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Experimental modelling of subclinical zinc deficiency in weaned piglets

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To my family,

and in loving memory of my private life. . . . . .

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List of Abbreviations

AAS	atomic absorption spectroscopy
ACTB	β-actin
ANOVA	analysis of variance
AP	plasma alkaline phosphatase
app.	apparent(ly)
BAX	B-cell lymphoma 2–associated X protein
bp	base pair(s)
BLAST	basic local alignment search tool
C	carbon
°C	degree(s) Celsius
CA	crude ash
CASP3	caspase 3
CASP8	caspase 8
CASP9	caspase 9
CAT	catalase
CDKN1A	cyclin-dependent kinase inhibitor 1A
cDNA	copy DNA
CF	crude fibre
Cl	chloride
CLUSTER	cluster analysis procedure in SAS
CP	crude protein
Cq	quantificatioin cycle (syn. Ct)
Ct	cycle threshold (syn. Cq)
Cu	copper
d	day(s)
DLG	Deutsche Landwirtschafts-Gesellschaft e.V.
DM	dry matter
DMT1	divalent metal transporter 1
DNA	deoxyribonucleic acid
oligo-dT	oligonucleotide consisting only of thymine
E	amplification efficiency
EC	efficiency-corrected
EDTA	Ethylenediaminetetraacetic acid

EFSA	European Food Safety Authority
e.g.	*exempli gratia* (engl.: for example)
EI24	etoposide-induced 2.4
engl.	zeigt die englisch Übersetzung an
etc.	et cetera
FAS	Fas cell-surface death receptor
g	gram or measure of centrifuge acceleration, respectively
GADD45A	growth arrest and DNA damage–inducible α
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GfE	Gesellschaft für Ernährungsphysiologie (engl.: Society of Nutrition Physiology)
GIT	gastrointestinal tract
GLM	general linear model procedure in SAS
GPX1	glutathione peroxidase 1
GSH	reduced glutathione
GSR	glutathione reductase
GSSG	oxidised glutathione
GUSB	β-glucuronidase
h	hour(s)
H	hydrogen
H3	histone 3
HDA	H_2O_2-detoxification activity
HPLC	high-performance liquid chromatography
ICP-MS	inductively coupled plasma mass spectrometry
IGFBP3	insulin-like growth factor-binding protein 3
IPC	interplate calibrator
K	potassium
kD	kilodalton
kg	kilogram
KLF4	krueppel-like factor 4
L	litre
LC	liquid chromatography
Log_{10}	decadic logarithm
M	mol

ME	metabolizable energy
mg	milligram
min	minute(s)
miRNA	micro RNA
MJ	megajoule
mL	millilitre
mM	millimole
Mn	manganese
mRNA	messenger RNA
MT	metallothionein
MT1A	metallothionein 1A
MT2B	metallothionein 2B
MTF1	metal regulatory transcription factor 1
µg	microgram
µL	microlitre
µM	micromole
n	number of individuals per treatment group
N	nitrogen or total sample size, respectively
N/A	not assessed, not measured, not available, not detectable
Na	sodium
ng	nanogram
nL	nanolitre
NLIN	nonlinear procedure in SAS
NLMIXED	nonlinear mixed model procedure in SAS
nm	nanometre
nM	nanomole
NRC	National Research Council of the United States of America
O	oxygen
P	probability of a statistical type-I error
PA	phytic acid
pcd	precaecal (syn. ~ileal) digestibility
PCR	polymerase chain reaction
pH	negative decadic logarithm of hydron (H^+) concentration
PRDX1	peroxiredoxin 1

PRDX3	peroxiredoxin 3
PRDX4	peroxiredoxin 4
qPCR	quantitative PCR
r	Pearson correlation coefficient
R^2	coefficient of determination
redox	reduction-oxidation
Refseq	reference sequence
REG	linear regression procedure in SAS
RNA	ribonucleic acid
RNAlater	total RNA stabilising reagent
ROS	reactive oxygen species
RPS18	ribosomal protein subunit 18
RQI	RNA quality indicator
RT-qPCR	reverse transcriptase qPCR
s	second(s)
SAS	Statistical Analysis System
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	standard error
SEM	standard error of means
SFN	stratifin
SHISA5	shisa family member 5
SLC	solute carrier family
SOD1	Cu/Zn-superoxide dismutase
SOD2	Mn-superoxide dismutase
syn.	synonymous
SZD	subclinical zinc deficiency
SZM	subklinischer Zinkmangel
Ti	titanium
TL	total lipids (ether extract)
TP53	tumour protein p53
U	Unit(s) of enzyme activity
UBC	polyubiquitin-C
V	volt(s)

vgl.	vergleiche
z.B.	zum Beispiel
ZBC	zinc-binding capacity
ZIP1-14	solute carrier family 39 members 1 to 14
Zn	zinc
ZnT1-10	solute carrier family 30 members 1 to 10
-/-	indicates homozygous transgenic knockout of a gene

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1st Chapter

General introduction

The first report upon the importance of zinc (Zn) for the pig organism was published in 1955 by Tucker and Salmon (1). The authors recognised severe dermatitis, reduced growth development, anorexia, diarrhoea, vomiting and, in extreme cases, weight loss and death of growing pigs, which were fed diets containing total (native) Zn contents between 34-44 mg/kg. A supplementation of up to 2% of $ZnCO_3$ on top of these diets was effective to cure this condition. During the following decades, scientific investigations on the Zn metabolism of monogastric livestock revealed a clear connection between the dietary composition and the necessity for Zn supplementation.

Most sources of animal protein are banned from livestock diets due to European Law (2). This forces producers to maintain sufficient amino acid supply to their herds through plant biomass. In case of pigs and poultry, it means that oilseeds and legume meals are used to achieve sufficient protein supply, together with cereal protein and crystalline amino acids. Therefore, diets for monogastric livestock are mainly represented by milled or crushed plant seeds as well as associated by-products. However, such raw materials contain significant amounts of phytic acid (PA), of which the oilseed and legume meals can account for up to ~50% of the total PA content in the diets of pigs and poultry. Phytic acid represents the major storage form of phosphorus within the seed (3). Furthermore, it can bind divalent cations at its phosphorus residues, thereby forming insoluble chelates (so-called phytates) (4, 5). In the course of its transfer through the gastrointestinal tract (GIT), PA dissociates under the acidic conditions within the stomach (6). Within the small intestinal lumen, however, the neutral conditions (pH 7) render PA again capable of binding divalent cations, leading to *de novo* complexation of luminal Zn^{2+} (7-9). This is exacerbated by the fact that the native phytase[1] activity within the digestive systems of pigs and poultry is negligible

[1] Phytases (or myo-inositol hexakisphosphate phosphohydrolases) are a group of enzymes which catalyse the hydrolysis (cleavage) of phytic acid.

under the terms of high dietary supplementation with available phosphorus (10, 11). Hence, most phytate-associated minerals within the monogastric intestine are subject of faecal excretion under practical feeding conditions. Phytate levels in complete feed for monogastric animals are currently rather high and can be >8 g/kg (3, 12). Dose-response studies with ^{65}Zn-labelled[2] rats suggest a drop in true Zn absorption to literally 0% in the presence of such phytate contents at given dietary Zn concentration (14). Therefore, it is inevitable to compensate such antagonistic effects by supplementation of Zn (and phytase) . According to studies which aimed to estimate the gross Zn requirements[3] of growing piglets, the necessary minimum amount of Zn in practical complete feed lies at ~50 mg/kg (15-23). On the contrary, feeding piglets with diets that are free of antagonistic substances drastically reduces the necessary dietary Zn concentration to ~15 mg/kg (21, 23). There is still great uncertainty regarding the necessary amounts of dietary Zn supplementation to high yielding livestock, which is highlighted by the current use of quite generous safety margins (24-27). This is due to a lack of data regarding changes of gross Zn requirements, as affected by varying dietary composition and potentially higher metabolic demands during stressful situations like infections.

The current recommendations for the feeding of high Zn safety margins to pigs and poultry should maintain sufficient Zn supply during all phases of the production cycle. For example, the German Society of Nutrition Physiology (GfE) as well as the National Research Council of the United States of America (NRC) recommend a minimum concentration of Zn in complete feed for growing piglets (7-11 kg body weight) of 90 and 100 mg/kg (under the assumption of 90% dietary dry matter), respectively (24, 27). This corresponds to the ~2fold gross Zn requirement (~50 mg/kg) based on earlier

[2] The ^{65}Zn is a radioisotope, which decay can be detected using a gamma counter. It has been previously used as a tracer substance to assess the net Zn fluxes through animal organisms. See Kirchgessner et al. (13) for further details on the methodology.

[3] The gross requirement represents the minimum dietary amount of a nutrient that is necessary to meet current metabolic requirements, considering incomplete absorption and metabolic utilisation.

published data (15-22)). High dietary amounts of surplus Zn, however, are associated with a linear increase of faecal Zn (28, 29). This represents a potential threat for the environment, as Zn has also a considerable toxic potential if its concentration within a biological system exceeds basal levels (30, 31). This also accounts to soils of agricultural land, which Zn loads have been directly associated with the Zn loads in manure. Furthermore, Zn may be transferred to ground water when the sorption capacity of the soil aggregates is exceeded. (32). As an attempt to reduce Zn emissions from livestock production systems, the European Commission (33) most recently reduced the allowed upper limits of Zn in complete feed (150 mg/kg for piglets and sows, 120 mg/kg for fattening pigs, boars, poultry and ruminants). This decision was based on an opinion of the European Food Safety Authority (EFSA) (34), in which even stricter upper limits were recommended in the presence of phytase supplements. Therefore, the allowed upper limits for Zn in complete feed might be further reduced on a mid-term scale. As a result, the permissible range for Zn safety margins in animal feeding is shrinking, which will force livestock producers to feed much closer to the actual gross Zn requirements. This is currently complicated by the aforementioned lack of reliable data on the change of the gross Zn requirement under varying rearing conditions. Without this information, it is critical to postulate a further decline of the current total Zn loads in livestock diets. Temporal phases of marginal supply can impair the biological performance (for example growth, milk yield, egg yield etc.) of animals quite quickly (35). This must not necessarily be associated with pathologies but might reduce the economic success of livestock production.

Another problem relates to the diagnosis of Zn deficiency under practical conditions in the field. This accounts especially to subclinical events, which are supposed to be the predominant phenotype of Zn malnutrition in livestock herds. Although there have been

intense research activities to identify suitable biomarkers, the results obtained so far are unsatisfactory (36). Indeed, under experimental conditions that involve a standardisation of sex, genetic background as well as environmental and dietary conditions, it is possible to correlate certain parameters to the alimentary Zn supply and even estimate requirement thresholds using factorial approaches[4]. Although, even under such conditions the control levels of status parameters can differ significantly between experiments, not only when comparing different laboratories but also within the same institute (for example weaned piglets, same breed, 50% female, 50% male-castrated, ~8.5 kg average body weight, ~13.5 mg Zn intake/day: average plasma Zn concentration 184 and 206 µg/L, average bone Zn concentration 151 and 180 mg/kg ash, according to own unpublished data and *Chapter 2, page 65*, respectively). Such pronounced interindividual differences in the expression of "classical" Zn status parameters make it difficult to establish reliable reference thresholds that indicate the Zn supply status in the field. In anticipation of a further tightening of the legal framework for the feeding of Zn, it will become more urgent to intensify the search for biomarker patterns that reflect an individuals' Zn status under practical feeding conditions. Therefore, we must deepen our understanding of regulative mechanisms of Zn homeostasis[5] within single body compartments as well as the whole organism.

Whether it is the derivation of gross Zn requirements or the study of Zn metabolism, both presuppose an appropriate experimental approach which reflects the metabolic situation under practical feeding conditions in respective animal models.

[4] Factorial approaches are characterised by regression analysis of the response of performance and/or status parameters to finely-graded changes in dietary nutrient supply levels, in order to highlight the transition from deficient to sufficient supply.

[5] Homeostasis describes the sum of mechanisms through which a dynamic system maintains its inner environment against changing external conditions.

Preliminary considerations on the experimental derivation of gross zinc requirements of monogastric livestock

The collection of data on feed Zn bioavailability or Zn requirements, respectively, must occur by following the response of suitable status parameters within precise dose-response studies. Appropriate experimental setups should span the range between deficient and potentially sufficient supply levels, to gain information on the Zn supply status of treatment groups. The overall goal is to estimate the point of sufficient dietary Zn supply as well as the intensity of the slope in response of status parameters to changes in the alimentary supply level. This can be achieved, for example, by using broken-line regression models (37). In this context, a suitable status parameter must significantly change its dose-response behaviour when the Zn requirements are satisfied, compared to its response over deficiently supplied treatment groups. The "ideal" response pattern includes a linear increase or decrease, respectively, over deficient alimentary Zn doses and a plateau in response over sufficient dose-levels (Figure 1). Thereby it is possible to differ between deficiently and sufficiently supplied groups, for example by estimating the statistical breakpoint in response (37). This threshold may represent the gross Zn requirement at given experimental conditions.

The percentage amount of dietary Zn that can be absorbed from the GIT, and which is therefore available for metabolic processes, results from its chemical availability within the gut lumen and the physiological status of the animal. The first is affected by the chemical form(s) (chemical species) in which Zn is present within the diet as well as the interaction with other dietary ingredients as well as endogenous secretions (38). Actively regulated Zn-transport pathways can only transfer divalent Zn ions through biological membranes (39, 40). Therefore, luminal Zn is absorbable if it is either present as free Zn^{2+} or loosely bound to water-soluble molecules (38). During the transfer of diets based on cereal and oilseed meals through the GIT, a significant amount of

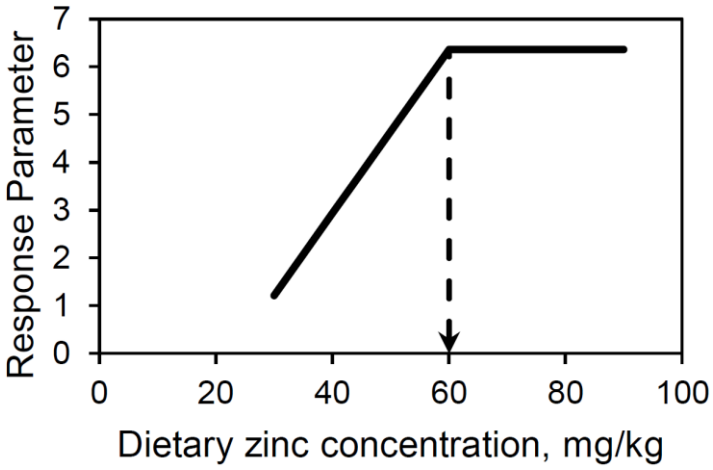

Figure 1. Theoretical broken-line response of a random zinc status parameter to changes in dietary zinc concentration. Notes: In this example, the parameter (for example apparently-digested feed zinc) exhibits a plateau in response above a dietary threshold of 60 mg zinc/kg diet, below which it decreases by a slope of 0.17/mg reduction in dietary zinc. The dietary threshold (breakpoint) represents the gross zinc requirement at given experimental conditions.

luminal Zn seems to be immobilised by its association with PA (4). Therefore, growing piglets fed practical diets without Zn supplementation develop first signs of clinical Zn deficiency within 2 weeks (35). It has been already pointed out that the gross Zn requirement of growing piglets was earlier estimated to lie at ~50 mg/kg diet under practical feeding conditions (15-23). Such diets have native Zn contents of 30-40 mg/kg (for example *Chapter 2, page 52*). Given a gross Zn requirement of ~15 mg/kg when feeding diets excluding antagonistic substances (21, 23), this implies that under practical conditions a significant amount of the supplemented Zn is required to satiate the Zn binding sites of PA rather than metabolic requirements. Hence, Zn supplementation strategies aim at increasing the total Zn load within the GIT until enough mobile luminal Zn is present to meet the net Zn requirement[6] of an animal.

In summary, this illustrates how high dietary phytate contents increase the necessary total Zn amount in modern diets for monogastric livestock. Furthermore, it indicates the

[6] The net requirement represents the necessary nutrient amount that must be present behind the gut barrier at a given timepoint to supply all metabolic processes.

optimal range of dietary Zn concentrations that must be applied in a dose-response study aiming at the Zn metabolism (spanning between deficient dose-levels and potentially sufficient supply at ~≥50 mg Zn/kg diet under practical feeding conditions).

Earlier studies on ^{65}Zn-labelled rats demonstrated the importance of the animals' physiological status for the Zn absorption from the GIT. Under the conditions of a systemic Zn deficiency (depletion of body Zn stores), the organism seems to drastically increase the efficiency of Zn absorption from the GIT lumen. This is necessary, because a replenishment of bone Zn stores entails a rise in metabolic Zn requirements. Simultaneously, endogenous Zn losses into the GIT are reduced to an inevitable amount (41, 42). During the last two decades, it has become clear that these effects are based on a complex regulative network, at the end of which the expression and presentation of certain Zn transport proteins at biological membranes is modulated (namely members of the solute carrier (SLC) families 30 (ZnT) and 39 (ZIP)). Today, 10 members of the ZnT and 14 members of the ZIP family have been described in mammal models. Especially the presence and activity of specific transporters at the plasma membranes[7] of gut mucosal cells (ZnT1, ZIP4) as well as at the plasma and vesicular[8] membranes of pancreatic acinar cells[9] (ZnT1, ZnT2, ZIP5), have been discussed to be essential for the adaption of the organism's Zn homeostasis to changing alimentary supply levels (40, 43-45).

