The response of zinc transporter gene expression of selected tissues in a pig model of subclinical zinc deficiency

Daniel Brugger[a*], Martin Hanauer[b], Johanna Ortner[b], Wilhelm M. Windisch[b]

[a]Institute of Animal Nutrition, Vetsuisse-Faculty, University of Zurich, Winterthurerstrasse 270, 8057 Zurich, Switzerland

[b]Chair of Animal Nutrition, TUM School of Life Sciences Weihenstephan, Technical University of Munich, Liesel-Beckmann-Strasse 2, 85354 Freising, Germany

[*]Corresponding author: Institute of Animal Nutrition, Vetsuisse-Faculty, Winterthurerstrasse 270, 8057 Zurich (Switzerland), phone: +41 446 35 8827,

dbrugger@nutrivet.uzh
m.hanauer@outlook.de
JohannaOrtner@gmx.de
wilhelm.windisch@wzw.tum.de

Running title: Zinc transporter expression in zinc deficient pigs

Funding source: This work has been generously supported by Bayerische Arbeitsgemeinschaft Tierernährung e.V.

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 regulated under the present experimental conditions by two distinct homeostatic 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 benefit 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 transporter genes 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 [3-6]. Liuzzi, Bobo [7] suggested an intestinal-pancreatic axis along which the abundance and activity of certain Zn transporters is regulated on the transcriptional and post-transcriptional level to maintain whole-body Zn homeostasis.

On the one hand, this involves the intestinal regulation of ZIP4 and ZnT1. Current knowledge suggests that ZIP4 is presented on the apical side and ZnT1 on the basolateral side of enterocytes, especially within the jejunum. As a result, these transporters represent the most important active route of luminal Zn transfer into the circulation [7-14]. The regulation of intestinal ZIP4 activity is subject to mechanisms of transcriptional

and post-transcriptional regulation. An inadequate dietary Zn supply triggers an increased synthesis of the peptide and its subsequent presentation on the apical plasma membrane. Conversely, body Zn repletion causes ZIP4 endocytosis and breakdown of the protein and its mRNA [15-17]. The amount of ZIP4 mRNA in the enterocyte is a product of changes in its stability and the body Zn status-dependent activity of kruppel-like-factor 4 (KLF4) [18, 19]. In contrast, the expression of *ZnT1* mRNA is a result of the amount of free cytosolic Zn^{2+}, which rising concentrations promote the activity of metal-responsive transcription factor 1 (MTF1) [13, 20, 21]. Therefore, *ZnT1* gene expression is no marker for the body Zn status but the cytosolic Zn^{2+} levels in certain tissues.

Most endogenous Zn losses occur in the gastrointestinal tract [22], mainly via exogenous pancreatic secretions of Zn-metallopeptides (e.g. zymogens like carboxypeptidase A and B) [23]. The differential uptake of Zn in pancreatic acinar cells and the excretion back into the circulation are due to the activity of ZIP5 and ZnT1 at the basolateral membrane. A Zn deficiency reduces the ZIP5-dependent uptake from the circulation and at the same time promotes the activity of ZnT1, presumably as a measure to bring Zn back into the periphery for the benefit of other tissues. Zinc repletion and excess promote the opposite response [7, 16, 24]. The transporter that loads Zn into zymogen granules of pancreatic acinar cells is ZnT2. A systemic Zn deficiency decreases the pancreatic ZnT2 abundance, which has associated with a decrease in the Zn concentrations in these granules. Otherwise, a systemic Zn overload triggers the opposite reaction, as a measure in favor of an increased excretion of excess Zn quantities from the system into the gastrointestinal tract [25].

These aforementioned molecular changes in the intestine and exogenous pancreas nicely reflect the organisms attempts to adjust gastrointestinal absorption and endogenous Zn losses in times of deficiency and oversupply, respectively, and directly correspond to classical data on quantitative Zn fluxes in ^{65}Zn labelled rats fed varying dietary Zn concentrations [26].

Subclinical zinc deficiency is probably the most common form of zinc malnutrition in humans and animals [27]. However, it has not yet been thoroughly investigated. In fact, many of the original *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

88 the mobilizable Zn pools in the skeleton and the soft tissues, are exhausted [28-31]. This results in the quite
89 unspecific set of visible symptoms of Zn malnutrition (e.g. growth depression, anorexia, developmental
90 disorders, tissue necrosis etc. in pigs [32]) and, as a result, an increased background noise from metabolic
91 measurements. In addition, this represents the endpoint in adapting metabolic processes to an ongoing dietary
92 zinc deficiency, which can reduce the informative value of quantitative measurements of Zn-homeostatic
93 mechanisms at the level of the whole organism.

94 To promote translational research on SZD, we have developed an experimental model to promote this
95 phenotype in pigs [33]. It allows high-resolution analyses of the kinetics and dynamics of zinc in the growing
96 pig organism, from the level of the quantitative metabolism to the subcellular level. At the same time, it does
97 not promote any change in the health status of the animals based on continuous veterinary surveillance. The
98 previous findings revealed the adjustment of the quantitative zinc metabolism to a short-term (8d), finely
99 graded reduction in the alimentary zinc intake. Based on this, we were able to derive the gross zinc requirement
100 under the given experimental conditions at 60 mg / kg diet [33]. Already in this early phase of zinc deficiency,
101 various pathophysiological reactions at the metabolic and subclinical level became evident. These included a
102 reduction in pancreatic digestive capacity [34] and cardiac redox capacity [35]. The latter was accompanied
103 by an increased need for the detoxification of reactive oxygen species and the activation of pro-apoptotic
104 signaling pathways when the alimentary zinc supply was ~40 mg / kg diet and below for a period of 8 days
105 [35]. Furthermore, we were able to show that certain tissues that are important for the acute survival of the
106 developing organism (heart, skeletal muscle, thymus, mesenteric lymph nodes, pancreas) partly or fully
107 replenished their initially depleted zinc concentrations at the expense of other organs or even accumulated Zn
108 above the level of the control group. The critical threshold for these compensatory reactions was also ~40 mg
109 Zn / kg diet [36]. Overall, these findings suggested two independent Zn-homeostatic regulatory pathways that
110 act in this early phase of developing zinc deficiency: 1) the regulation of the absorption, redistribution and
111 excretion of zinc for the maintenance of whole-body Zn homeostasis (critical threshold: ~60 mg / kg diet for
112 8d) and 2) the compensation of increased oxidative stress and perhaps inflammation in certain tissues as a
113 result of an ongoing nutritional zinc deficiency (critical threshold; ~40 mg / kg diet for 8d).

So far, no study has dealt with the qualitative and quantitative expression of all known Zn transporter genes in a large animal model. In addition, current data sets on Zn transporters are mostly limited to very specific roles related to biomedical research questions, and only a handful of studies looked at the situation under non-clinical conditions in and between tissues in response to finely graded differences in dietary Zn supply. Finally, the regulation of Zn transporters under conditions of SZD is yet unclear. We believe that the comparative analysis of the Zn transporter gene expression in and between tissues allows a deeper insight into the adaptation of the body to different Zn supply levels. Such a dataset could also be suitable to test our hypothesis of two different homeostatic regulation pathways in the early stages of a developing Zn deficiency. The present study therefore examined the expression of the known ZnT and ZIP genes in potentially important tissues of zinc homeostasis (jejunal and colonic mucosa, liver, kidney). To the best of our knowledge, this dataset represents the first comprehensive examination of ZnT and ZIP gene expression in the translational model of the pig. Furthermore, it appears to be the first investigation of zinc transporter genes in the context 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 [37]. The dietary model applied for this investigation was carefully developed to allow in-depth 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 [33] and the subsection on statistical analyses, respectively.

2.1 Animals and diets

This study applied the experimental SZD model originally proposed by Brugger, Buffler [33], 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. 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. 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 ± 0.24, 33.6 ± 0.56, 38.8 ± 0.82, 42.7 ± 0.48, 47.5 ± 0.97, 58.2 ± 0.29, 67.8 ± 1.87, 88.0 ± 1.65 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 total experimental period during which the varying zinc feeding occurred lasted a total of 8d. Figure 1 shows a scheme of the study time-course.

The basal diet was designed to meet all the recommendations of the National Research Council regarding the feeding of weaned piglets except for Zn [38]. 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 (tap water) *ad libitum* during all times of this study and were subject to continuous veterinary surveillance. The zinc background in drinking water was analyzed regularly to ensure negligible background levels.

2.2 Sampling conditions

Diet samples were collected and processed as described previously [33]. 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.

180 ## 2.3 Analyses of dry matter and total zinc concentration in diets

181 Analyses of dry matter and Zn in diets occurred as described earlier [33]. Each experimental diet was sampled
182 in triplicate and milled through a 0.5 mm screen. Subsequently, each of the triplicates was weighed in triplicate
183 for chemical extraction procedures, producing n = 9 data points for nutrient and Zn concentrations of each
184 individual experimental diet). Zn concentrations were measured by atomic absorption spectrometry (AAS)
185 (NovAA 350; Analytik Jena AG) applying a certified AAS Zn standard material (Merck 109953, Merck
186 Millipore) after microwave wet digestion (Ethos 1, MLS GmbH) with 65% HNO_3, 30% H_2O_2 and Type I
187 ultrapure water.

