / N/A[4] |
| Zip11 | XM_003131247.3 | CCATCCGGATAGACAAGAGTGAG | TGCACTAGGTTCCCCTGAGA | 531 → 645 | 115 | 58.6 / 58.6 / 64.1 / 60.0 |
| Zip12 | XM_003130728.1 | CCGGAAGCCAGTGTATGGAA | CCGCCAACTGAGGAAGTGTA | 533 → 628 | 96 | N/A / N/A / N/A / N/A[4] |
| Zip13 | XM_003122808.3 | AAGACGATCCGTGGCACTCC | GGTGTCAGGGATGGTTACTCAA | 1047 → 1149 | 103 | 61.3 / 60.0 / 62.5 / 63.4 |
| Zip14 | XM_001925697.2 | GAACCTCTCAACGTGCTTCA | CAGAACTCCTGGAACTCAGGT | 421 → 516 | 96 | 60.0 / 60.0 / 64.1 / 64.1 |

Notes: [1]Sequence information was provided by the National Center for Biotechnology Information (NCBI) RefSeq database; O´Leary NA, Wright MW, Brister JR, Ciufo S, Haddad D, McVeigh R, Rajput B, Robbertse B, Smith-White B, Ako-Adjei D et al. 2016. Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion and functional annotation. Nucleic Acids Research. 44(D1):D733-D745. [2]Position on template indicates first base at 5' and last base at 3' side (5'→3') at which the respective primer pair binds. [3]Product length presented as base pairs. [4]The optimal annealing temperature in murine brain cDNA preparations was estimated to be 60.0°C for ZIP10 and ZIP12, respectively. bp, base pairs; PCR, polymerase chain reaction; ZIP1-14, solute carrier (SLC) family 39 member 1-14.