It is yet unclear, how members of the ZIP family are transporting Zn^{2+} through biological membranes (44). This is due to methodological boundaries regarding their *in vitro* synthesis. Two possible modes-of-action are currently discussed to represent the

[7] The plasma membrane (or cell membrane) is the biochemical barrier, which shields the cellular content against the surrounding environment.
[8] Cellular vesicles are small membrane-enclosed structures within cells and extracellular space, respectively, that form during the transport of materials into or from the cytosol.
[9] Acinar cells (or centroacinar cells) are secretory cells of the exocrine pancreas.

most likely scenarios, with ZIP transporters acting either as electrodiffusion[10]-channels (46) or as HCO_3^- symporters[11] (47). However, it is well accepted that the ZIP family members are transporting Zn^{2+} towards the cytosol[12], either from the extracellular space or from separated cellular compartments (40, 43, 44). In contrast, there seems to be consensus that members of the ZnT family function as Zn^{2+}/H^+ exchangers[13], thereby transporting Zn^{2+} away from the cytosol into the extracellular space or into separated cellular compartments, respectively (48, 49).

The ZIP4 has been identified in various cell types including gut mucosal cells, where it seems to transport Zn from the gut lumen through the apical plasma membrane into the cytosol (50-53). It appears to be regulated by transcriptional[14] as well as post-transcriptional mechanisms. These involve its translocation to the plasma membrane during Zn deficiency and, on the contrary, its rapid endocytosis[15] combined with ubiquitination[16] and increased messenger ribonucleic acid (mRNA)[17] degradation during Zn repletion (54-56). Increased or decreased gene expression of ZIP4 seems to be a result of a variation in mRNA stability as well as modulation of the transcription factor[18] krueppel-like-factor 4 (KLF4) (57, 58). It appears that KLF4 is activated during

[10] Electrodiffusion describes the motion of ions alongside a gradient of opposite electrical charges.
[11] Symporters are plasma membrane-associated transport proteins, which move different kinds of chemicals from one side to the other side of the membrane at the same time (into or out of the cell, respectively).
[12] The cytosol represents the liquid cellular content of all biological cells, which can be further separated into compartments by individual membranes.
[13] Exchangers are plasma membrane-associated transport proteins, which move a certain chemical from one side to the other side of the membrane in exchange for a different chemical (into or out of the cell, respectively).
[14] Transcription (or gene expression) is the process by which mRNA molecules are synthesized complementary to respective DNA sequences.
[15] Endocytosis describes the process of internalisation of molecules from the plasma membrane or extracellular space, respectively, into the cytosol. The opposite process is called exocytosis.
[16] Ubiquitination (or ubiquitylation) is a process during which a substrate protein is marked with ubiquitin to determine its fate within a cell. Thereby it can subsequently be subject to degradation, translocation as well as changes in its activity or affinity to interact with other molecules.
[17] Messenger ribonucleic acid is a diverse group of RNA molecules that convey genetic information from the DNA to ribosomes during the process of protein biosynthesis.
[18] Transcription factors are proteins which affect the transcription of certain genes, which either promotes or inhibits mRNA biosynthesis.

Zn restriction. However, the precise mode of action has yet to be evaluated.

The ZnT1 has been described at the basolateral membrane of various cell types including gut mucosal cells, where it transfers cytosolic Zn^{2+} through the basolateral plasma membrane into the circulation (59-62). Its regulation seems to occur predominantly by the amount of free cytosolic Zn^{2+}, which activates the metal responsive transcription factor 1 (MTF1) (61, 63, 64). Hence, ZnT1 does not directly refer to the supply status of the organism but the activity of other Zn transporters that affect the levels of free cytosolic Zn^{2+}.

The pancreas is supposed to play a major role in Zn homeostatic regulation because it contributes significantly to the amount of Zn excreted into the GIT (39, 65). This excretion seems to occur by exocytosis of Zn peptides into the pancreatic duct[19] (for example Zn-enzymes like carboxypeptidases A and B) (66). The transporter responsible for the transfer of cytosolic Zn into respective vesicles of pancreatic acinar cells seems to be ZnT2. Its expression appears to be reduced during Zn deficiency, leading to a significant drop in the Zn loads of pancreatic zymogen[20] granules. Reversely, this response turns into the opposite under the terms of Zn overload (67). Furthermore, during Zn deficiency the pancreas upregulates basolateral ZnT1, obviously for the purpose to recycle pancreatic Zn in favour of other tissues (53). The uptake of Zn from the circulation into the pancreas seems to be facilitated by ZIP5, which localises to the basolateral membrane of a variety of cell types, including the pancreatic acinar cells during times of Zn repletion (68). In contrast, under the terms of Zn deficiency, pancreatic ZIP5 is removed from the basolateral membrane and gets degraded as an attempt to reduce pancreatic Zn uptake from the circulation. Interestingly, the current

[19] The pancreatic duct (or "Duct of Wirsung") joins the exocrine pancreas to the common bile duct.
[20] Zymogens (or proenzymes) represent inactive precursors of enzymes. For example, the spectrum of exogenous proteases within pancreatic tissue is inactive to prevent self-digestion. After their secretion into the GIT via the pancreatic duct, these zymogens undergo hydrolysis which renders them active.

state of knowledge dictates that ZIP5 seems to be predominantly regulated on level of its peptide pool rather than transcription. In fact, ZIP5 mRNA has been reported to be constantly associated to polysomes[21], thereby remaining ready to be quickly translated if ZIP5 activity is again needed at the basolateral plasma membrane (56).

Taken together, under the terms of sufficient and excess alimentary Zn supply, the organism regulates the activity of specific Zn transporters at the gut mucosa as well as within pancreatic acinar cells. This is supposed to represent a highly conserved protective mechanism to dampen the effects of systemic Zn deficiency and overload during fluctuations of the dietary Zn supply and/or daily Zn demands. Hence, the overall goal of Zn homeostasis seems to be the maintenance of body Zn levels in a range that benefits undisturbed metabolic processes and prevents cellular stress and associated damage to the organism.

The above described homeostatic processes must be considered when experiments are conducted that aim in the derivation of gross Zn requirements. The magnitude of the response to the dietary treatment is obviously affected by the severity of systemic Zn depletion or overload, respectively. In fact, under the terms of Zn deficiency a significantly higher ZIP4 activity has been recognised at the gut mucosa compared to basal physiological conditions (51, 68). This appears plausible because clinically deficient animals are not only in need of Zn to satisfy their basal metabolic requirements but also to replete stressed body Zn stores and maintain regenerative processes (69). However, in nature and especially in practical livestock feeding clinical events of Zn deficiency are quite rare (70). Therefore, a gross Zn requirement threshold that has been assessed by stepwise increase in Zn supply to clinically deficient individuals most likely

[21] A polysome (or polyribosome) represents a complex of an mRNA molecule and two or more ribosomes, thereby representing the molecular machinery responsible for the translation of nucleic acid sequences into amino acid sequences (70).

does not reflect practical conditions. Furthermore, such studies draw comparisons between sick and healthy individuals, which is obviously limited in its informative value. Otherwise, subclinical events of Zn deficiency are supposed to occur regularly, for example during phases of temporally reduced feed intake post weaning (71). Therefore, it appears plausible to mimic these physiological conditions in experiments that aim to estimate Zn requirements of farm animals.

Preliminary considerations on the experimental investigation of zinc homeostasis and associated metabolic processes under the terms of subclinical zinc deficiency

At least 10% of the human proteome[22] needs Zn as a structural or catalytic cofactor to maintain many biofunctions, including basic cellular processes like deoxyribonucleic acid (DNA) replication and transcription (72). This may be extrapolated on most eukaryotic[23] species. It is therefore not surprising that a clinical Zn deficiency is characterised by a complex of serious but unspecific symptoms, including growth retardation, anorexia, reduced immune function, impairment of fertility, tissue necrosis etc. (69, 73). As a consequence, the animal organism aims in a stabilisation of Zn load behind the gut barrier by a restriction of endogenous losses as well as a fine-tuned exchange of Zn between biomolecules (39).

The complex of unspecific symptoms highlighted above implies a multitude of secondary metabolic events during clinical Zn deficiency. It can be assumed that the quality of measurements obtained under such conditions is limited due to significant „background noise“. This may complicate a differentiation between effects that are directly regulated by changes in the Zn availability behind the gut barrier and secondary „artefacts“. Such „artefacts“ result from secondary physiological events, which are indirectly linked to Zn homeostatic regulation patterns. A prominent example is the interaction between an individuals' Zn status and redox (reduction-oxidation) metabolism[24]. Although Zn deficiency promotes oxidative stress, this is not directly associated with the adaption of Zn buffering and muffling within the organism. Zinc

[22] The proteome represents the qualitative and quantitative spectrum of proteins, which are present in a cell or multicellular system, respectively, at a given timepoint.
[23] Eukaryotic species consist of cells that have a nucleus and various other functional compartments (organelles) that are separated from the remaining cytosol (liquid cellular content) by individual membranes.
[24] Redox metabolism is the sum of metabolic processes that enable reduction-oxidation reactions within biological cells under tightly controlled conditions.

ions are not redox-active like, for example, iron or copper that are educts of Fenton[25] (-like) reactions (74). Hence, the effects of Zn homeostasis on redox metabolism are of secondary nature (for example by changes in the abundance and/or activity of Zn-dependent antioxidative molecules) (75). As the majority of Zn peptides are involved in the regulation of basic cellular processes like transcription and replication of DNA (72), it appears that all cellular physiological processes are directly or indirectly affected by a Zn-peptide.

In summary, to deepen our understanding of Zn homeostatic regulation and associated body functions, an experimental modelling of subclinical rather than clinical Zn deficiency may be beneficial. This phenotype is characterised by a dose-dependent decline in the organisms Zn status but, at the same time, no visible symptoms of Zn deficiency (76). It represents a situation of basal physiological regulation, during which the Zn homeostasis as well as associated metabolic pathways are still able to compensate alimentary insufficiencies and avoid serious degenerative processes. Hence, the amplitudes in biological variation of measurements may be expected to be comparably lower than in clinically Zn deficient animals, yielding an overall reduced „background noise“. Furthermore, during this early stage of Zn deficiency mainly the direct Zn-dependent parameters are affected, which allows a discrimination of true effects of Zn-homeostatic adaption from secondary events.

To modulate a short-term subclinical Zn deficiency (SZD) under standardised experimental conditions, it is crucial to recall the basic kinetics of Zn metabolism and the timeframe in the development of clinical Zn deficiency. On the one hand, the dietary treatment must be long and intense enough to promote a reaction of the Zn homeostasis. On the other hand, the development of clinical symptoms must be avoided, which limits

[25] Fenton and Fenton-like reactions, respectively, represent the oxidation of organic substrates by H_2O_2. Among other elements, these reactions are catalysed by iron (classical Fenton reaction) and copper.

the maximum duration of the experiment. Earlier basic research on Zn metabolism of monogastric mammals indicate that the Zn homeostasis fully reacts within a timeframe of ~3-5 d (42, 77). It is noteworthy that this timeframe equals the half-life of gut mucosal cells (78). This represents the minimum duration of experiments to monitor the adaption of Zn homeostasis. However, the capacities of the organism to maintain normal metabolic function through a mobilisation of body Zn stores should not be exceeded. Based on earlier studies, the mobilizable body Zn stores account for ~15% of whole body Zn contents (79-82). Under the assumption of a daily growth rate of piglets at ~3% of body weight and a deficient Zn supply at ~50% of the daily metabolic requirement, this would account for a daily loss in whole body Zn of ~1.5%. Therefore, the critical value of ~15% loss in whole-body Zn would be reached after ~10 d. This hypothesis is supported by data of Windisch (35), who demonstrated that weaned piglets receiving cereal-soybean-based diets without Zn fortification develop first signs of clinical Zn deficiency (reduced feed intake) already after 10-12 d. In conclusion, an experimental period of at least 3 d and no more than 10 d may be capable to induce a short-term SZD when applying comparable diets to growing monogastric mammals.

Outline of the thesis

This doctoral project aimed in the experimental modelling of short-term SZD in weaned piglets. The approach should provide a useful tool for the estimation of reliable measures of the gross Zn requirement of monogastric livestock under practical feeding conditions. Further research interest was related to the physiological adaption under the terms of SZD, namely effects on digestive function, redox metabolism, body Zn redistribution and Zn transporter gene expression. On base of this comprehensive dataset, five manuscripts were submitted to peer-reviewed journals (*Chapters 2-6*). At the time of submission of this thesis, three manuscripts were already published (*Chapters 2-4*; please see the respective chapters' title pages *45, 83 and 115* for links to the respective publisher websites) and two were still under review (*Chapters 5 and 6*).

Chapter 2 presents the development of the experimental approach and the identification of suitable biomarkers of the Zn supply status under experimental conditions. The promotion of short-term SZD occurred by varying dietary Zn supply to 48 weaned piglets under practical feeding conditions. The Zn doses applied during the experimental period spanned the range from deficient alimentary supply levels to mild oversupply based on published requirement thresholds (28.1 to 88.0 mg Zn/kg diet) (24, 27). A total absence of clinical Zn deficiency symptoms (for example growth depression, feed refusal, necrosis of the skin and organs; (69)) and, at the same time, finely graded differences in the response of certain Zn status parameters proved the induction of SZD in animals fed <58 mg Zn/kg diet. Furthermore, the response of apparently-digested feed Zn, liver Zn concentration as well as relative hepatic metallothionein gene expression followed a Zn status-dependent pattern, which allowed a differentiation between deficiently and sufficiently supplied animals. This was evident by breakpoints in response of all these parameters close to ~60 mg Zn/kg diet.

The effects of SZD on the digestive capacity of weaned piglets are discussed in *Chapter 3*. By monitoring the activity of activated zymogens and α-amylase within pancreatic tissue homogenates, a direct connection between the Zn supply status and digestive function became evident, which seemed to be partly connected to a decrease in total pancreatic Zn concentration. Furthermore, this decrease in digestive enzymes significantly reduced the coefficients of apparent dry matter and crude nutrient digestion, already after just 8 d of insufficient alimentary Zn supply.

Chapter 4 highlights the response of cardiac redox metabolism to SZD in weaned piglets. A Zn supply status-dependent decrease in total cardiac glutathione led to a decrease in anti-oxidative capacity as indicated by a reduced ability of the heart muscle to detoxify H_2O_2. This was accompanied by cardiac α-tocopherol depletion and the promotion of antioxidative and proapoptotic gene expression. Monitoring the cardiac Zn concentrations indicated a change in behaviour, which became evident by stepwise repletion of cadiac Zn in animals fed <42.7 mg Zn/kg diet. This further promoted stress-responsive gene expression following rising cardiac Zn concentrations.

Patterns of body Zn redistribution during short-term SZD in weaned piglets were analysed in *Chapter 5*. This data illustrates a clear hierarchy of Zn redistribution between tissues as a result of Zn homeostatic regulation. Within this hierarchy, Zn donors (for example bone, intestine, liver, kidney) contrasted against Zn acceptors (for example heart muscle, skeletal muscle, immune tissue). The Zn-accepting tissues replenished their Zn concentrations during SZD or even increased them above the level assessed within control animals (receiving 88.0 mg Zn/kg diet). This hierarchy could have been confirmed by cluster analysis of tissue Zn concentrations between biological matrices. Moreover, a cluster analysis of tissue Zn concentrations between treatment groups allowed a discrimination between deficiently and sufficiently Zn-supplied

animals. This represents to our knowledge, the first report of a successful estimation of the Zn supply status according to clusters of tissue Zn concentrations.

Chapter 6 presents a comparative analysis of gene expression of all known members of the solute carrier families 30 (ZnT) and 39 (ZIP) of Zn transporters in jejunum, colon, liver and kidney during SZD in weaned piglets. The qualitative and quantitative expression patterns were analysed in and between tissues. It was recognised that many of the investigated gene expression patterns exhibited breakpoints in response to changes in dietary Zn supply. These thresholds either lay at ~40 or ~60 mg Zn/kg diet, thereby indicating clear differences in the respective stimuli to which these genes responded. A breakpoint close to ~60 mg Zn/kg diet (gross Zn requirement under given experimental conditions) highlighted a role of the respective gene in the regulation of Zn fluxes to meet the basal requirements. In contrast, a subset of the investigated Zn transporter genes seemed to be involved in the regulation of Zn fluxes for the compensation of stress and inflammatory processes. This was evident by breakpoints close to ~40 mg Zn/kg diet, which have been reported earlier to also apply to parameters of cardiac antioxidative capacity under these experimental conditions. This represents to our knowledge the first description of tissue-dependent expression patterns of all known Zn transporter genes in weaned piglets.