188

189 ## 2.4 Gene expression analysis

190 Primer design, assay quality control and chemical procedures (total RNA extraction, reverse transcription,
191 quantitative PCR (qPCR)) were performed as described earlier[33, 35]. Purity (measured with the NanoDrop
192 2000 system, Thermo Scientific) and integrity (measured with the Experion system, Biorad) of all total RNA
193 extracts from all tissues met or exceeded the minimum thresholds necessary for gene expression profiling
194 applying qPCR methodology[39]. Primer pairs (Eurofins Scientific) were designed with Primer Blast [40] for
195 the potential reference transcripts glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-glucuronidase
196 (GUSB), histone H3 (H3), ubiquitin C (UBC), β-actin (ACTB) and divalent metal transporter 1 (DMT1) as
197 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,
198 10, 11, 12, 13, 14, based on published porcine sequence information [41] (Supplementary Tables 1 and 2). All
199 oligonucleotides bind to homologous regions of respective transcripts to amplify the pool of potential
200 transcript variants within one reaction. Reference gens were selected for each individual tissue by applying
201 the RefFinder tool for the respective complete Ct datasets from each tissue [42]. This tool combines the major
202 computational approaches for reference evaluation (geNorm, Normfinder, BestKeeper, comparative Delta-Ct
203 method) to compare and rank the tested genes. In this way we identified the following suitable reference genes:
204 *ZnT5, ZnT6* and *ZIP9* in the jejunum, *DMT1, ZIP1* and *ZnT5* in the colon, *DMT1, ZIP7* and *ZIP13* in the liver
205 as well as *ZnT4, ZnT5* and *ZnT6* in the kidney. The $2^{-\Delta\Delta Ct}$ method [43] was used to normalize the gene

206 expression data, because the determination of the amplification efficiency revealed comparable values
207 between 95% and 100% of applied RT-qPCR assays. A detailed description of the procedure for the
208 determination of amplification efficiency has been provided earlier [33].
209 *ZIP10* and *ZIP12* transcripts were not detected in any of the porcine tissues examined within the present study.
210 These assays amplify sequences, which appear to be highly conserved between mammal species. Therefore,
211 murine brain and liver cDNA preparations were used for testing.
212

213 2.5 Statistical analyses

214 Data analysis was performed with SAS 9.4 (SAS Institute Inc.) applying the procedure NLMIXED to estimate
215 linear broken-line regression models ($y = a + bx + cx$) based on independent group means relative to dietary
216 Zn concentration ($n = 8$). The decision for linear broken-line models over non-linear models was made by
217 following the approach proposed by McDonald [44] by which the goodness-of-fit of linear vs. polynomial
218 models is statistically compared using F-statistics. In case of our dataset we found that applying non-linear
219 models over the linear broken-line models yielded no significant increase in the quality of the curve-fitting.
220 Furthermore, using single data points from individual animals instead of the group mean values was not
221 advisable in light of the present experimental design because the imbalance in the ratio of X (eight dietary
222 treatment groups) to Y (six response values per treatment group) coordinates would have caused a severe
223 overestimation of the degrees of freedom and, therefore, false results. Broken-line regression is an iterative
224 procedure to estimate a potential statistical threshold (breakpoint) within non-linear data sets above and below
225 which a significant difference in the response behavior of a certain parameter to the dietary treatment is evident
226 [45]. If no significant breakpoint in parameter response could be estimated from a certain data set, a linear
227 regression model was tested instead (y= a + bx) (procedure REG). It is noteworthy, that those gene expression
228 patterns, which yielded no significant broken-line regression model, also did not fit significant linear models.
229 Only significant regression models were applied for data presentation and interpretation in the present
230 manuscript. A threshold of $P \leq 0.05$ was considered to indicate statistical significance. All $2^{-\Delta\Delta Ct}$ gene

expression values [43] 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 [46] 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 $1 - \beta = 0.8$ [47]. 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 in swine (for example growth retardation, anorexia as earlier reported by Tucker and Salmon [32]) evident at any time [33]. The experimental model introduced finely-graded adaptions in Zn status parameters as well as the Zn concentrations in the tissues under study (Supplementary Figure 1). This data has been presented earlier [36] and will therefore not been described in more detail in the present manuscript.

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 analyzed 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 *ZnT5* 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 [48]).

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

266 *ZIP2* and *ZIP3* in colonic tissue as well as the candidate genes that served as reference genes for data
267 normalisation within respective tissues. Significant dietary thresholds either lay at ~40 or ~60 mg Zn/kg diet,
268 respectively. However, the slopes of the respective segments within many broken-line regression models were
269 not significant and coefficients of determination of respective models were low (R^2). Subsequently, only
270 models expressing at least one significant slope over changes in dietary Zn supply are described within figures
271 and tables.

272 Figure 2 presents the broken-line response of jejunal Zn transporter gene expression as affected by varying
273 dietary Zn supply. Table 3 presents the corresponding statistical measures of the respective regression curves.
274 Above significant dietary thresholds of 57.1, 62.3, 38.8, 41.6, 62.6 and 52.3 mg Zn/kg diet ($P < 0.0001$,
275 respectively) jejunal *ZIP5* and *ZIP11* significantly increased or decreased, respectively, in response to changes
276 in dietary Zn ($P \leq 0.05$, respectively) whereas *ZIP1* and *ZIP13* did not change in a significant manner. Below
277 these thresholds, the relative mRNA abundance of *ZIP1* and *ZIP13* significantly increased whereas *ZIP11*
278 significantly decreased with further reduction in dietary Zn supply ($P \leq 0.001, \leq 0.05$ and ≤ 0.0001,
279 respectively). The *ZIP5* gene expression did not change significantly with stepwise decrease in dietary Zn
280 concentration below its respective breakpoint.

281 Figure 3 presents the broken-line response of colonic Zn transporter gene expression as affected by varying
282 dietary Zn supply. Table 4 presents the corresponding statistical measures of the respective regression curves.
283 Relative mRNA abundance of *ZnT4, ZnT9, ZIP4, ZIP5, ZIP7, ZIP11* and *ZIP13* showed significant
284 breakpoints in response to a finely graded reduction in dietary Zn concentration at 60.6, 63.9, 59.6, 39.0, 42.7,
285 44.8 and 68.3 mg Zn/kg diet, respectively ($P \leq 0.0001$, respectively). Above the respective dietary thresholds,
286 *ZIP4, ZIP5* and *ZIP7* plateaued in response to changes in dietary Zn. These genes significantly increased their
287 relative expression levels in response to further reduction in dietary Zn below these breakpoints ($P \leq 0.001$,
288 ≤ 0.001 and ≤ 0.05 for *ZIP4, ZIP5* and *ZIP7*, respectively). On the contrary, colonic *ZnT4* and *ZIP11*
289 significantly increased ($P \leq 0.05$ and ≤ 0.001, respectively) whereas *ZnT9* and *ZIP13* significantly decreased
290 ($P \leq 0.05$, respectively) with reduction in dietary Zn concentration from 88.0 mg Zn/kg to their respective

291 breakpoints. Below these dietary thresholds, *ZnT4* and *ZIP11* significantly decreased ($P \leq 0.001$ and ≤ 0.05,
292 respectively) whereas *ZnT9* and *ZIP13* did not change significantly.

293 Figure 4 presents the broken-line response of hepatic Zn transporter gene expression as affected by varying
294 dietary Zn supply. Table 5 presents the corresponding statistical measures of the respective regression curves.
295 Gene expression patterns of *ZnT4, ZnT6, ZnT8, ZIP1* and *ZIP14* exhibited significant changes in their response
296 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
297 ≤ 0.0001). Above the respective dietary thresholds, *ZnT4, ZnT8, ZIP1* and *ZIP14* did not change significantly
298 in response to changes in dietary Zn supply whereas *ZnT6* significantly decreased directly to a reduction in
299 dietary Zn from 88.0 mg Zn/kg diet to the respective breakpoint ($P \leq 0.001$). On the contrary, *ZnT4* and *ZnT6*
300 significantly decreased ($P \leq 0.001$ and ≤ 0.05, respectively) whereas *ZnT8, ZIP1* and *ZIP14* significantly
301 increased ($P \leq 0.05$, ≤ 0.05 and ≤ 0.0001, respectively) with reduction in dietary Zn concentration below the
302 respective dietary thresholds.

303 Figure 5 presents the broken-line response of nephric Zn transporter gene expression as affected by varying
304 dietary Zn supply. Table 6 presents the corresponding statistical measures of the respective regression curves.
305 Gene expression of *ZnT1, ZnT3, ZnT7* and *ZIP4* changed significantly around dietary thresholds of 70.4, 42.6,
306 35.2 and 41.9 mg Zn/kg diet ($P < 0.0001$, respectively). All these genes plateaued in response to a reduction
307 of dietary Zn concentration from 88.0 mg/kg to the respective breakpoints. Further reduction in dietary Zn
308 below these thresholds promoted a significant increase of *ZnT3, ZnT7* and *ZIP4* ($P \leq 0.05$, ≤ 0.0001 and $\leq$
309 0.05, respectively) as well as a significant decrease of *ZnT1* gene expression ($P \leq 0.05$).

310 Figure 6 summarizes the aforementioned breakpoint estimates from Tables 3, 4, 5 and 6 including respective
311 standard errors of the statistical estimation. Across tissues, observed breakpoints of gene expression response
312 to varying dietary Zn supply scattered around mean values of 42.9 ±2.08 or 63.1 ±6.19 mg Zn/kg diet,
313 respectively.

4 Discussion

We investigated the expression of ZnT (SLC30) and ZIP (SLC39) transporter genes in weaned piglets exposed to different Zn concentrations in the diet (28.1 to 88.0 mg Zn/kg). The tissues examined were jejunal and colonic mucosa, liver and kidney, as the play key roles in in the uptake, (re)distribution and excretion of Zn in the body [23]. The gene expression of the pancreas was not investigated for technical reasons. The experimental setup included both sexes and the results of the present study were comparable between them.

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

Most transcripts were present in all examined tissues, which seems to agree with the available literature (summarized in [3-6]). The only exceptions were *ZIP10* and *ZIP12*, which were not recognized at all. In addition, *ZnT3* mRNA was only expressed in nephric tissue.

RNA sequencing experiments in humans and *Drosophila* confirmed *ZIP12* gene expression only in the brain [49, 50]. In addition, studies on its functional genomics and proteomics have highlighted its role in neuronal development and function [51, 52] and in the schizophrenic brain [53]. Hence, the absence of *ZIP12* cDNA in any tissue from the present study agrees well with these findings. Otherwise, *ZIP10* transcription has been identified in various tissues, including those investigated in the present study [49, 50]. Aside from its role in cancer progression, its function as a zinc transporter within the renal collecting duct system has been studied in various species [54-56], highlighting its possible role in reabsorption of Zn^{2+} from primary urine. Hence, the question arises why we could not identify its mRNA signature in any of our samples, including those from the kidney? Our *ZIP10* qPCR assay amplifies an mRNA sequence that is conserved in mammals, including rodents and humans. It worked successfully with murine brain and liver preparations (data not shown). Furthermore, we used a representative cross-section of each porcine kidney for the total RNA preparations. We therefore conclude that the lack of *ZIP10* transcripts in our tissue samples was not due to technical bias and that the growing pig does not express this gene in respective tissues under the given experimental conditions. However, future studies should apply microdissection of different parts of the porcine kidney to confirm our results.