Contribution statement

The doctoral candidate's contribution included experimental design, collection of zootechnical data, collecting and processing of biological samples, supervision and conduction of chemical and molecular biology analyses, data analyses and interpretation as well as authoring of all manuscripts.

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2nd Chapter

Development of an experimental model to assess the bioavailability of zinc in practical piglet diets

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Abstract

Sufficient zinc (Zn) supply is a key element of successful animal husbandry. Proper use of dietary Zn sources, however, demands knowledge of Zn requirement and bioavailability, reflecting practical feeding systems.

In this study, an experimental model is presented where 48 fully weaned and individually housed piglets received a fine differentiated alimentary Zn supply. The basal diet consisted mainly of corn and soybean meal (native Zn: 28.1 mg/kg feed) and was fortified with Zn from Zn sulphate at eight levels (0, 5, 10, 15, 20, 30, 40 and 60 mg Zn/kg). All animals were pretreated uniformly with the highest Zn supply (88 mg total Zn/kg feed) for 2-weeks (feeding *ad libitum*). Subsequently, animals were switched to the eight experimental diets (six animals per group, restricted feeding at 450 g/d). This period was limited to 8 d in order to avoid clinical Zn deficiency symptoms. Measurements included amounts of apparently-digested Zn, final levels of plasma Zn, plasma Zn-binding capacity, plasma alkaline phosphatase activity, femur Zn, liver Zn as well as hepatic metallothionein (MT) 1A and MT2B gene expression and hepatic MT protein abundance.

Clinical signs of Zn deficiency were completely absent through the entire study. All the analysed parameters except for MT protein abundance responded sensitively to graduations in dietary Zn contents and indicated the presence of Zn deficiency at lower dietary Zn additions. Amounts of apparently-digested Zn, liver Zn as well as hepatic MT1A and MT2B gene expression indicated transition from deficient to sufficient Zn supply between 47.5 and 58.2 mg of total Zn per kg of diet as assessed by broken-line response techniques. Analysed blood and bone parameters responded linearly to graduations in dietary Zn supply even within sufficient Zn supply levels.

Taken together, the results indicate the suitability of our experimental model to determine Zn requirement in piglets and hence to also assess bioavailability of dietary Zn sources. The latter may be done by comparing the slope of the amounts of apparently-digested Zn as well as by determining the response of blood and bone parameters to graduations in dietary Zn at insufficient Zn supply.

Introduction

Sufficient supply with zinc (Zn) is inevitable for a proper functioning organism. Even small alimentary deficiencies can cause growth depression and impairment of animal welfare when occurring longer than the organism is capable of maintaining Zn homoeostasis through mobilisation of whole-body Zn stores. In order to avoid such situations of marginal (and severe) alimentary deficiency in piglet rearing, Zn is supplemented with large safety margins to compensate for fluctuations in daily demands and factors modulating bioavailability (1, 2). However, every milligram that exceeds the body's net demand is excreted mainly via faeces and may contribute to undesirable accumulations of Zn in the soil of agricultural land (3, 4). In order to cover the animals' demand as precise as possible, reliable data on the bioavailability of feed Zn under various dietary and physiologic conditions are indispensable. Hence, precise and reproducible experimental approaches are needed to create such data sets. The present study aimed to develop an adequate model in weaned piglets under practical conditions, by inducing fine-scaled differences in Zn status without promotion of pathological symptoms of Zn deficiency. In this context, several Zn-dependent biofactors were monitored and screened for their suitability as Zn status parameters under the conditions of latent Zn deficiency.

Material and Methods

This animal study was registered and approved by the responsible animal welfare authorities (district government of Upper Bavaria, federal state of Bavaria, Az. 55.2.1.54-2532.3.63-11).

Animals and diets

The study included six litters of eight fully weaned piglets each (four male castrated and four female, initial average body weight 8.6 kg). Animals were housed in separate flat deck boxes, which allowed individual feeding and faeces collection. All flat deck boxes were in the same room and separated by bars, allowing interindividual contact 24 h a day. Initial room temperature (start of acclimatisation phase) was 30°C and weekly reduced by 1°C until the end of study. Mean humidity ranged between 45% and 55%. The light programme consisted of 12 h daylight and 12 h of crepuscular light during night time. Animals had *ad libitum* access to drinking water.

Within the six litters, animals were randomly assigned to one of eight treatments (complete randomised block design; all animals of one litter represented an experimental block with balanced distribution of gender and life weight). The treatments consisted of different levels of dietary additions of Zn from Zn sulphate ($ZnSO_4$ * $7H_2O$; analytical grade; Merck 108883; Merck KGaA) to a basal diet composed mainly of corn and soybean meal (Table 1). The basal diet was designed to meet all nutrient demands of growing piglets according to GfE (1) except for Zn. Furthermore, the basal diet was supplemented with 0.3% TiO_2 as indigestible marker for proximate estimation of faecal digestibility of ingested Zn. Native dietary Zn accounted for 28.1 mg/kg.

The components of the basal diet except for Zn addition were mixed in one single batch, pelleted (70°C, steam) in order to reduce native phytase activity and ground again. From this basal batch, eight aliquots of 1 kg each were taken, finely ground and mixed up with increasing amounts of Zn from Zn sulphate. These premixes were added to sub-batches of the basal feed preparation (60 kg each), homogenised and pelleted again. Zn inclusions into the eight sub-batches were adjusted to produce alimentary Zn additions of 0, 5, 10, 15, 20, 30, 40 and 60 mg Zn/kg, respectively. Total Zn concentrations of the eight final feed mixtures (including native dietary Zn) accounted for 28.1, 33.6, 38.8, 42.7, 47.5, 58.2, 67.8 and 88.0 mg/kg, respectively (Table 2). Analysis of phytase activity (Method: ISO 30024) after the second pelleting revealed very low native phytase activities in final feed mixtures of 140 U/kg on average. Prior to the onset of the study, all animals were fed the diet with the highest Zn addition (60 mg/kg of added Zn and 88 mg of total dietary Zn) *ad libitum* during a 2-week acclimatisation period. Subsequently, animals were switched to the eight treatments and were fed the diets with different Zn contents at restricted amounts of daily 450 g per head for a total experimental period of 8 d. This amount represented the average daily feed consumption under *ad libitum* conditions during the last week of the acclimatisation period.

Assessment of performance parameters

All animals were weighed at the start of acclimatisation (day 0) as well as at experimental days 1 and 8. The life weight data from the experimental period was used to calculate the daily weight gain. Feed residuals (if any) were reweighed on a daily base to assess the daily feed intake during the experiment. The feed conversion ratio was subsequently calculated as the quotient of total feed intake (kg) and total weight gain (kg) during the experimental phase.

Table 1. Composition, metabolizable energy and analysed crude nutrient contents of the basal diet.

Dietary Component, %		Chemical composition	
Corn	46.0	[1]Metabolizable energy, MJ ME/kg	13.3
Soybean meal (40% crude protein)	26.0	Dry matter, g/kg	902
Potato protein	10.0	Crude protein, g/kg	238
Wheat bran	5.00	Crude fat, g/kg	46.0
Sugar beet pulp	3.00	Crude fibre, g/kg	51.0
Feeding sugar	2.00	Crude ash, g/kg	61.0
Soybean oil	1.50	Lysine, g/kg	13.8
[2]Premix	3.00	Methionine, g/kg	4.10
$Ca(H_2PO_4)_2$	1.60	Threonine, g/kg	10.3
$CaCO_3$	1.40	Tryptophan, g/kg	2.90
NaCl	0.50		

Notes: [1]ME contents as well as contents of essential (pcd) amino acids were estimated from feed table information (http://datenbank.futtermittel.net/); [2]Premix: 2.8% MgO; 0.08% $CuSO_4$ * $5H_2O$; 2.0% $FeSO_4$ * $7H_2O$; 0.20% $MnSO_4$ * H_2O; 0.002% Na_2SeO_3 * $5H_2O$; 0.002% KI; 0.05% vitamin A; 0.007% vitamin D3; 0.2% vitamin E; 0.002% vitamin K3; 0.01% vitamin B1; 0.03% vitamin B2; 0.1% niacin; 0.02% pantothenic acid; 0.02% vitamin B6; 0.15% vitamin B12; 0.03% biotin; 0.002% folic acid; 6.7% choline chloride; 10% TiO_2 and 77.6% corn meal. Vitamin and trace element contents (except Zn) met the requirements according to GfE (1).

Table 2. Analysed total Zn content of the diets three independent samples per feed mixture).

Dietary Zn supplementation (mg/kg)	Zn in final feed mixtures (mg/kg)
0	28.1^{h}
5	33.6^{g}
10	38.8^{f}
15	42.7^{e}
20	47.5^{d}
30	58.2^{c}
40	67.8^{b}
60	88.0^{a}
SEM	0.71
P-value	*<0.0001*

Notes: SEM, standard error of means; Zn, zinc; mean values not sharing a common superscript differ at $P \leq 0.05$.

Sampling conditions

Three independent feed samples were derived from each of the eight feed mixtures and stored in airtight polyethylene bottles at −20°C. Faeces samples from every animal were collected during the last three experimental days, pooled and stored in plastic bags at −20°C. At the morning of day 8, all animals were sacrificed by bleeding under anaesthesia (Azaperon, Ketamin) 10 h after the last feeding.

During bleeding, blood was collected in Li-heparin tubes; blood plasma was derived by centrifugation (1100 g, 10 min, 4°C) and stored in 1 ml Eppendorf tubes at −80°C. Tissue samples were taken from bone (left femoral head) and liver (*Lobus hepatis sinister lateralis*). Liver samples for total RNA extraction were incubated in RNAlater (Life Technologies GmbH) at 5°C overnight and subsequently stored at −80°C in cryotubes. Liver samples for western blot analysis were snap frozen and also stored at −80°C. Bone and liver tissue for Zn analysis was vacuumed and stored at −20°C.

Zinc content analysis

All Zn contents were measured by atomic absorption spectrometry (AAS; novAA 350, Analytik Jena AG) after sample type-dependent preparation steps. Feed samples were mixed with 65% HNO_3, 30% H_2O_2 and double-distilled H_2O and wet digested through heating via microwave (Ethos 1, MLS GmbH). Faeces and liver samples were weighed, freeze-dried and reweighed prior to microwave wet digestion with the same reagents as for the feed samples. One half of each femoral head (cut lengthwise) was dried (105°C, 1 d), reweighed and mineralised (470°C, 2 d) in platinum dishes. The ash was subsequently dissolved and wet digested via microwave as all other sample types. Zn content of blood plasma was directly measured via AAS. Calibration of Zn analysis was based on certified AAS standard solutions (Merck 109953, Merck Millipore).

Crude nutrient analysis and apparently-digested zinc

Dietary crude nutrient and dry matter contents were assessed by standard protocols (5) (Table 1). Metabolizable energy (ME) contents as well as contents of essential amino acids were estimated by feed tables of the Deutsche Landwirtschafts-Gesellschaft e.V. (DLG) (http://datenbank.futtermittel.net) (Table 1).

Contents of TiO_2 in feeds and faecal samples were determined according to Brandt and Allam (6). The apparent digestibility of dietary Zn (%) was calculated on the basis of the respective ratios of Zn to TiO_2 in feeds and faeces and was used to quantify the total amounts of apparently-digested Zn per kg consumed feed (mg Zn/kg feed).

Assessment of Zn-binding capacity and alkaline phosphatase activity in blood plasma

The Zn-binding capacity (ZBC) in blood plasma was assessed according to Roth and Kirchgessner (7). In brief, ZBC denotes the percentage of free plasma Zn-binding sites relative to its total Zn-binding potential. The activity of alkaline phosphatase in blood plasma (U/L) was determined with a commercial kit (AP 307; Randox Laboratories Ltd.) according to manufacturer's instructions.

Reverse transcription-quantitative polymerase chain reaction

Total RNA extraction and quality control

Total RNA was extracted from liver using 50 mg tissue with the miRNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Tissue homogenisation was performed with the FastPrep System (MP Biomedicals). Total RNA yield and purity were determined spectrophotometrically (Biophotometer, Eppendorf). $OD^{260nm/280nm}$ and $OD^{260nm/230nm}$ values of >2.0 were considered as indicators of high purity extracts with regard to contaminations with proteins and extraction reagents (8). Total RNA integrity

was assessed by automated capillary gel electrophoresis (Experion system, Biorad). An RNA quality indicator (RQI) of >5.0 indicated sufficient integrity for reverse transcription quantitative polymerase chain reaction (RT-qPCR) experiments (8). Total RNA solution was stored at −80°C until further usage.

Reverse transcription

Total RNA samples were reverse transcripted in duplicate using the iScript™ Reverse Transcription Kit (Biorad) according to manufacturer's instructions (500 ng of total RNA per reaction). This kit uses a mixture of random hexamer and oligo-dT primers. The block cycler (T100, Biorad) was programmed with 25°C for 5 min, 40°C for 30 min and 85°C for 5 min. All copy DNA (cDNA) samples were diluted 1:5 with nuclease-free water prior to RT-qPCR experiments. cDNA solution was stored at −20°C until further usage.

Primer design and optimisation

Primer pairs for the target transcripts metallothionein (MT) 1a and MT 2b as well as for the potential reference transcripts glyceraldehyde 3-phosphate dehydrogenase (GAPDH), beta-glucuronidase (GUSB), histone 3 (H3) and ubiquitin c (UBC) were designed and tested for target specificity with primer blast (9) (Table 3). Default modifications of the tool were kept, except for amplicon length (100–200 bp), primer position on the transcript – which should span an exon–exon junction where possible – total GC content (between 40% and 60%) and GC content within the first five 3′ bases (<3). Transcript sequences for *Sus scrofa* were taken from the NCBI Refseq database (10). Primer pairs were ordered at Eurofins and tested for the optimal annealing temperature (earliest quantification cycle (Cq) with acceptable melting curve) via gradient PCR using the SYBR No-Rox Kit (Bioline) on the Mastercycler gradient S

(Eppendorf). PCR products were checked for product length on a 1.7% agarose gel using a 50 bp ladder (Invitrogen) as marker.

Relative quantification of gene expression

RT-qPCR was performed in a 96-well format on the Mastercycler gradient S (Eppendorf). The cDNA duplicates of all samples were distributed on two plates using an interplate calibrator (IPC) to correct for interplate variation. The real-time cycler was programmed as follows: 95°C for 2 min, 40 cycles of 95°C for 5 s, primer pair-specific annealing temperature (Table 3) for 10 s and 72°C for 8 s. In the end of each RT-qPCR run, a melting curve analysis was performed.

Normalisation of gene expression data

All mean Cq data were corrected for interplate variation with the equation:

$$Cq^{IPC\ corrected} = Cq_i^{uncorrected} - Cq_i^{IPC} + \frac{1}{number\ of\ plates} * \sum_{i=1}^{number\ of\ plates} Cq_i^{IPC}$$

The whole IPC-corrected mean Cq data set (target transcripts + potential reference transcripts) was screened with GenEx and Normfinder algorithms using the software GenEx (Multi D Analysis) to identify the most stable expressed transcripts over all treatment groups in order to calculate the reference gene index (11, 12).

The so-called $^{\Delta\Delta}$Ct method is a very common approach of RT-qPCR data normalisation for relative quantification of gene expression (13). It assumes a 100% amplification efficiency of every assay (doubling of PCR product amount per PCR cycle, $copy\ number = 2^n$). Although, amplification efficiency of target and/or reference gene assays may vary substantially, possibly leading to artefacts in target gene expression results due to interactions between treatment effects and amplification efficiency. In

such a case, it is necessary to take assay-specific differences into account. This can be done by using a normalisation model which corrects for such differences in amplification efficiency (14). In order to ensure that both normalisation strategies provide approximately equivalent results under the present experimental conditions, the $^{\Delta\Delta}$Ct method as well as the relative quantification model by Pfaffl (14) was used. The results of both normalisation strategies are subsequently discussed in this manuscript. Amplification efficiency (E) was calculated according to following equation:

$$E = \left(10^{-\left(\frac{1}{\text{standard curve slope}}\right)}\right) - 1$$

Amplification efficiency was measured using standard curves derived from reverse transcribed liver total RNA dilution series (200, 150, 100, 50, 10 and 1 ng/µl) of the three samples with the highest and lowest total RNA quality, respectively. The group with the highest Zn supplement (60 mg/kg of added Zn) served as control for both normalisation strategies as it represents the baseline Zn supply during acclimatisation phase. RT-qPCR data are presented as xfold regulation relative to a mean expression level of the control group of 1.0.

Table 3. PCR primer and PCR product specification.

Gene	Accession number	Forward sequence	Reverse sequence	[1]Position on template (5'→3')	Product length bp	Annealing temperature °C
GAPDH	NM_001206359.1	CACATGGCCTCCAAGGAGTAA	GGAGATGCTCGGTGTGTTGG	1082 → 1210	129	58.6
GUSB	NM_001123121.1	TCACGAGGATCCACCTCTCAT	CCTATGGCCCTCTGAGGTGA	1647 → 1808	162	58.6
H3	NM_213930.1	CTTTGCAGGAGGCAAGTGAG	GCGTGCTAGCTGGATGTCT	333 → 445	113	58.6
UBC	XM_003483411.1	AGTGATGGCCAGTGAAGCAA	GCAGGCCACTGAGAGCTAAT	2306 → 2442	137	64.1
MT1A	NM_001001266.2	TCTCACCTGCCTCCACTCAT	AGCAGCAGCTCTTCTTGCAG	33 → 153	121	60.0
MT2B	XM_003355808.2	GCAAATGCAAAGACTGCAAA	TACATCTGGGGCAGGGTCT	154 → 311	158	64.1

Notes: [1]Position on template indicates first base at 5′ and last base at 3′ side at which the respective primer pair binds, yielding the product length presented in the next column; GAPDH, glycerinaldehyde-3-phosphatedehydrogenase; GUSB, beta-glucuronidase; H3, histone 3; UBC, polyubiquitin-c; MT1A, metallothionein 1A; MT2B, Metallothionein 2B.

Western blot

Semi-quantitative determination of MT protein abundance was performed by western blot analysis using β-actin as reference protein. Liver tissues (100 mg) were homogenised twice in 1 mL RIPA-lysis buffer (0.05 M Tris-HCl, 0.15 M NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA, pH 7.4), containing protease inhibitor (cOmplete ULTRA Tablets, EDTA free, Roche Diagnostics International AG) for 20 s each, using the FastPrep System including matrix green beads (MP Biomedicals). Homogenates were incubated on ice for 45 min. Cell lysates were centrifuged at 13,000 g for 15 min at 4°C. Supernatants were used for SDS-PAGE. Total protein concentration was determined by bicinchoninic acid (BCA) assay (Smith et al. 1985). Fifteen microliters of protein extract (1.33 µg/µL protein) was incubated with reducing loading buffer (Roti Load 1, Carl Roth GmbH und Co. KG) at 90°C for 5 min. Samples and unstained protein marker (peqGOLD protein marker II, Peqlab Biotechnologie GmbH) were loaded on 8–16% polyacrylamide gel (nUView precast gel, NuSep, c/-LTF-Labortechnik GmbH & Co KG) and separated in SDS electrophoresis buffer (Carl Roth GmbH) at 200 V for 60 min. Proteins were transferred on polyvinylidene fluoride (PVDF) membranes (Immobilon P, Merck Millipore) using a wet transfer system (Peqlab Biotechnologie GmbH). Transfers were conducted at 90 V for 2 h 30 min with two blotting sandwiches in each transfer procedure. After transfer blots were dried at room temperature for 2 h, successful transfer was determined by Ponceau S red staining (0.5% Ponceau S red, 1% acetic acid).

MTs were detected immunochemically using polyclonal rabbit anti-human antibody (ABIN 675009, antibodies-online). Membrane was blocked with 3% milk powder in phosphate buffered saline with Tween-20 (PBST) buffer (0.032% NaH_2PO_4 * H_2O, 0.142% Na_2HPO_4 * $2H_2O$, 0.82% NaCl, 0.05% Tween-20, pH 7.4 at 25° C) for 60 min.

The blot was incubated in 2500-fold diluted primary antibody-containing blocking buffer overnight at 4°C. After three washing steps (PBST buffer, 10 min each), membrane was incubated in 5000fold diluted, horseradish peroxidase (HRP)-conjugated secondary antibody (mouse anti-rabbit, ABIN871126, antibodies-online) for 60 min at room temperature. Blot was washed three times again in PBST and then incubated in HRP substrate (LuminataClassico HRP, Merck Millipore) for 5 min. Chemiluminescence was detected using Fusion Fx imaging system (VilberLourmat GmbH). For reprobing membrane with reference protein, blot was stripped twice in stripping buffer (1.3% glycine, 1% SDS, 1% Tween-20, pH 2.2 at room temperature) for 10 min and subsequently washed in PBS buffer and tris buffered saline with Tween-20 (TBST) buffer containing 50 mM Tris, 150 mM NaCl and 0.05% Tween-20. For immunodetection of β-actin (ACTB), monoclonal HRP-conjugated mouse anti-pig antibody (NB600-501H, Novus Biologicals) was used. After blocking with 3% BSA in PBST buffer for 30 min at room temperature, membrane was incubated in 5000fold diluted antibody for 1 h 15 min at room temperature. Subsequently, membrane was washed three times in PBST buffer (10 min each) and then incubated in HRP substrate for 5 min. Detected quantities of MTs were standardised by calculating the MT/ACTB ratio.

Statistical analysis

Data analysis was performed as two-way ANOVA (treatment, block) using the general linear model (GLM) procedure of the software package SAS 9.3 (SAS Institute Inc.). Significantly different treatment means ($P \leq 0.05$) were identified with Tukey test. The variation within a data set was expressed as the standard error of means (SEM). Furthermore, orthogonal contrasts (linear trend, quadratic trend) were calculated in

order to decide which parameters are worthwhile candidates for broken-line regression analysis (function contrast within the GLM procedure). On the basis of this statistical evaluation, straight broken-line models were estimated for the amounts of apparently-digested Zn per kg feed consumption ($y = a + bx$), liver Zn content ($y = a + bx + cx$) as well as relative gene expression of MT1A and MT2B in liver ($y = a + bx + cx$) using the nonlinear (NLIN) procedure (SAS 9.3).

Results

Animals and diets

Analysis of dietary Zn contents in the eight final feed mixtures indicated a recovery rate of virtually 100% of intended Zn additions (Table 2; $r = 1.00$; $slope = 0.99$). Furthermore, analysis of three independent feed samples per treatment revealed a high degree of homogeneity within final feed mixtures (Table 2; $SEM = 0.71$ mg/kg).

Life weight of animals did not differ between treatment groups at the beginning of acclimatisation (day 0) nor at the beginning and end of the experimental phase, days 1 and 8, respectively (Table 4). Daily weight gain, daily feed intake and feed conversion ratio were not affected by treatment (on average: 0.465 kg/d, 0.442 kg/d, 1.07). In total, all animals performed with good health, and there was no indication of Zn deficiency symptoms visible at any time.

Zinc metabolic parameters in blood plasma and femur

Blood plasma Zn content, activity of plasma alkaline phosphatase and femoral Zn content were strongly correlated to dietary Zn ($r = 0.96$, $r = 0.95$ and $r = 0.95$, respectively) (Table 5). On the contrary, the ZBC (%) showed an inverse linear relationship to alimentary Zn supply ($r = -0.96$) (Table 5). The linear dose–response behaviour of all measured plasma parameters covered the whole range of dietary treatment as indicated by highly significant linear trends and the absence of significant quadratic trends.

Zinc metabolic parameters in faeces and liver

Increasing dietary Zn content led to a straight linear rise of faeces Zn content ($r = 0.99$) (Table 6). Starting from native dietary Zn concentrations, rising Zn additions resulted

in a linear increase of the coefficient of apparent Zn digestibility as well as the amounts of apparently-digested Zn per kg of consumed feed (Table 6). Both factors showed turnarounds in dose–response behaviour once the dietary Zn content passed the fifth level of Zn addition (20 mg/kg of added Zn, 47.5 mg/kg of total dietary Zn). Respective quadratic trends were statistically significant as shown in Figure 1A; a broken-line model estimated the break point between this linear increase and a plateau of the amounts of apparently-digested Zn to range at a dietary Zn content of 58 ± 4 mg/kg ($R^2 = 0.96$). Since animals were fed restrictively and consumed equal amounts of feed, the break point in behaviour of the amounts of apparently absorbed Zn may be used also as an estimate for the transition from deficient to sufficient dietary Zn concentrations.

The behaviour of liver Zn concentration was opposite to that of apparent Zn digestibility and amounts of apparently-digested Zn (Table 6). Up to the fifth level of Zn addition (20 mg/kg of added Zn, 47.5 mg/kg of total dietary Zn), liver Zn showed almost no reaction to rising dietary Zn, while increasing strongly at higher dietary Zn. This turnaround in behaviour was confirmed by a significant quadratic trend as well as by a broken-line regression model (Figure 1B), which estimated the break point in behaviour to range at a dietary Zn content of 51 ± 8 mg/kg ($R^2 = 0.94$).

Relative transcript abundance of MT1A and MT2B in liver showed a similar response to liver Zn. The relative xfold regulation tended to zero over the first five treatment levels (0 –20 mg/kg of added Zn, 28.1–47.5 mg/kg of total dietary Zn) and increased massively from a total dietary Zn content of 58.2 mg/kg (Table 6) for the $^{\Delta\Delta}$Ct-normalised as well as for the efficiency-corrected data (Table 6). In both cases, significant quadratic trends confirmed turnarounds in a dose–response behaviour. In the present investigation, both normalisation models led to almost the same results, indicating high quality and reproducibility of RT-qPCR assay setup ($r = 1.0$ for the

correlation between single $^{\Delta\Delta}$Ct-normalised and efficiency-corrected data points of MT1A and MT2B, respectively).

Figure 2 presents the relative transcript abundance of hepatic MT1A and MT2B, as both $^{\Delta\Delta}$Ct-normalised and efficiency-corrected data in relation to feed Zn contents. Applying a broken-line model revealed break points in behaviour at dietary Zn contents of 58 ± 3 mg/kg ($R^2 = 0.99$) and 55 ± 4 mg/kg ($R^2 = 0.97$), respectively, for MT1A (Figure 2A and B) and 50 ± 6 mg/kg ($R^2 = 0.96$) and 49 ± 5 mg/kg ($R^2 = 0.96$), respectively, for MT2B (Figure 2C and D).

Western blot analysis revealed a 3.5–14 kD and a 42 kD protein for MT and beta-actin, respectively. In the present study, relative MT protein abundance did not reveal a statistically significant trend over treatment groups (Table 6).

Table 4. Effect of graded levels of dietary Zn supply on animal performance parameters.

	Dietary Zn supplementation, mg/kg / Zn concentration in feed, mg/kg									Treatment effects (*P*-value)		
	0/28.1	5/33.6	10/38.8	15/42.7	20/47.5	30/58.2	40/67.8	60/88.0	SEM	ANOVA	[1]Linear	[2]Quadratic
Live weight, kg												
[3]Day 0	8.58	8.80	8.58	8.74	8.11	8.68	8.14	8.76	0.35	*0.65*	*0.84*	*0.28*
Experimental day 1	13.5	13.1	13.2	13.3	13.0	13.6	13.4	13.9	0.68	*0.98*	*0.40*	*0.66*
Experimental day 8	16.7	16.8	16.7	16.6	16.5	17.4	16.7	17.2	0.60	*0.94*	*0.41*	*0.95*
Weight gain, kg/d	0.40	0.46	0.44	0.42	0.43	0.47	0.41	0.41	0.03	*0.63*	*0.77*	*0.33*
Feed intake, kg/d	0.44	0.44	0.44	0.44	0.44	0.44	0.44	0.45	0.005	*0.95*	*0.33*	*0.99*
Feed conversion, feed : gain	1.11	0.95	1.05	1.14	1.07	0.96	1.16	1.09	0.09	*0.58*	*0.61*	*0.83*

Notes: [1]linear trend (orthogonal contrast); [2]quadratic trend (orthogonal contrast); [3]start of acclimatisation; ANOVA, analysis of variance; SEM, Standard error of means; Zn, zinc; *P ≤ 0.05* indicates significance.

Table 5. Effect of graded levels of dietary Zn supply on Zn status parameter in blood plasma and femur Zn.

	Dietary Zn supplementation, mg/kg / Zn concentration in feed, mg/kg									Treatment effects (*P*-value)		
	0/28.1	5/33.6	10/38.8	15/42.7	20/47.5	30/58.2	40/67.8	60/88.0	SEM	ANOVA	[1]Linear	[2]Quadratic
Plasma Zn, mg/L	0.21^{d}	0.32^{c}	0.27^{dc}	0.34^{c}	0.34^{c}	0.47^{b}	0.45^{b}	0.63^{a}	0.03	*≤0.0001*	*≤0.0001*	*0.96*
Plasma ZBC, %	85.7^{a}	78.0^{b}	81.4^{ba}	77.2^{b}	77.7^{b}	69.4^{c}	69.3^{c}	61.9^{d}	4.28	*≤0.0001*	*≤0.0001*	*0.32*
Plasma AP, U/L	128^{d}	202^{dc}	192^{dc}	189^{dc}	238^{bc}	292^{ba}	252^{bc}	356^{a}	18.7	*≤0.0001*	*≤0.0001*	*0.38*
Femur Zn, mg/kg ash	180^{d}	198^{dc}	197^{dc}	190^{dc}	208^{bc}	210^{bc}	229^{ba}	239^{a}	6.54	*≤0.0001*	*≤0.0001*	*0.65*

Notes: [1]linear trend (orthogonal contrast); [2]quadratic trend (orthogonal contrast); AP, alkaline phosphatase; ANOVA, analysis of variance; SEM, Standard error of means; ZBC, percentage Zn binding capacity; Zn, zinc; mean values not sharing a common superscript differ at *P ≤ 0.05*.

Table 6. Effect of graded levels of dietary Zn supply on Zn in faeces, apparently-digested Zn, liver Zn and hepatic metallothionein (MT1A, MT2B) mRNA expression ($^{\Delta\Delta}$Ct and efficiency-corrected (EC) normalisation).

	Dietary Zn supplementation, mg/kg / Zn concentration in feed, mg/kg									Treatment effects (*P*-value)		
	0/28.1	5/33.6	10/38.8	15/42.7	20/47.5	30/58.2	40/67.8	60/88.0	SEM	ANOVA	[1]Linear	[2]Quadratic
Faecal Zn, mg/kg DM	212^{g}	253^{f}	282^{ef}	308^{ed}	342^{d}	435^{c}	499^{b}	638^{a}	13.1	*<0.0001*	*<0.0001*	*0.93*
App. Zn digestibility, %	-9.61^{c}	-2.52^{bc}	0.64^{ba}	0.93^{ba}	4.55^{ba}	6.07^{ba}	7.66^{a}	5.02^{ba}	2.22	*<0.0001*	*<0.0001*	*<0.0001*
App.-digested Zn, mg/kg FI	-2.69^{d}	-0.85^{dc}	0.25^{bdc}	0.40^{bdc}	2.16^{bac}	3.52^{ba}	5.19^{a}	4.42^{ba}	1.01	*<0.0001*	*<0.0001*	*0.003*
Liver Zn, mg/kg DM	83.2^{cd}	87.1^{cd}	77.0^{d}	87.6^{cd}	88.8^{cd}	94.9^{cb}	106^{b}	127^{a}	8.13	*<0.0001*	*<0.0001*	*0.003*
Liver MT1A gene expression, [3] $^{\Delta\Delta}$Ct [5]xfold	0.003^{d}	0.004^{d}	0.02^{dc}	0.02^{c}	0.01^{dc}	0.09^{b}	0.25^{b}	1.00^{a}	0.11	*<0.0001*	*<0.0001*	*0.0005*
Liver MT1A gene expression, [4]EC [5]xfold	0.003^{d}	0.004^{d}	0.02^{dc}	0.02^{c}	0.01^{dc}	0.19^{b}	0.25^{b}	1.00^{a}	0.16	*<0.0001*	*<0.0001*	*0.0007*
Liver MT2B gene expression, [3] $^{\Delta\Delta}$Ct [5]xfold	0.03^{c}	0.02^{c}	0.02^{c}	0.04^{c}	0.04^{c}	0.33^{b}	0.33^{b}	1.00^{a}	0.10	*<0.0001*	*<0.0001*	*0.003*
Liver MT2B gene expression, [4]EC [5]xfold	0.03^{c}	0.03^{c}	0.02^{c}	0.05^{c}	0.05^{c}	0.35^{b}	0.35^{b}	1.00^{a}	0.11	*<0.0001*	*<0.0001*	*0.003*
Liver MT protein abundance, [5]xfold	1.10	1.12	1.37	1.08	0.88	0.60	1.15	1.00	0.24	*0.39*	*0.41*	*0.35*

Notes: [1]linear trend (orthogonal contrast); [2]quadratic trend (orthogonal contrast); [3] $^{\Delta\Delta}$Ct, gene expression data normalised according to Livak and Schmittgen (13); [4]EC; gene expression data normalised according to Pfaffl (14); [5]xfold; xfold difference in mRNA or protein abundance, respectively, relative to a level of 1.00 in the control group (88.0 mg Zn/kg diet); ANOVA, analysis of variance; App., apparent(ly); DM, dry matter; FI, feed intake; MT1A, metallothionein 1A; MT2B, metallothionein 2B; MT, metallothionein peptide (antibody not isoform specific); SEM, Standard error of means; Zn, zinc; mean values not sharing a common superscript differ at $P \leq 0.05$.