340 *ZnT3* mRNA was found in brain, adipose tissue, pancreatic beta-cells, epithelial cells, testis, prostate and retina
341 [57]. To date, most studies have focused on its role in transferring cytosolic Zn into synaptic vesicles [58].
342 Interestingly, this was also the case in gastrointestinal enteric neurons from pigs [59, 60]. The lack of *ZnT3*
343 gene expression in pig jejunum and colon from the present study may be due to the fact that these samples
344 represented the mucosa. To our knowledge, no study has yet investigated *ZnT3* gene expression in the livers
345 and kidneys of pigs. Therefore, this could be the first report on the lack of *ZnT3* gene expression in the liver
346 and its simultaneous detection in the kidney of the growing pig, at least under SZD conditions.
347

348 4.2 Response of *ZnT* and *ZIP* family member gene expression to dietary zinc

349 It has previously been shown that some Zn transporter genes respond to deficient dietary Zn supply (*ZnT1,*
350 *ZnT2, ZnT4, ZnT5, ZnT6, ZIP4, ZIP10*) [13, 18, 55, 61]. We have confirmed this for *ZnT1* (kidney), *ZnT4*
351 (colon, liver), *ZnT6* (liver) and *ZIP4* (colon, kidney) but not for *ZnT2, ZnT5* and *ZIP10*. In addition, other
352 transcripts also responded to deficient dietary Zn supply, which apparently have not yet been reported,
353 including *ZnT3* (kidney), *ZnT7* (kidney), *ZnT8* (liver), *ZnT9* (colon), *ZIP1* (jejunum, liver), *ZIP5* (jejunum,
354 colon), *ZIP7* (colon), *ZIP11* (jejunum, colon), *ZIP13* (jejunum, colon) and *ZIP14* (liver). Most earlier studies
355 used clinical Zn deficiency models. Therefore, our data seem to highlight differences in the adaption of Zn
356 transporter gene expression to short-term SZD. Clinical Zn deficiency is associated with many secondary
357 metabolic events during which tissue integrity can be compromised [32]. This reduces the resolution of
358 measurements due to increased background noise. Finally, clinical Zn deficiency is the endpoint in
359 physiological adaption to body Zn depletion. Hence, early response patterns have already changed, which
360 could explain why some results from the present short-term study were not described earlier. Several of the
361 *ZnT* and *ZIP* gene expression patterns showed significant breakpoints in response to diet Zn. However, only
362 the reaction of the genes shown in the figures and tables allowed a significant curve fitting. We assume that
363 only these genes were directly regulated by changes in the dietary Zn supply. On the contrary, the Zn
364 transporter genes with only significant breakpoints may have just adapted indirectly to the changes in Zn
365 fluxes triggered by these key transporters and are therefore not discussed further in this manuscript.

366 Genes showing a significant dose-response to dietary Zn could be divided into two groups, with statistical
367 breakpoints at either ~40 or ~60 mg Zn/kg diet. The gross Zn requirement under given conditions was earlier
368 estimated at ~60 mg Zn/kg diet [33]. We therefore assume that *ZnT* and *ZIP* genes, with a statistical breakpoint
369 of ~60 mg Zn/kg diet, are involved in maintaining whole-body Zn homeostasis. A prominent example was
370 colonic *ZIP4* gene expression, which plateaued in groups receiving ≥60 mg Zn/kg diet. A gradual decrease in
371 dietary Zn below this threshold induced a linear upregulation of *ZIP4* gene expression. In fact, this response
372 pattern correlates significantly with earlier data on the apparently digested feed Zn under the present
373 experimental conditions ($r = -0,91$; $P = 0.002$; data not shown) [33]. This reflects the classic concept of Zn
374 homeostasis, according to which the organism increases its Zn absorption capacity at the gut barrier during
375 periods of Zn malnutrition [26]. It also agrees with earlier published work in which ZIP4 was identified as the
376 main active Zn transporter at the apical membrane of enterocytes [16, 18, 24]. Our data confirms the role of
377 this gene within the homeostatic network that controls Zn levels in the body and particularly Zn absorption in
378 pigs and other mammals.
379 Regarding genes with a statistical breakpoint at ~40 mg Zn/kg diet, we postulate that they may be involved in
380 regulation of tissue Zn in connection with redox and immune functions. We have shown earlier under the
381 present experimental conditions that the heart muscle of Zn-deficient piglets restored its initially depleted total
382 Zn concentration. This was probably due to an up-regulation of the transporters that import Zn from the
383 circulation into the tissue. It occurred as a reaction to increased cardiac oxidative stress during SZD in groups
384 fed ≤40 mg Zn/kg diet [35]. Therefore, breakpoints of Zn transporter genes at ~40 mg Zn/kg diet could indicate
385 similar events in other tissues. A prominent example is the hepatic ZIP14. It was previously identified as an
386 acute-phase protein that transports circulating Zn and non-transferrin iron into the liver during times of
387 systemic inflammation [62, 63]. In the present study, its gene expression increased in piglets fed ≤42.7 mg
388 Zn/kg diet. Given the previous cardiac data, this could suggest that these animals developed an inflammatory
389 state during SZD. Earlier published data support this hypothesis by showing an inverse correlation between
390 systemic inflammatory activity and Zn status in the elderly [64]. The functional background has recently been
391 reviewed [65]. Indeed, we currently lack data on the adaption of stress and inflammatory pathways in the

392 tissues studied. These must be collected in future studies to allow a functional correlation to the regulation
393 and activity of certain Zn transporters.
394 It must be considered that the threshold of ~40 mg Zn/kg diet does not represent a minimum dietary Zn
395 concentration above which there are no adverse effects on the redox metabolism. Rather, it seems to be related
396 to the maximum tolerable bone Zn depletion during our experiment. In fact, animals that received ≤43 mg
397 Zn/kg diet showed a reduction in bone Zn between ~20-25% under given experimental conditions [33]. This
398 represents an exhaustion of the mobilizable skeletal Zn fraction, which was previously shown in ^{65}Zn-labelled
399 rats [66-68]. A continuation of the study >8 d until mobilizable body Zn stores were finally depleted, would
400 most likely have increased this threshold over time until it had reached the previously defined gross Zn
401 requirement [33].
402

403 ## 4.3 ZIP4 and ZnT1 expression in the intestine and kidney under SZD conditions

404 ZIP4 and ZnT1 are the active route of Zn transfer from the intestinal lumen to the circulation [7-14]. Previous
405 studies suggest that *ZIP4* transcription directly responds to the status of whole-body Zn homeostasis through
406 KLF4 activity [18, 19]. Colonic *ZIP4* expression has already been discussed. Its breakpoint reflects the gross
407 Zn requirement under given test conditions (60 mg/kg) [33] and confirms regulation for the benefit of whole-
408 body Zn homeostasis. Interestingly, we did not find any significant response of jejunal *ZIP4* gene expression.
409 The jejunum is generally regarded as the main site of Zn absorption [69]. We therefore expected a significant
410 upregulation of *ZIP4* transcription in the jejunal before the colonic mucosa, like has been shown in mice fed
411 low Zn diets [7, 9, 16, 70]. This was not the case, which again highlights clear differences between subclinical
412 and clinical Zn deficiency. Given the response of colonic *ZIP4* transcription to changes in dietary Zn supply
413 and its positive correlation with apparent Zn absorption, we assume that the large intestine is the main site of
414 Zn acquisition under SZD conditions. This has already been suggested by other authors who found peaks in
415 the colonic expression of Zn-responsive genes under conditions of mild Zn deficiency in adult rats [21, 71].
416 In addition, there have been reports of a significant contribution of caecal and colonic Zn absorption in times
417 of impaired Zn acquisition from the small intestine [72, 73]. We have previously shown a decrease in

418 pancreatic digestive capacity under the present experimental conditions [34]. We therefore conclude that the
419 main site of Zn absorption may have shifted to the large intestine in favor of Zn-dependent digestive enzymes
420 to stabilize the already impaired protein digestion. This needs to be checked in future studies with Ussing
421 chambers and patch-clamp techniques.

422 Contrary to earlier data [13], *ZnT1* gene expression was not regulated in any gut tissue examined. This protein
423 is located on the basolateral side of enterocytes and transports cytosolic Zn into the circulation [11]. *ZnT1*
424 transcription reacts to free cytosolic Zn^{2+}, which activates metal-regulatory transcription factor 1 (MTF1) with
425 rising concentrations [13, 20, 21]. Although total jejunal and colonic Zn have been decreased under the present
426 experimental conditions[36], this did not seem to affected the free cytosolic Zn^{2+} according to the unchanged
427 *ZnT1* transcription. Given the aforementioned decrease in jejunal and colonic Zn, it can be speculated that the
428 ZnT1 transport activity was altered, but regulation may have occurred mainly at a post-transcriptional level.
429 The exact nature of this regulation has yet to be investigated. It is assumed that extending the experimental
430 phase >8 d would also have caused an adaption of the *ZnT1* gene expression, as has been shown under long-
431 term conditions [11].

432 Previous studies showed no significant reaction of kidney or urinary Zn to varying Zn feeding [26, 67]. In
433 fact, the importance of the kidney in maintaining whole-body Zn homeostasis tends to be underestimated. The
434 stabilization of Zn levels in a tissue and its excretions despite changing dietary conditions is, however, a clear
435 sign of active homeostatic regulation. Most previous studies conducted experiments for ≥2 weeks. Hence, the
436 published kidney data reflect an endpoint in adjusting to clinical Zn deficiency. Under the present conditions,
437 kidney Zn and bone Zn showed a strong correlation ($r = +0.91$, $P = 0.002$, data not shown). The kidney seemed
438 to give up its Zn in favor of other tissues (e.g. heart, immune tissue) [36]. The present study complements this
439 picture with the reponse patterns of Zn transporter genes including *ZnT1* and *ZIP4*. The first appeared to be
440 regulated in context to basal Zn homeostasis, given its breakpoint at ~60 mg/kg diet. This regulation pattern
441 significantly correlates to kidney Zn and bone Zn ($r = +0.77$ and 0.94, $P = 0.02$ and 0.0005, data not shown),
442 which could indicate that ZnT1 was responsible for the kidney Zn depletion in response to the gradual
443 exhaustion of bone Zn. This is in line with earlier reports on the presence of ZnT1 at the basolateral membrane

of kidney cells [12]. It further indicates that the renal free cytosolic Zn^{2+} levels correlated with total kidney Zn over a wide range of dietary Zn dosages (28 to 68 mg Zn/kg diet), which contrasts our findings from the intestines. Contrary to the renal *ZnT1* expression, *ZIP4* expression changed at a much lower dietary threshold (~40 mg/kg) and increased linearly with further decrease in dietary Zn. As mentioned above, this breakpoint may indicate the *ZIP4* expression was regulated to compensate for increased cellular stress. An earlier study confirmed ZIP4 gene expression in the kidney [8], but there is yet no data on its role or regulation in renal tissue. We suspect that ZIP4 is involved in recycling of Zn from primary urine under SZD conditions. This would be particularly interesting considering that *ZIP10* gene expression was absent in this tissue, although it was previously claimed to be involved in urinary Zn reabsorption [54-56]. Unfortunately, our data do not allow us to differentiate between kidney cell types. Future studies will need to use microdissections to further support our findings. The possible interaction of the kidney with the intestinal-pancreatic axis of Zn homeostatic regulation, as suggested by Liuzzi, Bobo [7], should also be clarified.

Other Zn transporter genes also showed a significant dose-response to changes in dietary Zn and were shown accordingly in result tables and figures. Their exact role in the examined tissues is currently unclear and requires further scientific studies. In contrast, some *ZnT* and *ZIP* genes showed a very stable expression level over dietary Zn dosages, including jejunal *ZnT5* and *ZnT6*, colonic *ZIP1* and *ZnT5*, hepatic *ZIP7* and *ZIP13* as well as nephric *ZnT4, ZnT5* and *ZnT6*. Therefore, these served as reference genes for data normalization. Future studies should investigate under what conditions the transcription of these genes changes in pigs.

In conclusion, we recognized significant differences in the expression of zinc transporter genes to SZD compared to previous studies on clinical Zn deficiency. Many of the transcripts examined showed significant breakpoints in response to a reduction in dietary Zn. These thresholds were either ~40 or ~60 mg Zn/kg diet, which suggests differences in the specific stimuli to which these genes respond. A breakpoint near ~60 mg Zn/kg diet corresponds to the gross Zn requirement threshold under the present experimental conditions and suggests a role of certain genes in the maintenance of the basal whole-body Zn homeostasis. Other genes showed breakpoints close to ~40 mg Zn/kg diet, which was previously associated with replenishment of tissue

Zn and the adjustment of compensation mechanisms in response to increased oxidative stress under SZD conditions. This manuscript presents the first comparative study of the effects of short-term finely-graded differences in dietary Zn supply on the expression of all known ZnT and ZIP genes in different tissues of weaned piglets. Future studies must address their regulation on the proteomic level, accompanied by studies on tissue transcriptomics, proteomics and metabolomics to complete our knowledge on Zn transporters in mammal biology. Our current findings can be translated from pigs into humans due to the high similarity between these species in terms of their nutrition physiology.

CRediT author statement

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

Acknowledgements

We would like to express our deepest gratitude to Dipl. Ing. (FH) Michael Gertitschke and Andrea Reichlmeir for excellent technical assistance. Special thanks are related to Dr. med. vet. Astrid Kunert for veterinary assistance and advice as well as Gavin Boerboom, M.Sc., and Brett Boden, M.Sc., for valuable advice on the manuscript.

References

[1] Andreini C, Banci L, Bertini I, Rosato A. Counting the zinc-proteins encoded in the human genome. J Proteome Res. 2006;5:196-201.
[2] Frassinetti S, Bronzetti G, Caltavuturo L, Cini M, Croce CD. The role of zinc in life: a review. J Environ Pathol Toxicol Oncol. 2006;25:597-610.
[3] Baltaci AK, Yuce K. Zinc Transporter Proteins. Neurochem Res. 2018;43:517-30.
[4] Lichten LA, Cousins RJ. Mammalian zinc transporters: Nutritional and physiologic regulation. Ann Rev Nutr. 2009;29:153-76.
[5] Fukada T, Kambe T. Molecular and genetic features of zinc transporters in physiology and pathogenesis. Metallomics. 2011;3:662-74.
[6] Schweigel-Röntgen M. The families of zinc (SLC30 and SLC39) and copper (SLC31) transporters. In: Bevensee MO, editor. Exchangers. Burlington: Academic Press; 2014. p. 321-55.
[7] Liuzzi JP, Bobo JA, Lichten LA, Samuelson DA, Cousins RJ. Responsive transporter genes within the murine intestinal-pancreatic axis form a basis of zinc homeostasis. Proc Natl Acad Sci USA. 2004;101:14355-60.
[8] Wang K, Zhou B, Kuo YM, Zemansky J, Gitschier J. A novel member of a zinc transporter family is defective in acrodermatitis enteropathica. Am J Hum Genet. 2002;71:66-73.
[9] Dufner-Beattie J, Wang F, Kuo YM, Gitschier J, Eide D, Andrews GK. The acrodermatitis enteropathica gene ZIP4 encodes a tissue-specific, zinc-regulated zinc transporter in mice. J Biol Chem. 2003;278:33474-81.
[10] Dufner-Beattie J, Weaver BP, Geiser J, Bilgen M, Larson M, Xu W, et al. The mouse acrodermatitis enteropathica gene Slc39a4 (Zip4) is essential for early development and heterozygosity causes hypersensitivity to zinc deficiency. Hum Mol Genet. 2007;16:1391-9.
[11] McMahon RJ, Cousins RJ. Regulation of the zinc transporter ZnT-1 by dietary zinc. Proc Natl Acad Sci USA. 1998;95:4841-6.
[12] Cousins RJ, McMahon RJ. Integrative aspects of zinc transporters. J Nutr. 2000;130:1384S-7S.
[13] Liuzzi JP, Blanchard RK, Cousins RJ. Differential regulation of zinc transporter 1, 2 and 4 mRNA expression by dietary zinc in rats. J Nutr. 2001;131:46-52.

[14] Andrews GK, Wang H, Dey SK, Palmiter RD. Mouse zinc transporter 1 gene provides an essential function during early embryonic development. Genesis. 2004;40:74-81.

[15] Mao X, Kim BE, Wang F, Eide DJ, Petris MJ. A histidine-rich cluster mediates the ubiquitination and degradation of the human zinc transporter, hZIP4, and protects against zinc cytotoxicity. J Biol Chem. 2007;282:6992-7000.

[16] Weaver BP, Dufner-Beattie J, Kambe T, Andrews GK. Novel zinc-responsive post-transcriptional mechanisms reciprocally regulate expression of the mouse Slc39a4 and Slc39a5 zinc transporters (Zip4 and Zip5). The Journal of Biological Chemistry. 2007;388:1301-12.

[17] Kambe T, Andrews GK. Novel proteolytic processing of the ectodomain of the zinc transporter ZIP4 (SLC39A4) during zinc deficiency is inhibited by acrodermatitis enteropathica mutations. Molecular Cell Biology. 2009;29:129-39.

[18] Liuzzi JP, Cousins RJ, Guo L, Chang SM. Kruppel-like factor 4 regulates adaptive expression of the zinc transporter ZIP4 (Slc39A4) in mouse small intestine. Amercian Journal of Physiology Gastrointestinal and Liver Physiology. 2009;296:G517-G23.

[19] Curry-McCoy TV, Guidot DM, Joshi PC. Chronic alcohol ingestion in rats decreases Krüppel-like factor 4 expression and intracellular zinc in the lung. Alcoholism: Clinical and Experimental Research. 2013;37:361-71.

[20] Brugnera E, Georgiev O, Radtke F, Heuchel R, Baker E, Sutherland GR, et al. Cloning, chromosomal mapping and characterization of the human metal-regulatory transcription factor MTF-1. Nucleic Acids Research. 1994;22:3167-73.

[21] Langmade SJ, Ravindra R, Daniels PJ, Andrews GK. The transcription factor MTF-1 mediates metal regulation of the mouse ZnT1 gene. J Biol Chem. 2000;275:34803-9.

[22] Matsuno S, Miyashita E, Ejiri T, Sato T. Zinc and magnesium output in pancreatic juice after pancreaticoduodenectomy. The Tohoku Journal of Experimental Medicine. 1982;136:11-22.

[23] Holt RR, Uiu-Adams JY, Keen CL. Zinc. In: Erdman JW, Macdonald IA, Zeisel SH, editors. Present Knowledge in Nutrition. 10th ed. Hoboken, New Jersey: Wiley-Blackwell; 2012. p. 521 - 39.

[24] Dufner-Beattie J, Kuo Y-M, Gitschier J, Andrews GK. The adaptive response to dietary zinc in mice involves the differential cellular localization and zinc regulation of the zinc transporters ZIP4 and ZIP5. J Biol Chem. 2004;279:49082-90.

[25] Guo L, Lichten LA, Ryu M-S, Liuzzi JP, Wang F, Cousins RJ. STAT5-glucocorticoid receptor interaction and MTF-1 regulate the expression of ZnT2 (Slc30a2) in pancreatic acinar cells. PNAS. 2010;107:2818-23.

[26] Weigand E, Kirchgessner M. Total true efficiency of zinc utilization: Determination and homeostatic dependence upon the zinc supply status in young rats. J Nutr. 1980;110:469 - 80.

[27] Nielsen FH. History of zinc in agriculture. Adv Nutr. 2012;3:783 - 9.

[28] Windisch W. Effect of microbial phytase on the bioavailability of zinc in piglet diets. Proc Soc Nutr Physiol. 2003;12:33.

[29] Windisch W. Development of zinc deficiency in ^{65}Zn labeled, fully grown rats as a model for adult individuals. J Trace Elem Med Biol. 2003;17:91 - 6.

[30] Windisch W. Homeostatic reactions of quantitative Zn metabolism on deficiency and subsequent repletion with Zn in ^{65}Zn-labeled adult rats. Trace Elem Elec. 2001;18:122 - 8.

[31] Brugger D, Schlattl M, Windisch W. Short-term kinetics of tissue zinc exchange in ^{65}Zn-labelled adult rats receiving sufficient dietary zinc supply. Proc Soc Nutr Physiol. 2018;27:96.

[32] Tucker HF, Salmon WD. Parakeratosis or zinc deficiency disease in the pig. Proc Soc Exp Biol Med. 1955;88:613-6.

[33] Brugger D, Buffler M, Windisch W. Development of an experimental model to assess the bioavailability of zinc in practical piglet diets. Arch Anim Nutr. 2014;68:73-92.

[34] Brugger D, Windisch W. Subclinical zinc deficiency impairs pancreatic digestive enzyme activity and digestive capacity of weaned piglets. Br J Nutr. 2016;116:425-33.