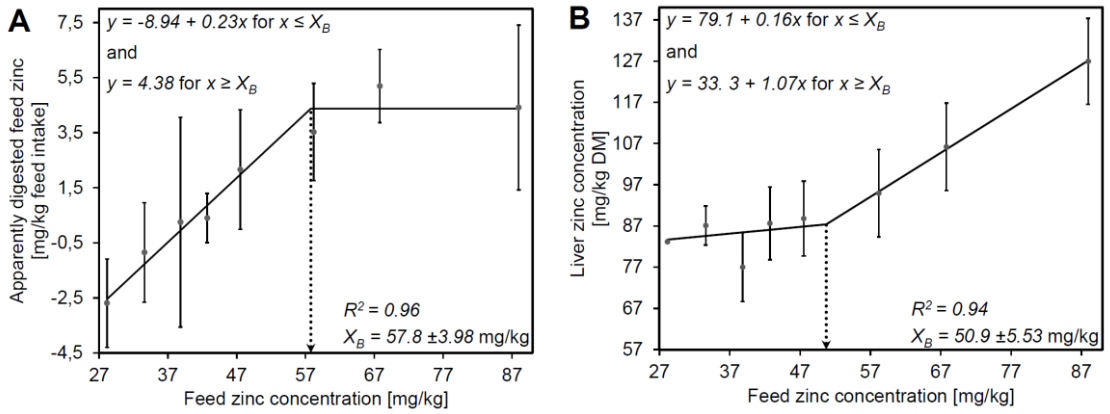

Figure 1. Broken-line analysis ($y = a + bx$ and $y = a + bx + cx$, respectively) on the effect of varying alimentary zinc supply on (A) apparently-digested amount of feed Zn and (B) liver Zn. Notes: DM, dry matter, R^2, coefficient of determination of the respective broken-line model, X_B, breakpoint of the respective broken-line model.

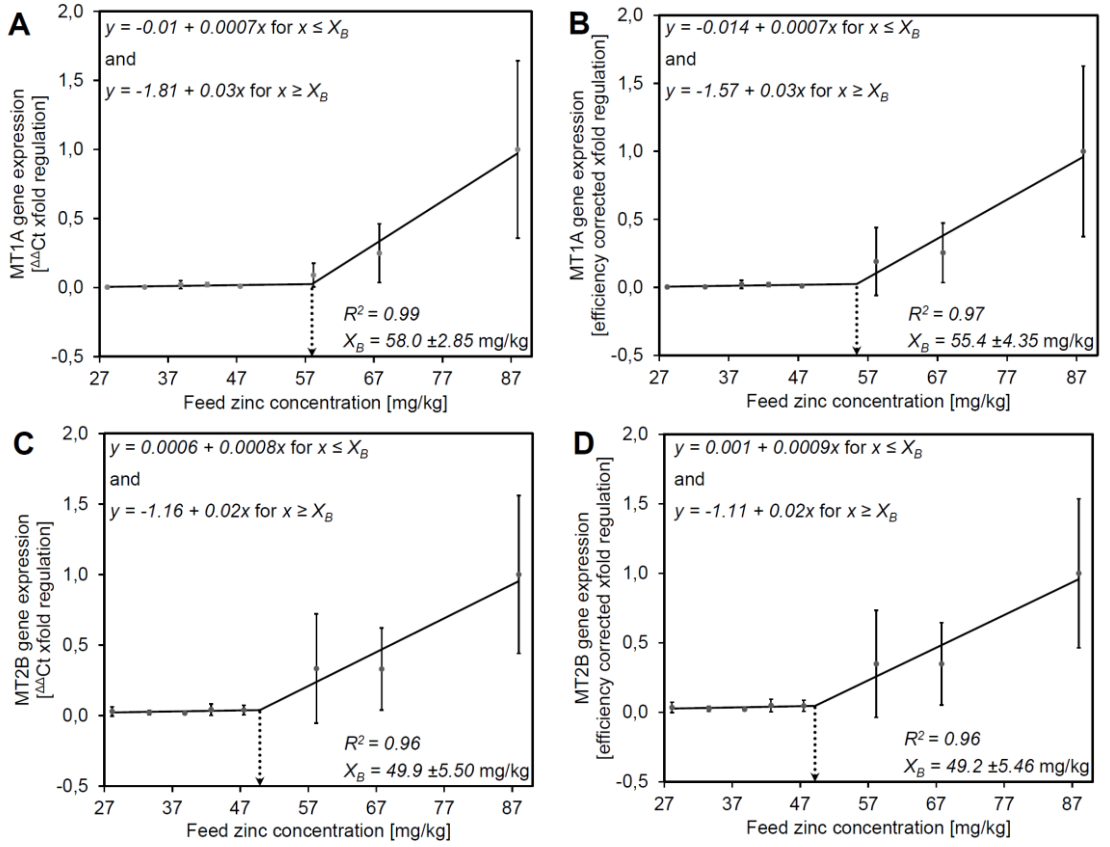

Figure 2. Broken-line analysis ($y = a + bx + cx$) on the effect of varying alimentary Zn supply on hepatic MT1A (A, B) and MT2B (C, D) expression, after $^{\Delta\Delta}$Ct and efficiency-corrected normalisation. Notes: R^2, coefficient of determination of the respective regression model, X_B, breakpoint of the respective regression model, $^{\Delta\Delta}$Ct gene expression normalisation according to Livak and Schmittgen (13), efficiency corrected gene expression normalisation according to Pfaffl (14), data is presented as xfold regulation compared to a relative mRNA abundance of 1.0 of the control group (88.0 mg Zn/kg diet).

Discussion

A major goal of this study was to develop an experimental model which induces finely-scaled dietary Zn deficiency under practical feeding conditions. Simultaneously, reactions on Zn deficiency should remain within physiological margins without inducing symptoms of pathological Zn deprivation. Furthermore, Zn supply should span a preferably large range from deficient to sufficient states in which quantitative, reproducible and reliable measurements on Zn metabolism can be conducted.

Methodological considerations

The basal diet was designed to provide high amounts of native phytate in order to generate an unfavourable situation to Zn bioavailability under practical feeding conditions. To accomplish this, the basic components were corn and soybean meal which are common in practical livestock feeding and have relatively low Zn contents and at the same time are rich in phytate (15). Moreover, the native phytase activity was repressed by pelleting at high temperatures (70°C) under the impact of steam. The high amount of soybean meal in the diet led to a high crude protein content of 23.8% which had to be tolerated in order to ensure high phytate intake and simultaneously sufficient quantity and quality of dietary protein supply at any time. As demonstrated by blood plasma parameters, bone parameters as well as negative values of apparently-digested dietary Zn, the diet used in this study was clearly able to produce Zn deficiency. The extent of Zn deficiency was much stronger compared to a previous study using practical feed components exhibiting high native phytase activities like wheat (16). Avoiding such feed components and minimising native phytase activities (for example through pelleting with steam) are obviously important factors to create a practical diet for Zn deficiency studies.

Zn deficiency studies often start with a depletion period over several weeks and a subsequent repletion phase in which the response of Zn metabolism is monitored. Indeed, such an experimental setup increases the gap between deficient and sufficient Zn supply and maximises the response of Zn metabolism to Zn supplementation from dietary sources with different bioavailability. However, replenishment of stressed Zn stores after the end of the depletion period induces a transient up-regulation of Zn absorption (17), which might add an uncontrolled side effect to measurements of Zn metabolism. Furthermore, animals maintained at Zn deficiency in such experimental setups ("negative controls") are exposed to long-term Zn deficiency with high risk of clinical disorders.

One major goal of the present study was to avoid symptoms of pathological Zn deficiency and secondary metabolic imbalances resulting thereof. For this reason, all animals were fed the basal diet with sufficient Zn content (60 mg/kg of added Zn, 88 mg/kg of total dietary Zn) for 2-weeks prior to the study in order to ensure fully replenished tissue Zn stores to all animals at experimental day 1. Furthermore, the experimental phase in which the animals faced fine-scaled alimentary Zn supply was limited to just 8 d. Whole-body Zn homoeostasis requires around 3 d to fully adapt to a change in dietary Zn supply levels as has been shown in rats (18, 19). On the other hand, the duration of Zn deficiency should not exceed the potential of Zn homoeostasis to maintain normal Zn metabolism through mobilisation of whole-body Zn stores. According to previous studies, the size of mobilizable Zn may be estimated to account for around 15% of whole-body Zn (17, 20-22). Assuming a daily growth rate of piglets of around 3% of body weight and a shortage of Zn supply to half of the metabolic requirement, normal growth should dilute whole-body Zn concentration by around 1.5%/d. Thus, the critical level of 15% loss of whole-body Zn concentration would be

reached after around 10 d of deficient Zn supply. In a previous study using a Zn-deficient diet similar to the present one, piglets showed normal zootechnical performance during the first week of treatment but exhibited a marked collapse in feed intake during experimental days 10 to 12 (23). This may be considered as indication of exhaustion of mobilizable whole-body Zn stores and the start of clinically relevant Zn deficiency. Since in the present study the duration of Zn supply was limited to 8 d and depressive effects on zootechnical performance or other evidence of clinical Zn deficiency were absent, the experimental setup may be considered to reflect a situation of Zn metabolism, which physiologically is still tolerable.

Parameters of zinc metabolism

Blood plasma Zn, AP activity and ZBC as well as bone Zn concentration reacted very sensitively to declining alimentary Zn supply and confirmed that the present diet was able to produce Zn deficiency. Interestingly, these parameters showed a straight linear response over the entire range of dietary Zn supply. There was no plateau when dietary Zn concentration reached that level which was to be expected to provide sufficient amounts of Zn to the metabolism (around 60 mg/kg of added Zn; (2)), as it is usually the case in long-term Zn supply studies (for example Weigand and Kirchgessner (24)). This might be explained by the fact that homoeostatic adaptation of Zn absorption due to a change in dietary Zn supply requires around 3 d to become fully active (17-23). Reducing the dietary Zn supply will therefore induce a short-term lack of absorbed dietary Zn even in diets which would provide sufficient Zn on a long-term basis. This short-term lack is compensated by Zn mobilisation mainly from the skeleton (22) and will be replenished during the days following homoeostatic adaptation to the new level of Zn supply (17). Short-term mobilisation of Zn stores may explain the strictly linear response of femur Zn to reduction of Zn supply along all levels of dietary Zn in the

present study. Furthermore, blood plasma Zn, AP activity and ZBC followed the behaviour of bone Zn obviously due to the nature of blood to serve as distribution media between storage (skeleton) and Zn-consuming tissues. In total, the analysed blood parameters and the bone Zn concentration revealed to be highly sensitive indicators of a change in dietary Zn supply but less suitable to distinguish between deficient and sufficient Zn supply in a short-term experimental setup.

In contrast to bone Zn and blood plasma parameters, the amounts of apparently-digested Zn per kg of feed consumption, liver Zn concentration as well as relative hepatic MT transcript abundance (MT1A and MT2B; $^{\Delta\Delta}$Ct and efficiency corrected) showed clear turnarounds of reaction to fine-scaled reductions of dietary Zn between 47.5 and 58.2 mg of Zn per kg feed. This marks the expected range of Zn demand coverage (around 60 mg/kg of added Zn, (2)). Obviously, the amounts of apparently-digested Zn increased in a linear fashion until the point of satisfactory supply at which homoeostatic counter regulation reduces relative true Zn absorption from the gut lumen (19, 25, 26). This reduction in absorption efficiency is an evolutionary conserved protection system against toxic Zn overload. Due to changes in the expression of certain Zn transporters, the organism is shutting down all energy-dependent Zn-specific transport mechanisms, leaving only passive non-saturable pathways active. This becomes apparent when supplementing pharmacological Zn doses (~2500 mg/kg) to piglets, as shown by Martin, et al. (27) and Martin, et al. (28). The negative amount of apparently-digested Zn in the group with the lowest feed Zn supply (0 mg/kg of added Zn; 28.1 mg/kg of total dietary Zn) indicates a loss of 2.69 mg Zn per kg feed consumption through faecal excretion. This marks the inevitable endogenous Zn losses which have been previously described (19, 24, 29). *Vice versa*, the liver avoids storing major amounts of Zn below the point of Zn requirement, obviously in favour of other tissues (22, 30, 31). This is

supported by a parallel response of hepatic MT mRNA expression. The lack of response at the proteomic level is probably due to the semi-quantitative nature of western blot analysis, which might be unable to detect fine-scaled differences in MT protein abundance. This is in concordance with findings of Martin, et al. (28) who were not able to detect differences in protein synthesis in piglets exposed to feed Zn concentrations of 57 and 164 mg/kg, respectively, but at a dietary Zn content of 2500 mg/kg. The hepatic MT protein turnover should be able to induce dose-dependent changes within the actual experimental period of 8 d as has been shown by Szczurek, et al. (32) in rats. After a 16-d Zn depletion period, animals were repleted with Zn (30 mg Zn per kg of semi synthetic diet). After 24 h of Zn repletion, significant increase in hepatic MT protein abundance was detected by a cadmium saturation assay as well as immunohistochemical localisation of MT. On the other hand, Cain and Holt (33) were able to show that hepatic MT has a half-life of up to ~4 d. Hence, our experimental approach should be appropriate to induce changes in hepatic MT abundance. Therefore, further investigations using more sensitive methods like high-performance liquid chromatography (HPLC) have to be performed in order to evaluate the potential of hepatic MT protein abundance as sensitive Zn status parameter. Furthermore, given the fact that proteomic response often shows poor correlation to transcriptomic response due to certain posttranscriptional mechanisms (34), investigations on the correlation of hepatic MT with certain micro-RNA (miRNA) molecules should be conducted. Those small and non-coding nucleic acids play an important role in post-transcriptional regulation of protein abundance, by reducing the translational processing of certain mRNA through interaction with the so-called “RNA-induced silencing complex”. To this day, hundreds of miRNA have been identified, of which some show tissue-specific expression patterns. Using state-of-the-art qPCR arrays for miRNA detection, highly specific and sensitive identification and quantification of those regulative molecules is

possible, yielding valuable information on this important level of physiological regulation (35).

Zinc deficiency has the potential to induce intracellular damage, possibly leading to major shifts in the expression of target genes as well as the so-called reference genes. This could result in treatment-dependent differences in amplification efficiency of certain transcripts, making an efficiency-corrected normalisation strategy necessary. In the present study, the two used normalisation strategies yielded almost the same results, as indicated by a high correlation of single $^{\Delta\Delta}$Ct to efficiency-corrected data points. Obviously, our animal model induced differential states of Zn status but did not introduce dose-dependent interferences with RT-qPCR analysis. This is further supported by the absence of severe Zn deficiency disorders in animals treated with Zn deficiency. In total, time consuming and costly amplification efficiency determination may not be necessary for future investigations on transcriptional changes under the terms of this experimental model.

Usability of the present experimental model in bioavailability studies

The most important precondition for direct assessment of bioavailability of Zn sources is deficient Zn supply because it excludes homoeostatic down-regulation of absorption and metabolic utilisation of dietary Zn (36, 37). This situation could be clearly induced by our experimental model since it was able to induce fine-scaled graduations in whole-body Zn status ranging from sufficient to deficient Zn supply levels without promoting pathological signs of Zn deficiency.

According to the broken-line calculations shown in Figures 1 and 2, the transition from deficient to sufficient Zn supply ranged between total dietary Zn of 47.5 and 58.2 mg/kg, which was equivalent to dietary additions of Zn from Zn sulphate of 20–30

mg/kg. In case of the amounts of apparently-digested Zn (Figure 1A), the response to rising dietary Zn within the range of deficient Zn supply showed a linear slope of 0.23 mg per mg of Zn added as Zn sulphate. When testing a Zn source with higher (lower) bioavailability than Zn sulphate, the respective slope should be higher (lower). Furthermore, the changes of the slopes should quantitatively reflect the relative differences of bioavailability compared to Zn sulphate since absorption of dietary Zn is the dominant factor determining bioavailability, while metabolic utilisation of absorbed dietary Zn is usually close to 100% (24, 36, 37). Indeed, the slope measured by our experimental approach will not reflect the true absorption of dietary Zn because endogenous faecal Zn losses are not specifically quantified. This applies also to Zn losses via urine. However, urinary Zn is usually negligible in quantitative terms (18, 24, 36). Hence, the amounts of dietary Zn apparently-digested under the condition of insufficient Zn supply reflect the Zn retention, being the dominant route of metabolic use of absorbed dietary Zn in the experimental model presented here.

However, the “window” of dietary Zn additions that still ensures an insufficient Zn supply will shrink with rising bioavailability of added dietary Zn. In order to detect the range of Zn supply which is still applicable for assessment of bioavailability, the Zn source under investigation should be added in fine graduations and in such a way that the transition from deficient to sufficient Zn supply may be surely detected. Simultaneously, enough supply levels need to remain within the range of deficient Zn supply in order to precisely calculate the slope of the amounts of apparently-digested Zn.

Besides apparent digestion of added dietary Zn, blood plasma Zn, ZBC, blood plasma AP activity and Zn concentration in bone seem to be potential parameters to derive relative differences in bioavailability by comparing the slopes of reaction to finely

graded levels of dietary Zn. These parameters responded linearly even above the transition from deficient to sufficient Zn supply levels. This seems to expand the "window" of dietary Zn additions for proper comparisons of bioavailability between different Zn sources. Nevertheless, linear responses above the point of sufficient Zn supply are to be considered as a short-term phenomenon, particularly when testing Zn sources with high bioavailability. Under condition of deficient Zn supply, however, the above-mentioned blood and bone parameters may be considered to serve as robust parameters to assess bioavailability. Nevertheless, proper use of such data still requires additional measurements proving that Zn supply is still deficient (for example amount of apparently-digested Zn, gene expression of hepatic MT).

Alternatively, bioavailability of different Zn sources may be assessed by comparing the respective transitions from deficient to sufficient Zn supply, for example through broken-line evaluations of responses of the amounts of apparently-digested Zn or hepatic MT gene expression to variations in dietary Zn supply. Again, this technique requires fine graduations of dietary Zn contents covering the range from deficient to sufficient supply levels as it is given by the experimental setup presented here.

Conclusion

The experimental model presented here induced fine-scaled differences in Zn status from deficient to sufficient supply as indicated by the response of above-mentioned blood, bone and faecal parameters. Furthermore, the apparently-digested amount of Zn, liver Zn as well as hepatic MT gene expression were identified as suitable markers for the point of transition from deficient to sufficient Zn supply. In combination with blood and bone parameters, the data can be used for relative comparisons of Zn bioavailability under the conditions of practical piglet rearing. Because of the absence of pathological symptoms of Zn deficiency, this approach can be further used for investigations on basal Zn homoeostatic regulation mechanisms and associated metabolic functions.

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3rd Chapter

Subclinical zinc deficiency impairs pancreatic digestive enzyme activity and digestive capacity of weaned piglets

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Abstract

This study investigated the effects of short-term subclinical Zn deficiency (SZD) on exocrine pancreatic activity and changes in digestive capacity.

A total of forty-eight weaned piglets were fed *ad libitum* a basal diet (maize and soybean meal) with adequate Zn supply (88mg Zn/kg diet) during a 2-week acclimatisation phase. Animals were then assigned to eight dietary treatment groups (n = 6) according to a complete randomised block design considering litter, live weight and sex. All pigs were fed restrictively (450 g diet/d) the basal diet but with varying $ZnSO_4 * 7H_2O$ additions, resulting in 28.1, 33.6, 38.8, 42.7, 47.5, 58.2, 67.8 and 88.0mg Zn/kg diet for a total experimental period of 8 d.

Pancreatic Zn concentrations and pancreatic activities of trypsin, chymotrypsin, carboxypeptidase A and B, elastase and α-amylase exhibited a broken-line response to stepwise reduction in dietary Zn by declining beneath thresholds of 39.0, 58.0, 58.0, 41.2, 47.5, 57.7 and 58.0 mg Zn/kg diet, respectively. Furthermore, carboxypeptidase B and α-amylase activities were significantly lower in samples with reduced pancreatic Zn contents. Coefficients of faecal digestibility of dry matter, crude protein, total lipids and crude ash responded similarly to pancreatic enzyme activities by declining below dietary thresholds of 54.7, 45.0, 46.9 and 58.2 mg Zn/kg diet, respectively.

In conclusion, SZD impaired pancreatic exocrine enzymes, this response was connected to pancreatic Zn metabolism and the decline in catalytic activity impaired faecal digestibility already after 1 week of insufficient alimentary Zn supply and very early before clinical deficiency symptoms arise.

Introduction

Clinically manifest Zn deficiency in growing individuals is associated with various unspecific symptoms such as growth depression, feed refusal and impaired immunity (1), highlighting the ubiquitous essentiality of this certain trace metal. However, this phenotype marks the end point in response to long-term insufficient supply at which the animals' mobile Zn stores are exhausted and the homoeostatic regulation is no longer capable of maintaining a stable equilibrium of body Zn. Moreover, this physiological state is rare in nature. Most likely, the predominant Zn malnutrition phenotype in men and animals is a subclinical deficiency associated with reduction of Zn status parameters without development of visible symptoms. Therefore, an experimental approach was recently developed that induces subclinical Zn deficiency (SZD) in weaned piglets. It has been shown that Zn status parameters in plasma, bone and liver respond very sensitively to graduations in analysed dietary Zn supply. At the same time, no overt signs of Zn deficiency were evident throughout the entire study (*Chapter 2, Table 4, page 65*). Using this approach, it is now possible to investigate the early and truly Zn-dependent metabolic adaptions in the development of a clinically manifest Zn deficiency.

The pancreas is essential for the regulation of feed digestion and energy homoeostasis (2). Moreover, significant amounts of endogenous Zn are secreted via the pancreatic duct into the gastrointestinal tract (GIT) in order to maintain a basal body Zn level (3, 4). Hence, it can be hypothesised that the regulation of pancreatic Zn metabolism and exocrine pancreatic digestive function could be somehow connected. Feed digestion is an important biological function, especially for growing livestock. In this context, the first few weeks after weaning are critical with regard to the maintenance of gut health and integrity (5). Hence, dietary fluctuations that negatively influence the GIT have to

be avoided. Indeed, there are already reports on the effects of clinically manifest Zn deficiency on digestive function (6, 7). However, as stated above, these findings may not be related to Zn homoeostatic adaption but a result of secondary metabolic imbalances arising in the course of clinical Zn deficiency. Data on the pancreatic response and related measures of digestive capacity under SZD are currently scarce.

Therefore, the goal of the present study was to investigate the effects of SZD on exocrine pancreatic enzyme activity in weaned piglets. Furthermore, coefficients of apparent faecal digestibility of crude nutrients were estimated in order to monitor the effects on digestive capacity.

Material and Methods

This animal study was registered and approved by the responsible animal welfare authorities (district government of Upper Bavaria, federal state of Bavaria (Germany) (Az. 55.2.1.54-2532.3.63-11).

Animals and diets

The experimental conditions of the present study, including animal housing conditions, diet design and trial conduction, have already been published in detail (*Chapter 2, pages 50-52*). In brief, forty-eight individually housed weaned piglets from six litters (eight animals per litter; 50% castrated male, 50% female, initial average body weight 8.5 ±0.27 kg, 28 d of age) were fed a Zn-adequate (added Zn amount from $ZnSO_4 * 7H_2O$ (analytical grade; Merck 108883; Merck KGaA): +60 mg Zn/kg diet; analysed dietary Zn content: 88 mg Zn/kg diet) basal diet *ad libitum*. The basal diet consisted mainly of maize and soybean meal and was fed for 2 weeks before the study for acclimatisation purposes and to ensure full body Zn stores at day 1 of the experimental period. The basal diet met all the recommendations for sufficient nutrient supply of growing piglets as published by the National Research Council (8). Tables 1 and 2 within *Chapter 2, page 52*, present detailed information on the composition and ingredients of the basal diet as well as the analysed dietary Zn concentrations as affected by varying Zn supplementation to the basal diet.

Following the acclimatisation period, all animals were assigned to eight dietary treatment groups according to a complete randomised block design. Each block represented eight animals from the same litter (six litters per blocks in total) consisting of four castrated male and four female piglets. An animal from each block was assigned to one of eight treatment groups (yielding six animals per treatment group). Thereby, it

was possible to establish a balanced distribution of life weight, genetics (litter mates) and sex over treatment groups. The total experimental period consisted of 8 d, during which all treatment groups received the same basal diet from the acclimatisation period restrictively (450 g diet/d; all animals consumed the total amount of feed) but with varying analysed dietary Zn contents as modulated by varying supplementation with $ZnSO_4$ * $7H_2O$ (added Zn amounts: +0, +5, +10, +15, +20, +30, +40, +60 mg Zn/kg diet; analysed 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 was considered to serve as control as it represented the feeding situation during the acclimatisation phase, from which the Zn contents of all other groups were gradually reduced. TiO_2 was admixed to all diets (3 g/kg diet) to serve as an indigestible marker for assessing apparent faecal crude nutrient digestibility.

Sampling conditions

Feed samples were stored at −20°C in airtight polyethylene bottles, whereas faeces samples from the last 3 experimental days (days 6–8) were pooled animal-wise in plastic bags and stored at −20°C until freeze-drying before chemical analyses.

At experimental day 8, all animals were killed without fasting and the pancreases were removed. Pancreatic samples for the measurement of enzyme kinetics and tissue Zn contents were immediately snap-frozen in liquid N_2 and stored at −80°C until further use.

Chemical analyses

In order to assess treatment-dependent shifts in digestive capacity, chemical analyses included Zn, crude nutrient and TiO_2 contents in feed and faeces, pancreatic Zn content

as well as pancreatic exocrine enzyme activities (trypsin, chymotrypsin, carboxypeptidase A, carboxypeptidase B, elastase, α-amylase).

Total zinc contents

Analyses of total Zn contents in feed, faeces and pancreatic tissue were performed as described earlier (*Chapter 2, page 53*) using atomic absorption spectrometry (AAS) (novAA 350; Analytik Jena AG) after wet digestion via microwave (Ethos 1; MLS GmbH). Calibration was based on certified AAS standard solutions (Merck 109953; Merck Millipore).

Contents of dry matter, crude nutrients and TiO_2 in feed and faeces

DM, crude nutrients and TiO_2 in feed and faeces were assessed by standard procedures (9, 10). In brief, all samples were milled through a 0.5 mm screen before analysis (faeces samples were freeze-dried before analysis). DM contents were assessed using 5 g of sample material by re-weighing after drying with heat (103°C) for 4 h. Crude ash (CA) was determined in 5 g of sample material by mineralising in platinum dishes at 550°C for 2 d. Crude protein (CP) contents were determined using 1 g of sample material according to the method of Kjeldahl, in which the sample was oxidised with H_2SO_4 (1.84 g/ml). After addition of 0.5 M NaOH, the released ammonia was measured through titration. In order to estimate the CP content of the sample, the amount of ammonia-associated N was multiplied with factor 6.25, assuming average N contents in protein of 16 %. Total lipid (TL) contents were analysed in 2.5 g of sample material by treating all samples with hot 3 M HCl for 3 h. The remaining solid residue was washed with double-distilled water, dried (1.5 h, 100°C) and used for lipid extraction with petroleum diethyl ether for 6 h in the Soxhlet apparatus. Crude fibre (CF) contents were assessed by treating 1 g of sample material for 30 min successively with boiling 0.13 M

H_2SO_4 and boiling 0.23 M KOH. The solid residue was mineralised in Pt dishes at 475°C for 30 min. The difference between the residue weight and the remaining ash represented the CF content.

Determination of TiO_2 contents was performed by extracting 1 g of sample in a hot solution consisting of 30 ml 96% H_2SO_4, 9.75 g K_2SO_4 and 0.25 g $CuSO_4$ * $5H_2O$ for 3 h. The supernatant (10 ml) was treated with 1ml of an aqueous solution consisting of 350 µl 96% H_2SO_4, 150 µl 85% H_3PO_4, 100 µl 35% H_2O_2 and 400 µl double-distilled H_2O and was incubated for 1 h at room temperature. The resulting yellow product was measured at 405nm on the UV MC^2 spectrophotometer (SAFAS Scientific Instruments) using plastic semi-micro cuvettes.

Pancreatic enzyme activities

Pancreatic tissue samples (100mg frozen sample/animal) were placed in 1 ml, ice-cold 154 mM NaCl and homogenised using the FastPrep System with Matrix Green Beads (MP Biomedicals). The homogenate was centrifuged at 13800 g for 20 min at 4°C. The supernatant was removed and stored at −80°C until further usage.

The protein concentration within pancreatic tissue lysates was determined using a bicinchoninic acid assay (11). This information was subsequently used to normalise the respective enzyme kinetics for the mg content of proteins within the reaction mix.

Activation of pancreatic trypsinogen to trypsin was performed by *in vitro* incubation with exocrine enteropeptidase E0885; Sigma-Aldrich) (12). All other zymogens (chymotrypsinogen, pro-carboxypeptidase A, pro-carboxypeptidase B, pro-elastase) were activated by *in vitro* incubation with exocrine trypsin (T4549; Sigma-Aldrich) (12-15).

Enzyme kinetics were measured using either 96-well microplates (Rotilab-microtest plates; Carl Roth GmbH & Co. KG) on the LEDETECT 96 system (Deelux Labortechnik GmbH) (trypsin, chymotrypsin, elastase) or quartz cuvettes (carboxypeptidase A and B) and plastic semi-micro cuvettes (α-amylase), respectively, on the UV MC^2 spectrophotometer.

Pancreatic enzyme activity was determined colorimetrically by monitoring the increase in absorption due to hydrolysis of respective substrates (trypsin: Nα-benzoyl-L-arginine 4-nitroanilide hydrochloride (B3133; Sigma-Aldrich) to p-nitroaniline at 405 nm; chymotrypsin: N-glutaryl-L-phenylalanine p-nitroanilide (G2505; Sigma-Aldrich) to p-nitroaniline at 405 nm; carboxypeptidase A: hippuryl-L-phenylalanine to hippuric acid at 254 nm; carboxypeptidase B: hippuryl-L-arginine to hippuric acid at 254 nm; elastase: N-succinyl-ala-ala-ala-p-nitroanilide (S4760; Sigma-Aldrich) to p-nitroaniline at 405 nm; α-amylase: reduction of 3,5-dinitrosalicylic acid by reducing groups of soluble starch (S9765; Sigma-Aldrich) at 540nm). Enzyme activities were defined as units (U) per mg protein and minute reaction time (U/mg per min); 1 U represented 1 nmol (trypsin, chymotrypsin, elastase) or 1 µmol (carboxypeptidase A, carboxypeptidase B, α-amylase) of released product, respectively. Reaction conditions for trypsin, chymotrypsin, carboxypeptidase A, carboxypeptidase B, elastase and α-amylase were pH 7.8/37°C, pH 7.6/25°C, pH 7.5/25°C, pH 7.65/25°C, pH 7.9/25°C and pH 6.9/25°C, respectively.

Estimation of apparent faecal digestibility coefficients for DM and crude nutrients

The percentage apparent faecal digestibility coefficients of DM, CP, diethyl ether extract (subsequently referred to as total lipids; TL), CA and CF were calculated on the

basis of the respective ratios of DM and crude nutrients to TiO_2 in feeds and faeces using the following formula:

$$Apparent\ digestibility\ (\%) = 100 - \left(\frac{\%\ TiO_2\ in\ feed}{\%\ TiO_2\ in\ faeces} * \frac{\%\ DM\ or\ nutrient\ in\ faeces}{\%\ DM\ or\ nutrient\ in\ feed} * 100\right)$$

Statistical analyses

All procedures were performed using SAS 9.4 (SAS Institute Inc.). The individual animal represented the experimental unit. All assessed parameters were subject to ANOVA (independent variables: analysed dietary Zn content with block as covariate) using the general linear model procedure. Significantly different treatment means were identified using the Student–Newman–Keuls test. Furthermore, an orthogonal contrast was estimated with the function CONTRAST between the groups with < 58 and ≥ 58mg Zn/kg diet, in order to highlight potential differences in response between insufficiently and sufficiently supplied Zn treatment groups. This threshold was chosen in light of an earlier study, in which the point of sufficient Zn supply under the present experimental conditions was recognised at 58 mg Zn/kg diet using broken-line response analysis of apparently-digested feed Zn data (*Chapter 2, page 67*).

In order to investigate potential relationships between pancreatic Zn metabolism and exocrine enzyme activity, an orthogonal contrast between animals with the 50% lowest pancreatic Zn contents compared with those with the 50% highest Zn contents was calculated.

In order to assess the mathematical relationships between feed Zn supply and the measured pancreatic and faecal parameters, broken-line ($y = a + bx + cx$) regression analysis was conducted using the procedure NLMIXED (nonlinear mixed model).

A threshold of $P \leq 0.05$ was considered as indicator of statistical significance.

Results

Effects of the dietary treatment on analysed pancreatic zinc and exocrine pancreatic activity

Table 1 presents the results of one-way ANOVA and orthogonal contrasting with the data on analysed pancreatic Zn and pancreatic enzyme activities relative to dietary Zn supply. Reduction of dietary Zn supply reduced the analysed pancreatic Zn contents. The minimum was achieved already at 38.8 mg Zn/kg diet. Significant differences have been determined between groups receiving 38.8 and 42.7 mg Zn/kg diet compared with the highest supplied group ($P = 0.01$). All other groups expressed no significant differences. Orthogonal contrasting revealed significantly lower pancreatic Zn contents in groups fed <58 mg Zn/kg diet ($P < 0.001$). The activity of all investigated pancreatic exocrine enzymes numerically declined with finely-graded reduction in alimentary Zn supply. This relationship occurred to be significant for chymotrypsin and α-amylase as indicated by ANOVA ($P = 0.05$ and $P = 0.01$, respectively) and orthogonal contrasting ($P < 0.05$ and $P < 0.01$, respectively) as well as for elastase based on orthogonal contrasting only ($P = 0.05$). However, the Student–Newman–Keuls test was not able to identify significant differences between treatment groups, except for α-amylase (significant difference between groups receiving 33.6 and 38.8mg Zn/kg diet compared with the control group receiving 88.0 mg Zn/kg diet).