[35] Brugger D, Windisch W. Short-term subclincial zinc deficiency in weaned piglets affects cardiac redox metabolism and zinc concentration. J Nutr. 2017;147:521-7.

[36] Brugger D, Windisch WM. Adaption of body zinc pools in weaned piglets challenged with subclinical zinc deficiency. Brit J Nutr. 2019;121:849-58.

[37] Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Impoving bioscience research reporting: The ARRIVE guidelines for reporting animal research. PLOS Biol. 2010;8:e1000412.

[38] NRC. Nutrient requirements of swine. 11th ed. Washington, D.C., USA: Nat. Acad. Press; 2012.

[39] Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009;55:611-22.

[40] Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden T. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinf. 2012;13:134.

[41] O´Leary NA, Wright MW, Brister JR, Ciufo S, Haddad D, McVeigh R, et al. Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion and functional annotation. Nucleic Acids Research. 2016;44:D733-D45.
[42] Xie F, Xiao P, Chen D, Xu L, Zhang B. miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. Plant Mol Biol. 2012;80:75-84.
[43] Livak K, Schmittgen T. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 2001;25:402-8.
[44] McDonald JH. Curvilinear Regression. In: McDonald JH, editor. Handbook of Biological Statistics, 3rd ed. Baltimore, MD, USA: Sparky House Publishing; 2014. p. 215-2.
[45] Robbins KR, Saxton AM, Southern LL. Estimation of nutrient requirements using broken-line regression analysis. Journal of Animal Science. 2006;84:E155-E65.
[46] Faul F, Erdfelder E, Lang A-G, Buchner A. G*Power 3: A flexible statistical power analysis program for the social, behavioural, and biomedical sciences. Behav Res Methods. 2007;39:175-91.
[47] McDonald JH. Power Analysis. In: McDonald JH, editor. Handbook of Biological Statistics, 3rd ed. Baltimore, MD, USA: Sparky House Publishing; 2014. p. 40-4.
[48] Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002;3:research0034-research.11.
[49] Duff MO, Olson S, Wei X, Garrett SC, Osman A, Bolisetty M, et al. Genome-wide identification of zero nucleotide recursive splicing in Drosophilla. Nature. 2015;521:376-9.
[50] Fagerberg L, Hallström BM, Oksvold P, Kampf C, Djureinovic D, Odeberg J, et al. Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics. Mol Cell Proteomics. 2014;13:397-406.
[51] Chowanadisai W, Graham DM, Keen CL, Rucker RB, Messerli MA. Neurulation and neurite extension require the zinc transporter ZIP12 (slc 39a12). PNAS. 2013;110:9903-8.
[52] Chowanadisai W. Comparative genomic analysis of slc39a12/ZIP12: insight into a zinc transporter required for vertebrate nervous system development. PLoS One. 2014;9:e111535.
[53] Bly M. Examination of the zinc transporter gene, SLC39A12. Schizophr Res. 2006;81:321-2.
[54] Kumar R, Prasad R. Functional characterization of purified zinc transporter from renal brush border membrane of rat. Biochem Biophys Acta. 1999;1419:23-32.
[55] Kaler P, Prasad R. Molecular cloning and functional characterization of novel zinc transporter rZip10 (Slc39a10) involved in zinc uptake across rat renal brush-border membrane. Am J Physiol Renal Physiol. 2007;292:F217-F29.
[56] Landry GM, Furrow E, Holmes HL, Hirata T, Kato A, Williams P, et al. Cloning, function, and localization of human, canine, and Drosophila ZIP10 (SLC39A10), a Zn2+ transporter. Am J Physiol Renal Physiol. 2019;316:F263-F73.
[57] Smidt K, Rungby J. ZnT3: a zinc transporter active in several organs. Biometals. 2012;25:1-8.
[58] McAllister BB, Dyck RH. Zinc transporter 3 (ZnT3) and vesicular zinc in central nervous system function. Neurosci Biobehav Rev. 2017;80:329-50.
[59] Wojtkiewicz J, Rytel L, Makowska K, Gonkowski S. Co-localization of zinc transporter 3 (ZnT3) with sensory neuromediators and/or neuromodulators in the enteric nervous system of the porcine esophagus. Biometals. 2017;30:393-403.
[60] Gonkowski S, Rowniak M, Wojtkiewicz J. Zinc transporter 3 (ZnT3) in the enteric nervous system of the porcine ileum in physiological conditions and during experimental inflammation. Int J Mol Sci. 2017;18:E338.
[61] Huang L, Kirschke CP, Gitschier J. Functional characterization of a novel mammalian zinc transporter, ZnT6. J Biol Chem. 2002;277:26389-95.
[62] Liuzzi JP, Lichten LA, Rivera S, Blanchard RK, Aydemir TB, Knutson MD, et al. Interleukin-6 regulates the zinc transporter Zip14 in liver and contributes to the hypozincemia of the acute-phase response. PNAS. 2005;102:6843-8.
[63] Liuzzi JP, Aydemir F, Nam H, Knutson MD, Cousins RJ. Zip14 (Slc39a14) mediates non-transferrin-bound iron uptake into cells. PNAS. 2006;103:13612-7.
[64] De Paula RCS, Aneni EC, Costa APR, Figeuiredo VN, Moura FA, Freitas WM, et al. Low zinc levels is associated with increased inflammatory activity but not with atherosclerosis, arteriosclerosis or endothelial dysfunction among the very elderly. BBA Clin. 2014;2:1-6.
[65] Maret W. The redox biology or redox-inert zinc ions. Free Radic Biol Med. 2019;134:311-26.
[66] Windisch W, Kirchgessner M. Zinc excretion and the kinetics of zinc exchange in the whole-body zinc at deficient and excessive zinc supply. 2. Effect of different zinc supply on quantitative zinc exchange in the metabolism of adult rats. J Anim Physiol Anim Nutr. 1994;71:123-30.

[67] Windisch W, Kirchgessner M. Tissue zinc distribution and exchange in adult rats at zinc deficiency induced by dietary phytate additions: II. Quantitative zinc metabolism of ^{65}Zn labelled adult rats at zinc deficiency. J Anim Physiol Anim Nutr. 1999;82:116 - 24.

[68] Windisch W, Wehr U, Rambeck W, Erben R. Effect of Zn deficiency and subsequent Zn repletion on bone mineral composition and markers of bone tissue metabolism in ^{65}Zn labelled, young-adult rats. J Anim Physiol Anim Nutr. 2002;86:214-21.

[69] Lee HH, Prasad AS, Brewer GJ, Owyang C. Zinc absorption in human small intestine. Am J Physiol. 1989;256:G87 - G91.

[70] Dufner-Beattie J, Langmade SJ, Wang F, Eide D, Andrews GK. Structure, function, and regulation of a subfamily of mouse zinc transporter genes. J Biol Chem. 2003;278:50142-50.

[71] Pfaffl MW, Windisch W. Influence of zinc deficiency on the mRNA expression of zinc transporters in adult rats. J Trace Elem Med Biol. 2003;17:97-106.

[72] Hara H, Konishi A, Kasai T. Contribution of the cecum and colon to zinc absorption in rats. J Nutr. 2000;130:83-9.

[73] Martin AB, Aydemir TB, Guthrie GJ, Samuelson DA, Chang SM, Cousins RJ. Gastric and colonic zinc transporter ZIP11 (Slc39a11) in mice responds to dietary zinc and exhibits nuclear localization. J Nutr. 2013;143:1882-8.

639 Table 1. Composition as well as concentrations of metabolizable energy and crude nutrients
640 of the basal diet [33]

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

641 [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
642 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%
643 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
644 of metabolizable energy and essential precaecal digestible (synonymous with "ileal digestible") amino acids were estimated according to feed table
645 information (http://datenbank.futtermittel.net/). Vitamin and trace element concentrations (except zinc) met the requirements according to NRC (10).

646

647 Table 2: Qualitative expression pattern of ZnT and ZIP genes within the jejunum, colon, liver and
648 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]	✓	✓[R]
ZnT6	✓[R]	✓	✓	✓[R]
ZnT7	✓	✓	✓	✓
ZnT8	✓	✓	✓	✓
ZnT9	✓	✓	✓	✓
ZnT10	✓	✓	✓	✓
ZIP1	✓	✓[R]	✓	✓
ZIP2	✓	✓	✓	✓
ZIP3	✓	✓	✓	✓
ZIP4	✓	✓	✓	✓
ZIP5	✓	✓	✓	✓
ZIP6	✓	✓	✓	✓
ZIP7	✓	✓	✓[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	✓	✓	✓	✓

649 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
650 gene using published mathematical procedures [48]. N/A, transcript not detected in respective tissue sample; SLC, solute carrier; ZIP1 to 14, SLC
651 family 39 members 1 to 14; ZnT1 to 10, SLC family 30 members 1 to 10.
652
653

654 Table 3. Broken-line regression analysis of relative jejunal gene expression (xfold) of ZIP1, 5, 11 and
655 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	

656 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
657 estimated based on independent arithmetic group means relative to dietary zinc concentration (*n = 8*). Parameter estimates are presented as means ±
658 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
659 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; SLC, solute
660 carrier; xfold, difference in the gene expression value according to Livak and Schmittgen [43] compared to a relative mRNA abundance of 1.0 in the
661 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
662 response; ZIP1, 5, 11, 13, SLC family 39 members 1, 5, 11, 13.
663

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; SLC, solute carrier; xfold, difference in the gene expression value according to Livak and Schmittgen [43] 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; ZIP4, 5, 7, 11, 13, SLC family 39 members 4, 5, 7, 11, 13 ZnT4, 9, solute carrier (SLC) family 30 members 4, 9.

674 Table 5. Broken-line regression analysis of relative hepatic gene expression (xfold) of ZnT4, 6 and 8
675 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	

676 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
677 estimated based on independent arithmetic group means relative to dietary zinc concentration (*n = 8*). Parameter estimates are presented as means ±
678 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
679 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; SLC, solute
680 carrier; xfold, difference in the gene expression value according to Livak and Schmittgen [43] compared to a relative mRNA abundance of 1.0 in the
681 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
682 response; ZIP1, 14, SLC family 39 members 1, 14; ZnT4, 6, 8 SLC family 30 members 4, 6, 8.