Table 2 shows the results of broken-line regression analyses of analysed pancreatic Zn and pancreatic enzyme activities relative to dietary Zn supply. A highly significant break point in pancreatic Zn response was evident at 39 mg Zn/kg diet as indicated by significant parameter estimates for the X and Y intercept of the break point ($P < 0.0001$, respectively). After a significant decrease of 0.41 mg pancreatic Zn/mg dietary Zn ($P < 0.0001$) over doses ≥ 39 mg Zn/kg diet, the behaviour changed towards

Table 1. Two-factorial ANOVA and orthogonal contrast of pancreatic enzyme activities relative to dietary zinc supply (Mean values and standard errors of the linear model).

										P	
Daily Zn uptake (mg/d)	12.7	15.1	17.5	19.2	21.3	26.2	30.5	39.6	SEM	ANOVA	[1]Orthogonal contrast
Analysed dietary Zn (mg/kg diet)	28.1	33.6	38.8	42.7	47.5	58.2	67.8	88.0			
Pancreas Zn (mg/kg DM)	76.2^{ab}	76.1^{ab}	70.3^{b}	71.8^{b}	79.0^{ab}	81.9^{ab}	85.8^{ab}	90.8^{a}	3.59	*0.01*	*<0.001*
Trypsin (U/mg * min^{-1})	157	144	171	180	161	167	196	240	25.7	*0.35*	*<0.10*
Chymotrypsin (U/mg * min^{-1})	628	648	564	833	633	784	746	751	51.7	*0.05*	*<0.05*
Carboxypeptidase A (mU/mg * min^{-1})	144	189	214	267	230	222	217	227	28.7	*0.41*	*0.56*
Carboxypeptidase B (U/mg * min^{-1})	26.8	29.1	27.1	30.6	32.8	30.4	30.4	30.3	2.12	*0.64*	*0.52*
Elastase (U/mg * min^{-1})	256	296	291	287	306	356	326	295	12.2	*0.26*	*0.05*
α-Amylase (mU/mg * min^{-1})	137^{ab}	119^{b}	131^{b}	174^{ab}	185^{ab}	152^{ab}	204^{ab}	230^{a}	20.3	*0.01*	*<0.01*

Notes: [1]orthogonal contrast between groups of animals receiving <58 or ≥58 mg Zn/kg diet; ANOVA, analysis of variance; DM, dry matter; SEM, standard error of means which represents the pooled standard error of the linear model; Zn, zinc; pancreatic enzyme activities are expressed as units of activity change per minute reaction time normalised to the total protein content within the sample; mean values not sharing a common superscript differ at $P \leq 0.05$.

Table 2. Broken-line regression analysis of analysed pancreatic zinc and pancreatic enzyme activity relative to dietary zinc supply (Parameter estimates with their standard errors).

	Models	Parameter estimates		SE	*P*	R^2
Pancreatic Zn (mg/kg DM)	$Y = 87.2 + b_1X$ for $X \leq X_B$	X_B	39.0	5.09	*<0.0001*	*0.92*
	$Y = 56.5 + b_2X$ for $X \geq X_B$	Y_B	72.6	1.39	*<0.0001*	
		b_1	-0.38	0.41	*0.39*	
		b_2	0.41	0.06	*<0.0001*	
Trypsin (U/mg * min^{-1})	$Y = 142 + b_1X$ for $X \leq X_B$	X_B	58.0	8.62	*0.0001*	*0.90*
	$Y = 39.9 + b_2X$ for $X \geq X_B$	Y_B	172	10.2	*<0.0001*	
		b_1	0.52	0.38	*0.21*	
		b_2	2.29	0.60	*0.005*	
Chymotrypsin (U/mg * min^{-1})	$Y = 484 + b_1X$ for $X \leq X_B$	X_B	58.0	17.7	*0.01*	*0.35*
	$Y = Y_B$ for $X \geq X_B$	Y_B	756	49.8	*<0.0001*	
		b_1	4.69	3.28	*0.19*	
Carboxypeptidase A (mU/mg * min^{-1})	$Y = -35.4 + b_1X$ for $X \leq X_B$	X_B	41.2	2.74	*<0.0001*	*0.82*
	$Y = Y_B$ for $X \geq X_B$	Y_B	233	6.33	*<0.0001*	
		b_1	6.50	1.87	*0.008*	
Carboxypeptidase B (mU/mg * min^{-1})	$Y = 4.22 + b_1X$ for $X \leq X_B$	X_B	47.5	0.009	*<0.0001*	*0.63*
	$Y = Y_B$ for $X \geq X_B$	Y_B	6.20	0.10	*<0.0001*	
		b_1	0.04	0.01	*0.006*	
Elastase (U/mg * min^{-1})	$Y = 212 + b_1X$ for $X \leq X_B$	X_B	57.7	12.9	*0.002*	*0.61*
	$Y = Y_B$ for $X \geq X_B$	Y_B	326	9.93	*<0.0001*	
		b_1	1.97	1.13	*0.12*	
α-Amylase (mU/mg * min^{-1})	$Y = 87.1 + b_1X$ for $X \leq X_B$	X_B	58.0	0.03	*<0.0001*	*0.77*
	$Y = 64.2 + b_2X$ for $X \geq X_B$	Y_B	176	12.3	*<0.0001*	
		b_1	1.53	0.67	*0.05*	
		b_2	1.92	0.73	*0.03*	

Notes: R^2, coefficient of determination of the respective broken-line regression model; X_B, X intercept of the respective breakpoint in parameter response; Y_B, Y intercept of the respective break point in parameter response; b_1, slope of the respective broken-line regression curve over dietary Zn doses lesser than or equal to the respective break point in parameter response; b_2, slope of the respective broken-line regression curve over dietary Zn doses greater than or equal to the respective break point in parameter response. Pancreatic enzyme activity is expressed as units of activity change per min reaction time normalised to the total protein content within the sample; $P \leq 0.05$ indicates statistical significance.

a replenishment of pancreatic Zn contents by 0.38 mg/mg dietary Zn at doses ≤39mg Zn/kg diet. However, the latter slope in response missed the threshold of statistical significance. The suitability of the model to explain the response of pancreatic Zn to dietary Zn was indicated by a high R^2 of *0.92*. All investigated pancreatic enzyme activities also exhibited highly significant breakpoints in parameter response to variations in dietary Zn levels. This was evident by significant parameter estimates for the respective X and Y intercepts of the break points in response to varying dietary Zn supply ($P = 0.0001$ and $P < 0.0001$ for trypsin, $P = 0.01$ and $P < 0.0001$ for chymotrypsin, $P < 0.0001$ and $P < 0.0001$ for carboxypeptidase A, $P < 0.0001$ and $P < 0.0001$ for carboxypeptidase B, $P = 0.002$ and $P < 0.0001$ for elastase, $P < 0.0001$ and $P < 0.0001$ for α-amylase). Trypsin and α-amylase declined by 2.29 U/mg per min and 1.92 mU/mg per min per mg reduction in dietary Zn, respectively, until a break point in response of 58.0 mg Zn/kg diet. Below this threshold, the slopes in response decreased to 0.52 U/mg per min and 1.53 mU/mg per min with every mg further reduction in dietary Zn supply, respectively. All other assessed enzyme activities exhibited plateaus in response above the respective dietary thresholds of 58.0, 41.2, 47.5 and 57.7 mg Zn/kg diet for chymotrypsin, carboxypeptidases A and B as well as elastase, respectively. Below these thresholds, a reduction in enzyme activities occurred by 4.69 U/mg per min, 6.50 mU/mg per min, 0.04 U/mg per min and 1.97 U/mg per min per mg reduction in dietary Zn supply, respectively. In most cases, the estimated slopes in response over dietary Zn doses were significant ($P = 0.005$ for b_2 of trypsin, $P = 0.008$ for b_1 of carboxypeptidase A, $P = 0.006$ for b_1 of carboxypeptidase B, $P = 0.05$ and $P = 0.03$ for b_1 and b_2 of α-amylase, respectively) except for b_1 of trypsin, chymotrypsin and elastase, respectively. Estimated broken-line models for trypsin, carboxypeptidases A and B, elastase as well as α-amylase were of high precision ($R^2 = 0.90, 0.82, 0.63, 0.61, 0.77$, respectively) apart from chymotrypsin (R^2 0.35).

Comparing exocrine enzyme activities between animals with the 50% lowest pancreatic Zn contents and their counterparts with the 50% highest pancreatic Zn contents indicated a decline in activity with reductions in tissue Zn (164 (±93) versus 190 (±68) U/mg per min for trypsin, 687 (±155) versus 719 (±157) U/mg per min for chymotrypsin, 204 (±78) versus 226 (±76) mU/mg per min for carboxypeptidase A, 28 (±7) versus 32 (±5) U/mg per min for carboxypeptidase B, 284 (±26) versus 324 (±50) U/mg per min for elastase and 147 (±56) versus 186 (±67) mU/mg per min for α-amylase) (data are not further shown in a table). However, these results were only significant for carboxypeptidase B and α-amylase ($P = 0.04$ and $P = 0.03$, respectively).

Effects of the dietary treatment on measures of digestive capacity

Table 3 presents the results of one-way ANOVA and orthogonal contrasting of apparent faecal digestibility coefficients relative to changes in dietary Zn supply.

Except for CF (average percentage faecal digestibility: 66.9 (± 1.64)%, CF data are not further shown in a table), all coefficients of apparent faecal digestibility exhibited a significant relationship with the analysed dietary Zn. This was characterised by a decline in response with finely-graded reduction in alimentary Zn supply. However, reductions were only significant for the lowest supplied group (28.1mg Zn/kg diet) according to ANOVA and post hoc testing in all assessed digestibility coefficients except for CF ($P < 0.01$, $P = 0,01$, $P < 0.0001$ and $P < 0.01$ for DM, CP, TL and CA, respectively). Orthogonal contrasting revealed significantly lower faecal digestibility coefficients for DM, CP, TL and CA in the groups receiving <58 mg Zn/kg diet ($P < 0.001$, $P < 0.01$, $P < 0.001$ and $P < 0.001$, respectively). Again, CF digestibility was not affected in a significant manner.

Table 4 presents results on broken-line regression analysis of apparent faecal digestibility coefficients relative to dietary Zn supply. Except for CF digestibility (data not shown), all assessed parameters revealed significant thresholds in parameter response as indicated by highly significant parameter estimates of X and Y intercepts of the respective break points ($P < 0.0001$ for X and Y intercepts of DM, CP, TL and CA, respectively). Above respective dietary break points of 54.7, 45.0, 46.9 and 58.2 mg Zn/kg diet, DM, CP, TL and CA digestibility coefficients exhibited a plateau in response to dietary Zn supply. Below these break points, significant reductions in digestibility by 0.07, 0.18, 0.54 and 0.12%/mg reduction in dietary Zn were evident for DM, CP, TL and CA digestibility coefficients, respectively ($P = 0.003$, $P = 0.006$, $P = 0.001$ and $P = 0.002$, respectively). All broken-line models were of high precision as indicated by R^2 values of *0.88, 0.80, 0.90* and *0.74* for DM, CP, TL and CA, respectively. In the case of faecal CF digestibility, no significant regression model could have been estimated.

Table 3. Two-factorial ANOVA and orthogonal contrast of apparent faecal DM and crude nutrient digestibility to dietary zinc supply (Mean values and standard errors of the linear model).

										P	
Daily Zn uptake (mg/d)	12.7	15.1	17.5	19.2	21.3	26.2	30.5	39.6	SEM	ANOVA	[1]Orthogonal contrast
Analysed dietary Zn (mg/kg diet)	28.1	33.6	38.8	42.7	47.5	58.2	67.8	88.0			
DM (%)	84.6^{b}	85.6^{ab}	85.6^{ab}	85.6^{ab}	86.1^{a}	86.7^{a}	86.8^{a}	86.2^{a}	0.33	*<0.01*	*<0.001*
Crude protein (%)	81.9^{b}	84.3^{ab}	84.5^{ab}	84.8^{ab}	84.8^{ab}	85.8^{a}	86.3^{a}	85.3^{a}	0.69	*0.01*	*<0.01*
Total lipids (%)	64.0^{b}	70.8^{a}	71.5^{a}	72.5^{a}	75.6^{a}	74.2^{a}	76.7^{a}	76.4^{a}	1.54	*<0.0001*	*<0.001*
Crude ash (%)	44.1^{c}	44.9^{bc}	47.2^{abc}	46.4^{abc}	46.1^{abc}	48.4^{ab}	49.3^{a}	47.1^{abc}	0.81	*<0.01*	*<0.001*

Notes: [1]orthogonal contrast between groups of animals receiving <58 or ≥58 mg Zn/kg diet; ANOVA, analysis of variance; DM, dry matter; SEM, standard error of means which represents the pooled standard error of the linear model; Zn, zinc; coefficients of apparent faecal digestibility are expressed as % of feed intake; mean values not sharing a common superscript differ at $P \leq 0.05$.

Table 4. Broken-line regression analysis of apparent faecal DM and crude nutrient digestibility relative to dietary zinc supply (Parameter estimates with their standard errors).

	Models	Parameter estimates		SE	*P*	R^2
Dry matter (%)	$Y = 83.0 + b_1X$ *for* $X \leq X_B$	X_B	54.7	4.66	*<0.0001*	*0.88*
	$Y = Y_B$ *for* $X \geq X_B$	Y_B	86.6	0.13	*<0.0001*	
		b_1	0.07	0.02	*0.003*	
Crude protein (%)	$Y = 77.2 + b_1X$ *for* $X \leq X_B$	X_B	45.0	3.28	*<0.0001*	*0.80*
	$Y = Y_B$ *for* $X \geq X_B$	Y_B	85.6	0.28	*<0.0001*	
		b_1	0.18	0.05	*0.006*	
Total lipids (%)	$Y = 50.2 + b_1X$ *for* $X \leq X_B$	X_B	46.9	2.79	*<0.0001*	*0.90*
	$Y = Y_B$ *for* $X \geq X_B$	Y_B	75.7	0.62	*<0.0001*	
		b_1	0.54	0.11	*0.001*	
Crude ash (%)	$Y = 41.0 + b_1X$ *for* $X \leq X_B$	X_B	58.2	0.03	*<0.0001*	*0.74*
	$Y = Y_B$ *for* $X \geq X_B$	Y_B	48.2	0.44	*<0.0001*	
		b_1	0.12	0.03	*0.002*	

Notes: R^2, coefficient of determination of the respective broken-line regression model; X_B, X intercept of the respective breakpoint in parameter response; Y_B, Y intercept of the respective break point in parameter response; b_1, slope of the respective broken-line regression curve over dietary Zn doses lesser than or equal to the respective break point in parameter response; coefficients of apparent faecal digestibility are expressed as % of feed intake; $P \leq 0.05$ indicates statistical significance.

Discussion

Methodological explications

At present, there is a lack of comprehensive data sets regarding the effects of SZD on the organism, as appropriate experimental approaches to induce this phenotype are missing. Therefore, an experimental model was recently developed to induce finely-graded differences in Zn supply status of weaned piglets, ranging from deficient states to mild oversupply (28.1-88.0 mg Zn/kg diet) under practical feeding conditions (*Chapter 2, page 50-52*). The basis of this approach was the short experimental period of 8 d during which the organism was able to respond to the insufficient alimentary Zn supply without exceeding its capacities in terms of bone Zn mobilisation. This time frame has been chosen on the basis of earlier published data, which stated that the mammalian Zn homoeostasis needs between 3 and 5 d to adapt to changes in dietary Zn supply (16, 17). Otherwise, the time during which growing piglets under practical feeding conditions develop clinically manifest Zn deficiency is approximately 10-12 d from which on the first visible symptoms (for example reduced feed intake) are evident (18-20). Previously published data obtained under the present experimental conditions clearly indicate finely-graded differences in the examined Zn supply status parameters (for example bone Zn, blood plasma Zn parameters, etc.) without promotion of Zn-deficiency symptoms. This was evident by complete absence of any changes in zootechnical performance data of all experimental groups (*Chapter 2, page 65*). Moreover, the point of gross Zn requirement was recognised to lie at 58 mg Zn/kg diet as indicated by broken-line response analysis of, for example, apparent Zn digestion (*Chapter 2, page 67*). This value corresponds to the gross Zn requirement threshold of growing pigs under practical feeding conditions as stated by the National Research Council (50 mg Zn/kg diet) (8).

However, it might be questioned whether an experimental approach that used restrictive feeding is comparable with an *ad libitum* feeding situation. Obviously, too intense feed restrictions could cause a stronger depletion of body Zn over time, and hence might change measurements of the gross Zn requirement. However, under the present experimental conditions, the level of feed restriction was adapted on the basis of the average *ad libitum* feed intake of all animals through the last 3 d of the acclimatisation period (450 g diet/animal and day). Thereby, it was possible to adjust the amount of diet provided per animal and day very closely to the average amount consumed under non-restrictive conditions. The success of this approach was highlighted by the fact that all animals were able to fully exploit their potential in terms of growth development during the experimental phase. Another possible point of criticism refers to the fact that none of the administered Zn doses met the recommendations for piglets within the life weight range monitored in the present investigation (100 mg Zn/kg diet; (8)). In light of the considerably long acclimatisation phase of 2 weeks, one might argue that the control group (88 mg Zn/kg diet) was insufficiently supplied, and hence all the animals were challenged with alimentary Zn deficiency. However, it must be clearly differed between net/gross Zn requirements and feeding recommendations. The first represents the amounts of Zn that have to be present behind the gut barrier and within complete feed, respectively, to enable the animal maintenance of its developmental stage and biological performance (growth, lactation, reproduction, etc.). The latter is the net/gross Zn requirement extended by a safety margin, which, in case of weaned piglets, represents a surplus of approximately 67% of the gross Zn requirement. This is a practical tool for pig feeders in order to establish a dietary Zn content that is always high enough to fulfil the animal's Zn demand also in times of higher requirements (for example increased immune activity). The extent of the safety margins is a result of uncertainties regarding feed Zn bioavailability as data on the matter are scarce. Given the good biological

performance of all animals throughout the whole study and the response of Zn status parameters (especially the broken-line response of apparently-digested feed Zn), it leads to the conclusion that five groups received insufficient alimentary supply (28.1–47.5 mg Zn/kg diet), and hence expressed finely-graded differences in the development of SZD. In contrast, the groups receiving ≥58 mg Zn/kg diet were considered as sufficiently Zn supplied.