683

684

685 Table 6. Broken-line regression analysis of relative nephric gene expression (xfold) of ZnT1, 3 and 7
686 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	

687 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
688 estimated based on independent arithmetic group means relative to dietary zinc concentration (n = 8). Parameter estimates are presented as means ±
689 SEs to indicate the precision of estimation. P ≤ 0.05 was considered to be significant. *, *** indicate P ≤ 0.05, 0.0001, respectively. b1, slope of the
690 broken-line regression curves over dietary zinc doses ≤XB; b2, slope of the broken-line regression curves over dietary zinc doses >XB; SLC, solute
691 carrier; xfold, difference in the gene expression value according to Livak and Schmittgen [43] compared to a relative mRNA abundance of 1.0 in the
692 control group (88.0 mg Zn/kg diet); XB, X intercept of the breakpoint in the parameter response; YB, Y intercept of the breakpoint in the parameter
693 response; ZIP4, SLC family 39 member 4; ZnT1, 3, 7, SLC family 30 members 1, 3, 7.

694

695 Figure 1. Time-course of the study. Piglets were assigned to eight treatment groups in a complete
696 randomized block design. Blocking parameters comprised live weight, litter mates and sex (50%
697 male-castrated, 50% female). During a 14d acclimatization period, all animals were fed a diet with
698 sufficient dietary Zn supply (+60 mg Zn/kg diet resulting in 88 mg/kg diet) *ad libitum*. During a
699 subsequent experimental phase of 8d, treatment groups were fed restrictively (450 g/d) the same
700 basal diet as during the acclimatization period but with varying dietary Zn concentrations (+0, +5,
701 +10, +15, +20, +30, +40, +60 mg Zn/kg diet resulting in 28.1, 33.6, 38.8, 42.7, 47.5, 58.2, 67.8, 88.0
702 mg Zn/kg diet). Analytical grade $ZnSO_4*7H_2O$ was used for varying Zn supplementation. In the end
703 of experimental d8, all animals were killed by exsanguination under anesthesia (azaperone and
704 ketamine) without fasting to obtain tissue samples. All diets met or exceeded published
705 recommendations for the feeding of weaned piglets according to NRC [21] except for Zn. d, day(s);
706 g, gram, kg, kilogram; mg, milligram, $ZnSO_4*7H_2O$, zinc sulfate heptahydrate; Zn, zinc.
707

708 Figure 2. Response of relative jejunal gene expression of ZIP1 (A), ZIP5 (B), ZIP11 (C) and ZIP13
709 (D) in weaned piglets fed diets with different zinc concentrations for 8 d (see Table 3 for detailed
710 information on the statistical measures of the respective regression models). Values are arithmetic
711 means ± SDs, $n = 6$. d, day; diet zinc, dietary zinc; xfold, difference in the gene expression value
712 according to Livak and Schmittgen [27] compared to a relative mRNA abundance of 1.0 in the
713 control group (88.0 mg Zn/kg diet); ZIP1, 5, 11, 13, solute carrier (SLC) family 39 members 1, 5, 11,
714 13.
715

716 Figure 3. Response of relative colonic gene expression of ZnT4 (A), ZnT9 (B), ZIP4 (C), ZIP5 (D),
717 ZIP7 (E), ZIP11 (F) and ZIP13 (G) in weaned piglets fed diets with different zinc concentrations for
718 8 d (see Table 4 for detailed information on the statistical measures of the respective regression
719 models). Values are arithmetic means ± SDs, n = 6. d, day; diet zinc, dietary zinc; xfold, difference
720 in the gene expression value according to Livak and Schmittgen [27] compared to a relative mRNA

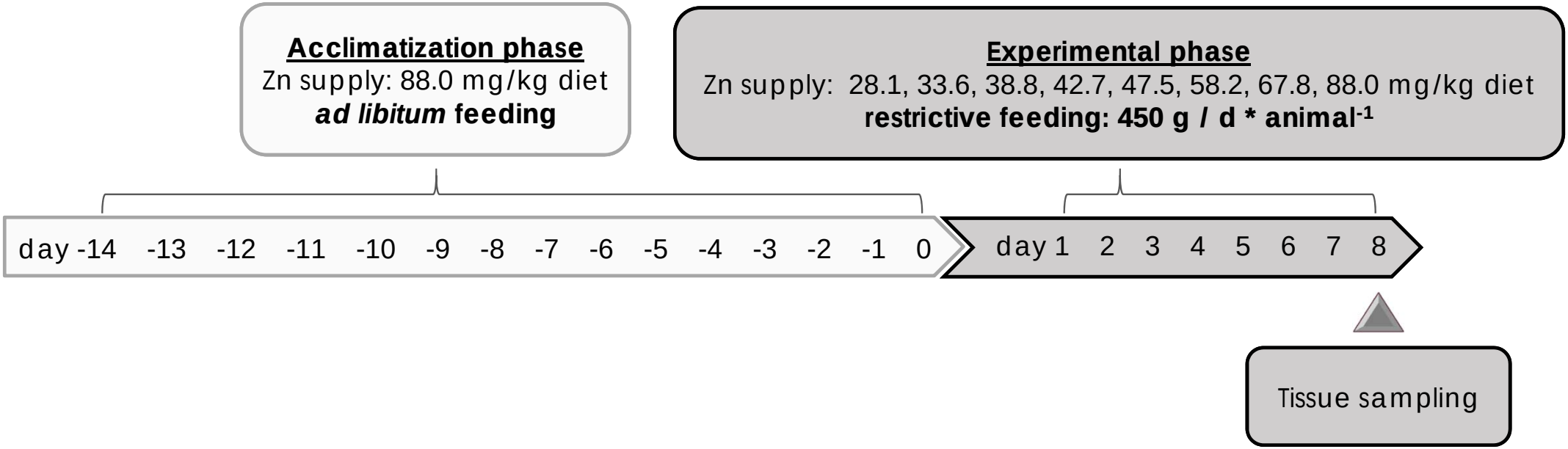
721 abundance of 1.0 in the control group (88.0 mg Zn/kg diet); ZnT4, 9, solute carrier (SLC) family 30
722 members 4, 9; ZIP4, 5, 7, 11, 13, SLC family 39 members 4, 5, 7, 11, 13.
723

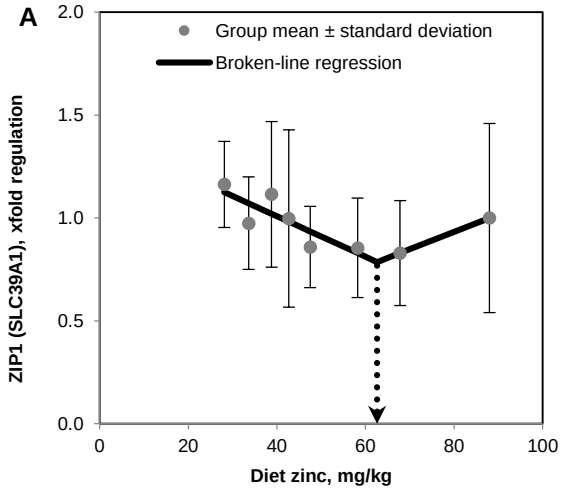
724 Figure 4. Response of relative hepatic gene expression of ZnT4 (A), ZnT6 (B), ZnT8 (C), ZIP1 (D)
725 and ZIP14 (E) in weaned piglets fed diets with different zinc concentrations for 8 d (see Table 5 for
726 detailed information on the statistical measures of the respective regression models). Values are
727 arithmetic means ± SDs, n = 6. d, day; diet zinc, dietary zinc; xfold, difference in the gene expression
728 value according to Livak and Schmittgen [27] compared to a relative mRNA abundance of 1.0 in the
729 control group (88.0 mg Zn/kg diet); ZnT4, 6, 8 solute carrier (SLC) family 30 members 4, 6, 8; ZIP1,
730 14, SLC family 39 members 1, 14.
731

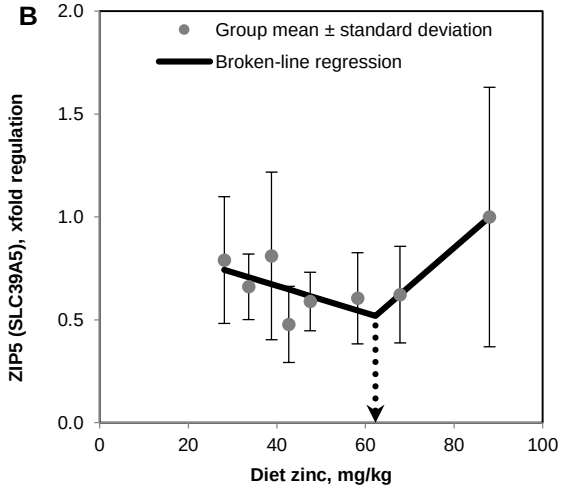
732 Figure 5. Response of relative nephric gene expression of ZnT1 (A), ZnT3 (B), ZnT7 (C) and ZIP4
733 (D) in weaned piglets fed diets with different zinc concentrations for 8 d (see Table 6 for detailed
734 information on the statistical measures of the respective regression models). Values are arithmetic
735 means ± SDs, n = 6. d, day; diet zinc, dietary zinc; xfold, difference in the gene expression value
736 according to Livak and Schmittgen [27] compared to a relative mRNA abundance of 1.0 in the control
737 group (88.0 mg Zn/kg diet); ZnT1, 3, 7, solute carrier (SLC) family 30 members 1, 3, 7, ZIP4, SLC
738 family 39 member 4.
739

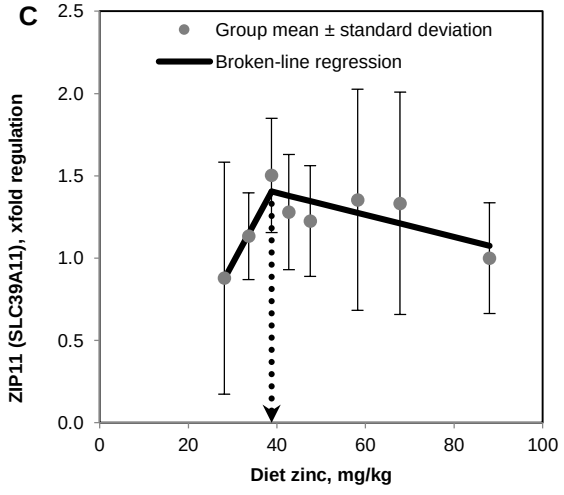
740 Figure 6. Comparative presentation of dietary breakpoint estimates for ZIP and ZnT gene expression
741 patterns in jejunum, colon, liver and kidney of weaned piglets fed diets with different zinc
742 concentrations for 8 d (see Figures 2, 3, 4 and 5 as well as Tables 3, 4, 5 and 6 for the respective
743 regression models). Across tissues, breakpoints scattered around dietary thresholds of either 42.9
744 ±2.08 or 63.1 ±6.19 mg Zn/kg diet, respectively, marked by horizontal dashed lines. Error bars
745 indicate standard errors of breakpoint estimation. Each breakpoint estimate was highly significant
746 ($P < 0.0001$ in any case). The presentation of breakpoints for each tissue was ordered in a way to

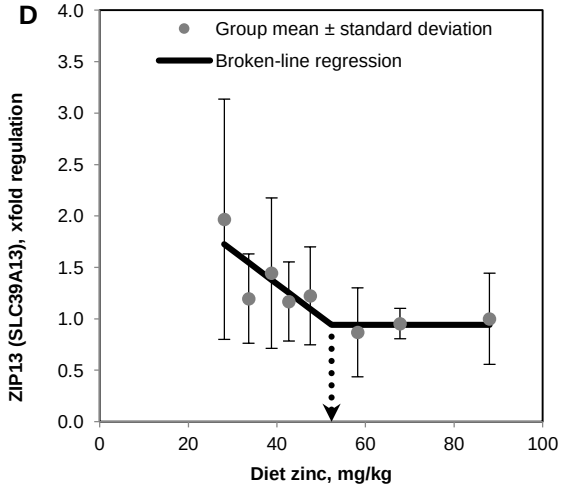
747 present the higher before the lower values. Breakpoints were obtained by broken-line regression
748 analysis over each eight treatment means, which were calculated based on single values of each
749 six animals per group fed restrictively (450 g/d) a diet with varying dietary Zn concentrations (28.1,
750 33.6, 38.8, 42.7, 47.5, 58.2, 67.8, 88.0 mg Zn/kg diet) in a complete randomized block design.
751 Analytical grade $ZnSO_4*7H_2O$ was used for varying Zn supplementation. ZnT, solute carrier (SLC)
752 family 30, ZIP, SLC family 39, Zn, zinc.

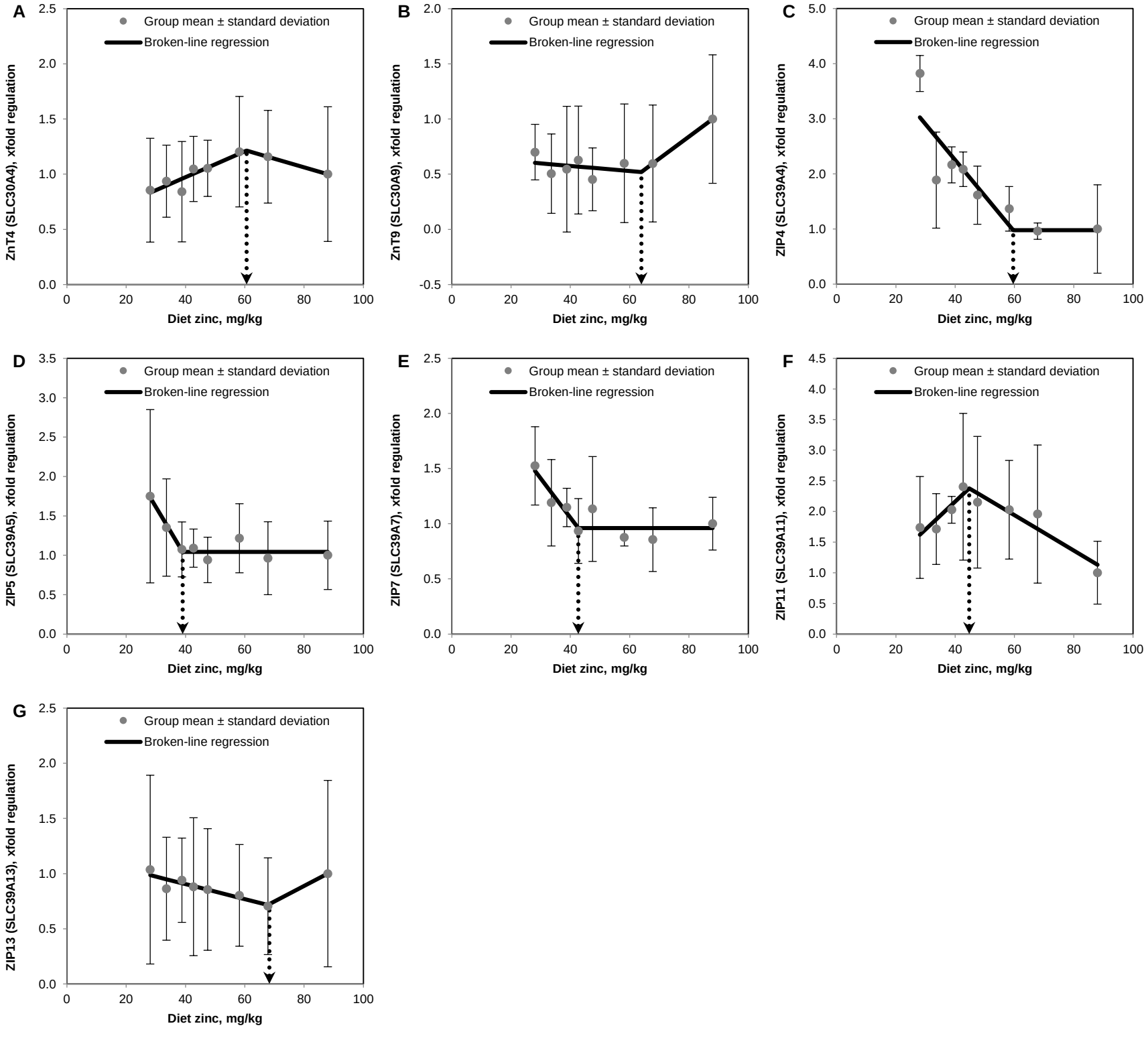

Acclimatization phase
Zn supply: 88.0 mg/kg diet
ad libitum feeding
Experimental phase
Zn supply: 28.1, 33.6, 38.8, 42.7, 47.5, 58.2, 67.8, 88.0 mg/kg diet
restrictive feeding: 450 g / d * animal-1
day -14 -13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0
day 1 2 3 4 5 6 7 8
Tissue sampling

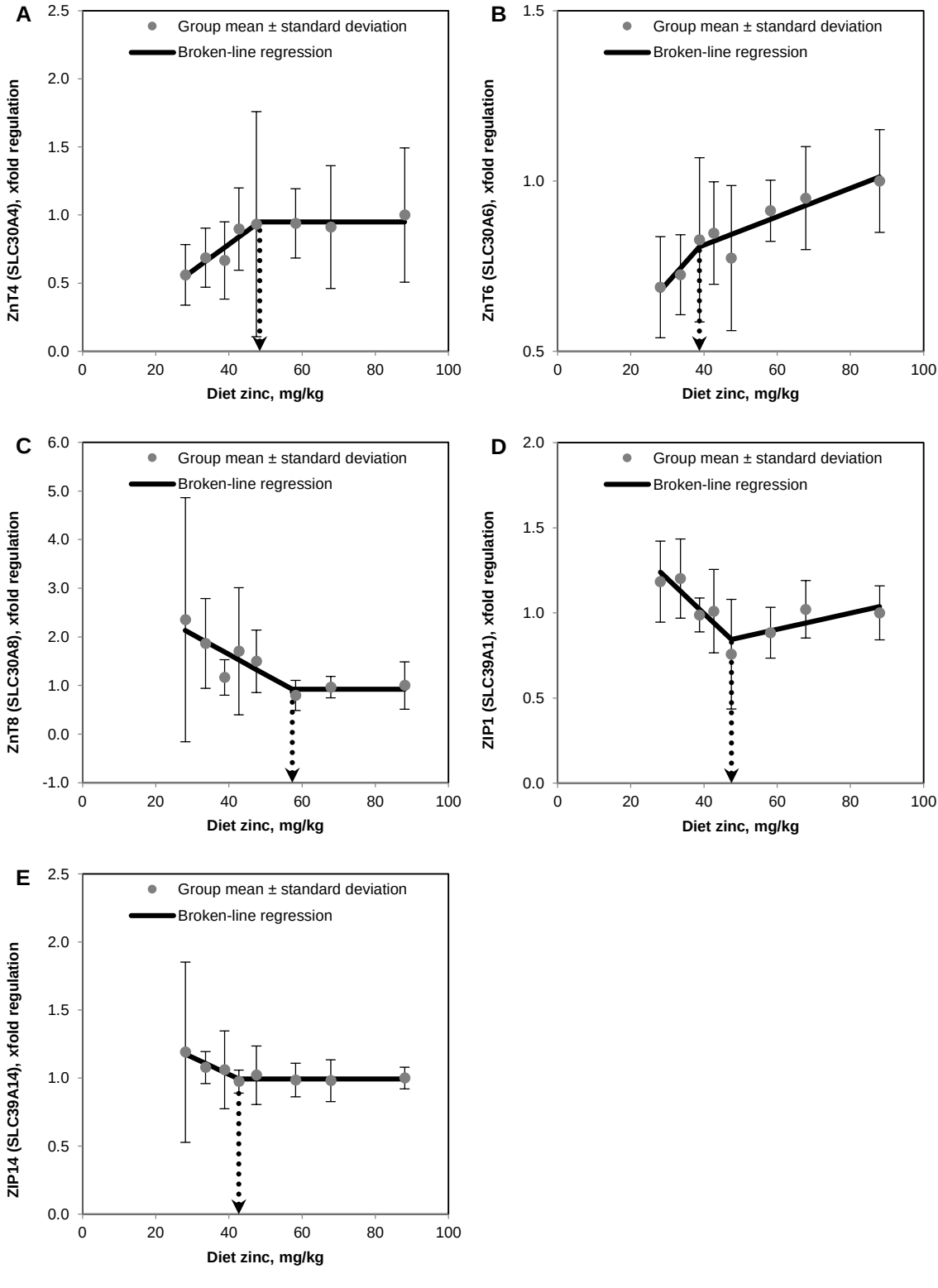

A
Group mean ± standard deviation
Broken-line regression
ZIP1 (SLC39A1), xfold regulation
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
Diet zinc, mg/kg

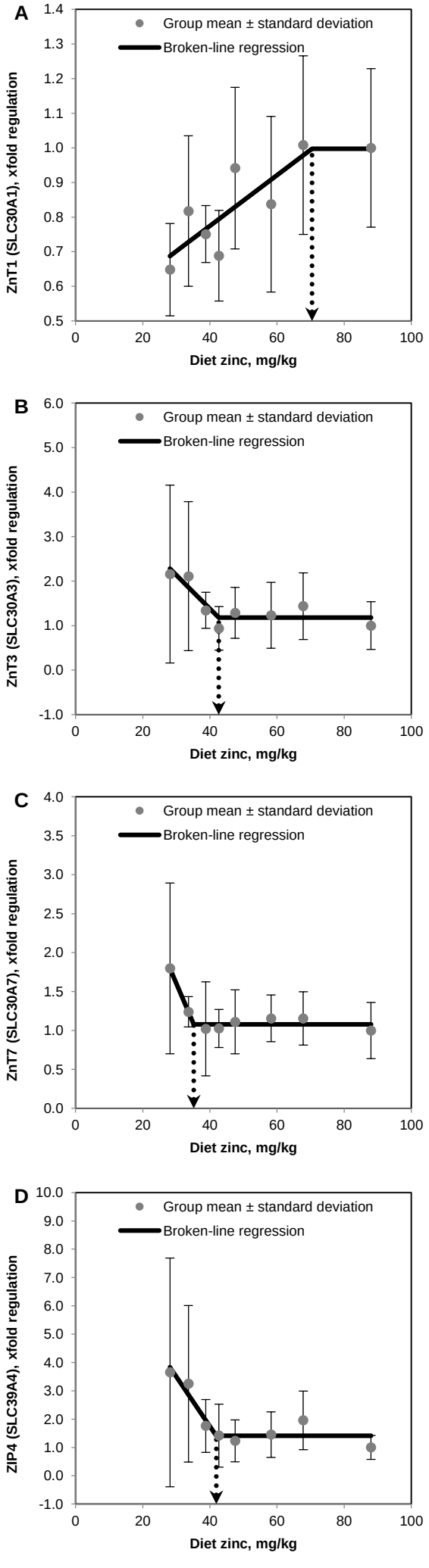

B
Group mean ± standard deviation
Broken-line regression
ZIP5 (SLC39A5), xfold regulation
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
Diet zinc, mg/kg

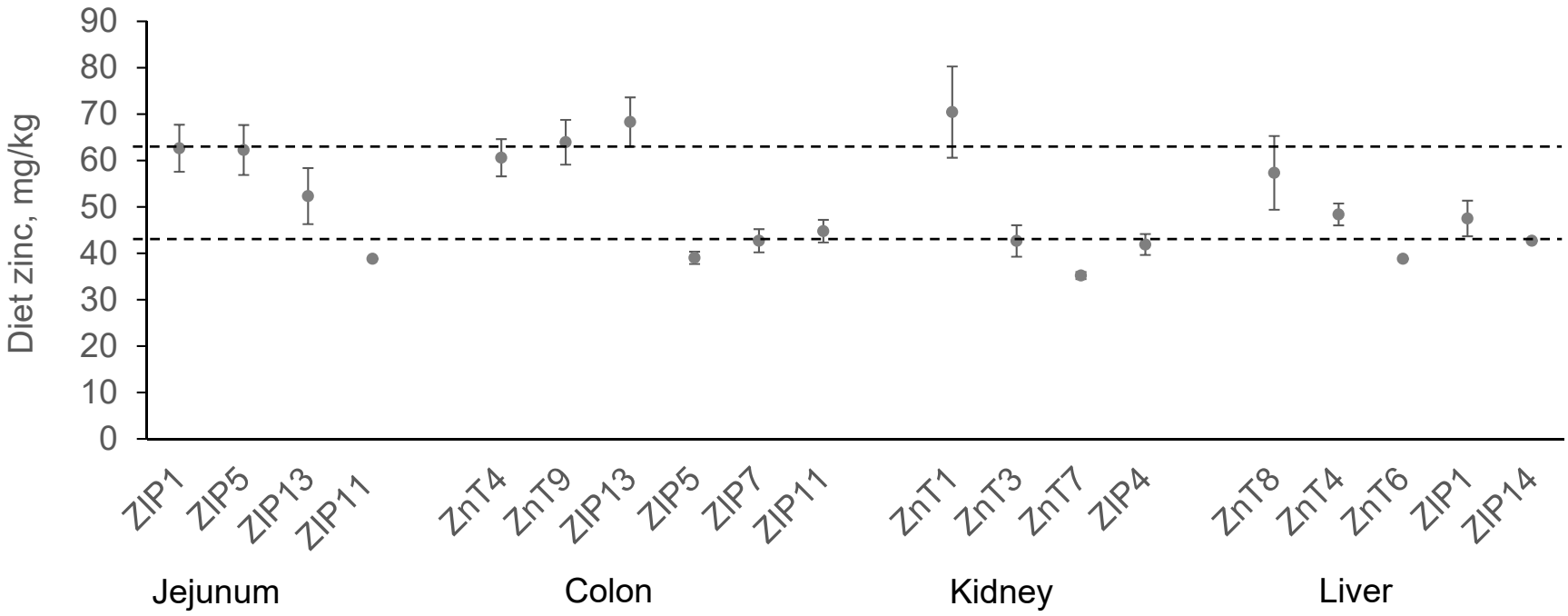

C
Group mean ± standard deviation
Broken-line regression
ZIP11 (SLC39A11), xfold regulation
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
Diet zinc, mg/kg


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


Diet zinc, mg/kg
90
80
70
60
50
40
30
20
10
0
ZIP1
ZIP5
ZIP13
ZIP11
ZnT4
ZnT9
ZIP13
ZIP5
ZIP7
ZIP11
ZnT1
ZnT3
ZnT7
ZIP4
ZnT8
ZnT4
ZnT6
ZIP1
ZIP14
Jejunum
Colon
Kidney
Liver

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

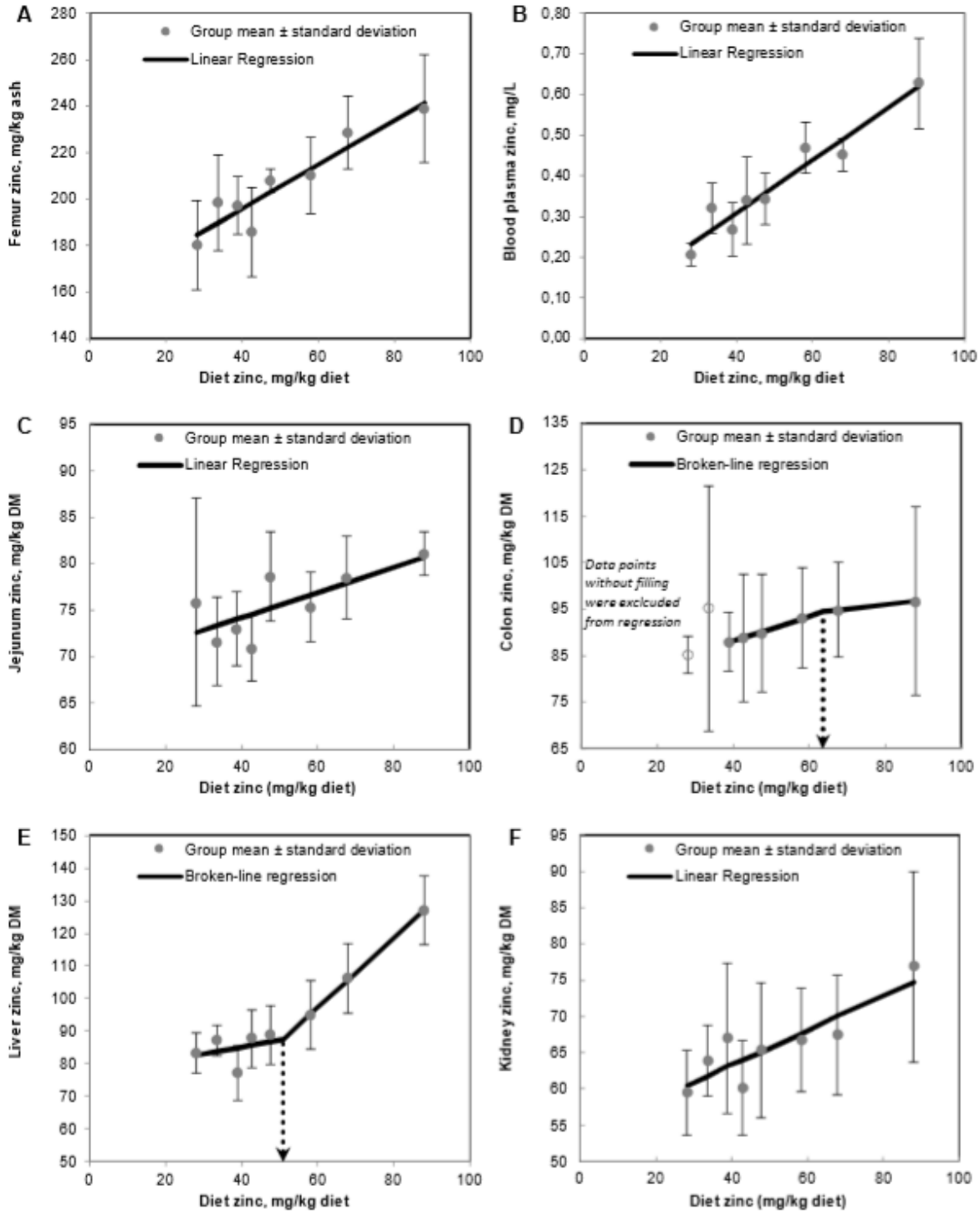
Gene	Accession number[1]	Forward sequence	Reverse sequence	Position on template[2] 5'→3'	Product Length[3] bp	Annealing temperature Jejunum/Colon/Liver/Kidney °C
GAPDH	NM_001206359.1	CACATGGCCTCCAAGGAGTAA	GGAGATGCTCGGTGTGTTGG	1082 → 1210	129	58.6 / 58.6 / 58.6 / 58.6
GUSB	NM_001123121.1	TCACGAGGATCCACCTCTCAT	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.



Supplementary Figure 1. Changes in total zinc pools of femur (A), blood plasma (B), jejunum (C), colon (D), liver (E) and kidney (F) of weaned piglets fed varying dietary Zn for 8 d. Data was originally presented by *Brugger, D., and W. M. Windisch. 2019. Brit. J. Nutr. 121: 849-858*. d, days; DM, dry matter; Values are arithmetic means ± SDs, n = 6 single values per mean.