Therefore, it is now possible to investigate the early metabolic shifts before the development of clinically manifest Zn deficiency during which the organism is still capable of compensating deficiency symptoms by mobilisation of body Zn stores. In a first attempt, the early effects of varying alimentary Zn supply on the exocrine pancreas and related measures of digestive capacity have been studied.

Effects of the dietary treatment on exocrine pancreatic activity

Exocrine proteases are present within the pancreatic tissue as inactive forms (so-called zymogens) to prevent the tissue from uncontrolled self-digestion (2). In order to assess the activities of these enzymes within a pancreatic tissue lysate, *in vitro* activation of zymogens had to be conducted before the enzymatic assay. The activity of digestive enzymes (activated zymogens as well as α-amylase) within tissue homogenates should serve as an indicator of the amount of enzymes within the pancreas. In the present study, a decline in the respective enzyme activities within tissue homogenates was evident in response to changes in dietary Zn supply. These findings are in line with earlier published data (21-26). Therefore, it can be proposed that one of the first pancreatic responses during the early states in the development of Zn deficiency is an increased degradation of exocrine enzymes and maybe enzyme-containing granules themselves, in order to decline zymogen secretion to reduce endogenous Zn losses. It

has yet to be evaluated whether this is accompanied by reduced transcription and/or translation of exocrine enzyme mRNA.

All assessed enzyme activities followed a broken line over dietary treatment groups, with significant break points in response between 41.2 and 58.2 mg Zn/kg diet, below which their activities declined in a linear fashion. This is in line with earlier findings under the present experimental conditions regarding apparent Zn digestion, liver Zn as well as hepatic metallothionein gene expression (*Chapter 2, pages 66-67*). On the basis of these data, the point of gross Zn requirement was estimated to lie at 58 mg Zn/kg diet. This corresponds to the threshold published by the National Research Council (8). Therefore, the assessed response patterns of enzyme activities indicate a relationship between the amount of pancreatic zymogens as well as α-amylase and the Zn supply status of the organism. In summary, a reduction in dietary Zn supply below the point of gross Zn requirement impaired pancreatic zymogen and α-amylase activity. Furthermore, this is to our knowledge the first report of a Zn-dependent regulation of pancreatic elastase activity.

Response of the analysed pancreatic zinc content and its potential role as a mediator of exocrine pancreatic activity

Regarding the response of exocrine enzyme activity over the range of dietary treatment groups, an involvement of Zn homoeostatic regulation would represent a possible explanation for the basic mode of action. Indeed, in the present study, a reduction in analysed dietary Zn supply caused a decline in the response of pancreatic Zn. Furthermore, based on the broken-line analysis, a replenishment of tissue Zn stores in groups fed dietary doses < 39.9mg Zn/kg diet was evident. Interestingly, comparable response patterns regarding the analysed Zn contents of other tissues (heart, muscle, lymph nodes, thymus) were observed within the same experiment (*Chapter 4, page 132-*

133, Chapter 5, page 156-157). Furthermore, cardiac stress metabolism was investigated, and a decrease in antioxidative capacity accompanied by an increase in stress-responsive proapoptotic gene expression became evident (*Chapter 4, pages 128-131*). Therefore, replenishment of certain tissue Zn stores at the expense of body Zn stores in the lowest supplied groups might represent a compensation mechanism to counteract increased stress levels in order to maintain tissue integrity. Indeed, whether comparable adaptions with regard to pancreatic stress metabolism are evident has to be investigated in follow-up studies. In order to prove whether decreased pancreatic Zn contents had an effect on the above-discussed measures of exocrine pancreatic enzyme activity, potential differences in response of enzyme activity between samples in the range of 50% higher and 50% lower pancreatic Zn contents have been investigated. On the basis of these secondary statistical analyses, a decrease in pancreatic Zn load reduced pancreatic enzyme activities. However, considerable variation within the data set was evident by the fact that only a few enzyme responses to changes in pancreatic Zn turned out to be statistically significant (carboxypeptidase B, α-amylase). This might indicate that there was some interference with other regulative stimuli. It has been shown that the small intestine communicates with the exocrine pancreas via a cholecystokinin-dependent signalling in order to increase its synthesis and secretory activity in times of reduced digestive capacity – for example, in the presence of enzyme inhibitors within the GIT (27). Presumably, there is a conflict of interest between Zn homoeostasis-dependent suppression and cholecystokinin-dependent stimulation of exocrine pancreatic activity. These potential interconnections should be addressed in appropriate follow-up studies.

Effects of the dietary treatment and exocrine pancreatic activity on measures of digestive capacity

The response of coefficients of apparent faecal digestion of DM, CP, TL and CA followed a broken line over the whole range of analysed dietary Zn doses, which is in good context to the response of pancreatic exocrine enzyme activity. Again, clear break points in response ranging between 47 and 58 mg Zn/kg diet were evident. This further confirms clear differences in the response of animals fed sufficient amounts of dietary Zn (≥ 58mg Zn/kg diet) and insufficiently supplemented diets (< 58mg Zn/kg diet). Moreover, these findings are in concordance with earlier published results, which suggest a decrease in apparent feed digestibility under the terms of clinically manifest Zn deficiency (6, 7). The clear relationship with certain pancreatic enzyme activities suggests a loss in luminal catalytic activity as a major cause of increased faecal DM and crude nutrient losses under the terms of short-term SZD. The efficiency by which the feed matrix is digested within the intestinal lumen determines the amount of soluble and hence absorbable nutrients at the gut barrier. Therefore, impairment of digestive capacity would foster a translocation of indigested feed components to lower intestinal segments. Indeed, concerning the TL digestibility, the activity of pancreatic lipase has not been assayed in the present study, as it was not possible to establish a suitable method in an appropriate amount of time at the laboratory responsible for these analyses. However, it has been shown that clinically manifest Zn deficiency can affect pancreatic lipase activity (28). Therefore, in light of our TL digestibility data, it seems plausible that pancreatic lipase was affected by the treatment similar to all other assessed enzymes.

The slope in response of TL digestibility to varying dietary Zn supply was approximately 3fold higher compared with CP digestibility. This might be interpreted as an effect of Zn metabolism being stronger in case of fat compared with protein.

However, apparent faecal digestibility reflects digestion of dietary protein only in part as most of faecal CP is of microbial origin (29). A higher influx of fermentable substrates into the hindgut promotes microbial growth, and hence reduces apparent faecal digestibility of CP (30). Reduction in faecal coefficients of CP digestibility is therefore indicative of impairment of precaecal nutrient digestion in general, including dietary protein and carbohydrates. Therefore, quantifying the effects of Zn metabolism on digestive capacity requires additional measurements such as pancreatic lipase activity, bile secretion, and, most importantly, *in vivo* experiments addressing prececal (=ileal) digestion.

The reductions in faecal digestibility coefficients, although statistically significant, are minor in terms of absolute numbers, and might question the biological significance of the data. However, it should not be forgotten that these data were evaluated in animals that were subclinically Zn deficient compared to animals with satisfied Zn demands. This means that we compared differences in Zn status and related metabolic response between healthy animals, as subclinical states are defined by a total absence of pathological symptoms. Therefore, we did not expect to see drastic differences in response of certain biological functions. The monitored shifts in digestive capacity represent early adaptations that contribute to the development of clinical deficiency on a mid-term scale. Indeed, based on earlier published data (18-20) we would expect the assessed differences to increase in the course of Zn deficiency and to promote the development of digestive depression.

Clinically manifest Zn deficiency has been associated with anorexia and decreased growth. The first intention would be to speculate that feed refusal promotes the reduction in growth development of Zn-deficient animals. Potential connections between Zn homoeostasis and leptin signalling, vagus nerve stimulation as well as

reduced ghrelin synthesis/secretion have been discussed as possible modes of action in order to explain the anorexia (31-33). However, some authors suggest the growth depression occurs earlier than the reduction in feed consumption (7, 34). This indicates both symptoms develop to some extent independent of each other in the time course of clinical Zn deficiency. In the present study, declines in digestive capacity were evident before the onset of Zn deficiency symptoms. A decrease in digestive efficiency may lead to an enrichment of indigested feed within the GIT in the course of Zn deficiency, thereby establishing a higher filling level and associated response of mechanoreceptors of the gut. This may be another plausible explanation for the repeatedly reported Zn deficiency-associated anorexia. However, as we neither assessed the filling level of the GIT in the present study nor the response of gastrointestinal mechanoreceptors, this hypothesis has to be proven in further studies.

Conclusion

Significant reductions in exocrine pancreatic enzyme activities under the terms of SZD have been recognised in weaned piglets. Remarkably, these shifts were evident after just 8 d of insufficient Zn supply under practical feeding conditions. These effects may be related to pancreatic Zn metabolism as analysed pancreatic Zn contents were also affected by the treatment, and the activities of carboxypeptidase B and α-amylase exhibited a significant reduction in animals with the 50% lowest pancreatic Zn contents. Furthermore, the reduction in exocrine pancreatic enzyme activities was accompanied by a decrease in faecal digestibility coefficients of DM, CP, TL and CA, indicating a direct impairment of feed digestion under the terms of SZD. The practical consequence of the present study is that even short periods of insufficient alimentary Zn supply have to be urgently avoided in order to maintain digestive function.

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4th Chapter

Short-term subclinical zinc deficiency in weaned piglets affects cardiac redox metabolism and zinc concentration

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Abstract

Subclinical zinc deficiency (SZD) represents the common zinc (Zn) malnutrition phenotype. However, its association with oxidative stress is not well understood. The heart muscle may be a promising target for studying early changes in redox metabolism.

We investigated the effects of short-term SZD on cardiac redox metabolism in weaned piglets. Forty-eight weaned German Large White x Landrace x Piétrain piglets (50% castrated males and 50% females; body weight of 8.5 kg) were fed diets with different Zn concentrations for 8 d. Measurements included cardiac parameters of antioxidative capacity, stress-associated gene expression, and tissue Zn status. Analyses comprised (linear, broken-line) regression models and Pearson correlation coefficients.

Glutathione and α-tocopherol concentrations as well as catalase, glutathione reductase, B-cell lymphoma 2–associated X protein, and caspase 9 gene expression plateaued in response to reduction in dietary Zn from 88.0 to 57.6, 36.0, 36.5, 41.3, 55.3, and 33.8 mg/kg, respectively ($P < 0.0001$). Further reduction in dietary Zn promoted a linear decrease of glutathione and α-tocopherol (30 and 0.6 nmol/mg dietary Zn, respectively; $P < 0.05$) and a linear increase of gene expression (0.02, 0.01, 0.003, and 0.02 $Log_{10}(2^{-\Delta\Delta Ct})$/mg dietary Zn, respectively; $P < 0.05$)). Tissue zinc declined linearly with reduction in dietary Zn (0.21 mg tissue Zn/mg dietary Zn; $P = 0.004$) from 88.0 to 42.7 mg/kg ($P < 0.0001$), below which it linearly increased inversely to further reduction in dietary Zn (0.57 mg tissue Zn/mg dietary Zn; $P = 0.006$). H_2O_2-detoxification activity and metallothionein 1A gene expression decreased linearly with reduction in dietary Zn from 88.0 to 28.1 mg/kg (0.02 mU and 0.008 $Log_{10}(2^{-\Delta\Delta Ct})$/mg dietary Zn, respectively; $P < 0.05$). Fas cell-surface death receptor, etoposide-induced 2.4 and cyclin-dependent kinase inhibitor 1A gene expression correlated positively to cardiac Zn in piglets fed ≤ 42.7 mg Zn/kg ($r \geq 0.97$; $P < 0.05$).

Short-term SZD decreased cardiac antioxidative capacity of weaned piglets while simultaneously increasing stress-associated gene expression and zinc concentration. This is the first report to our knowledge on the effects of SZD on redox metabolism.

Introduction

Apart from studies that have used cell cultures (1, 2), oxidative damage has been reported in response to insufficient dietary zinc (Zn) supply *in vivo* (3, 4). Most studies thus far have investigated the relation between Zn homeostasis and redox metabolism during clinical Zn deficiency. However, this condition is associated with secondary metabolic imbalance, which impairs the resolution of measurements. This complicates identifying the effects that result directly from Zn homeostatic regulation. Therefore, the basis for the association of Zn deficiency with oxidative stress is currently not well understood. The most common phenotype of Zn malnutrition in humans and animals is presumably a subclinical Zn deficiency (SZD) (5). SZD is characterised by a total absence of clinical symptoms (e.g., growth retardation, anorexia, tissue necrosis, (6)) and, at the same time, a decreased Zn status. We have recently published an experimental model of short-term SZD in weaned piglets (*Chapter 2, page 45*), thus giving us an opportunity to investigate the potential relation between the Zn supply status and redox metabolism with high resolution.

Because of its high metabolic turnover, cardiac tissue has the highest production of reactive oxygen species (ROS) per gram of tissue. At the same time, it has a lower antioxidative capacity than other organs. Hence, it is quite susceptible to imbalances in redox metabolism (7). This is especially true for growing individuals, who have considerably higher heart rates than adults (8). Therefore, early events in the development of Zn deficiency–dependent oxidative stress may be captured in the heart muscles of growing individuals.

We aimed to provide the first data to our knowledge on the adaption of cardiac redox metabolism to short-term SZD in weaned piglets. We hypothesised that this condition is associated with a decrease in cardiac antioxidative capacity and changes in stress-

associated cardiac gene expression that may be accompanied by a decrease in the Zn status of the heart muscle.

Material and Methods

This study was registered and approved by the responsible animal welfare authorities (Federal State of Bavaria: case number 55.2.1.54-2532.3.63-11).

Animals and Diets

Forty-eight weaned German Large White x German Landrace x Piétrain piglets from 6 litters (50% castrated males and 50% females) aged 4 wk and with an initial mean ± SD body weight of 8.5 ± 0.27 kg were purchased from Christian Hilgers (Upper Bavaria). The piglets were fed a basal diet *ad libitum* that consisted mainly of corn and soybean meal with adequate dietary Zn supplementation as analytical grade $ZnSO_4$ * $7H_2O$ (analysed final concentration of dietary Zn: 88.0 mg/kg) (Merck KGaA) within a 2-week acclimatisation phase. The basal diet contained all nutrients according to published piglet requirements (9). Table 1 within *Chapter 2, page 52*, presents detailed information on the composition and ingredients of the basal diet.

The piglets were assigned to 8 feeding groups in a completely randomised block design considering a balanced distribution of life weight, litter, and sex ($n = 6$/group). During a total experimental period of 8 d, all piglets were fed restrictively (450 g/d) the same diet as during the acclimatisation phase but with a varying supplementation of $ZnSO_4$ * $7H_2O$ (analysed final concentrations of dietary Zn: 28.1, 33.6, 38.8, 42.7, 47.5, 58.2, 67.8, and 88.0 mg/kg). Table 2 within *Chapter 2, page 52*, presents detailed information on the analysed dietary Zn concentrations as affected by varying Zn supplementation to the basal diet. The group that was supplied 88.0 mg/kg served as the control because it represented the initial feeding situation during acclimatisation. As reported in *Chapter 2, page 52*, one-way ANOVA combined with the Student-Newman-Keuls Test (with diet as the independent variable) identified no significant differences between

experimental and control diets except for the analysed dietary Zn concentration, which differed by $P < 0.0001$. These analyses were performed based on 3 separate samples/batch (diet) that were screened independently (duplicate weighing) for crude nutrient and trace metal (iron, zinc, copper, manganese) concentrations.

Sampling conditions

Diet samples were stored in airtight polyethylene bottles at -20°C and milled through a 0.5 mm screen before chemical analysis. All animals were killed by exsanguination under anaesthesia (azaperone and ketamine) without fasting after 8 experimental days, and the heart muscle was removed. Samples for chemical analyses were snap-frozen in liquid nitrogen and stored at -80°C. Samples for the gene expression analysis were incubated in RNAlater (Thermo Scientific) according to the manufacturer´s instructions and stored at -80°C.

Analyses of dry matter and total Zn in diets and cardiac tissue

Details on the dry matter and Zn analyses in diets and soft tissue have been already presented in *Chapter 2, pages 53-54*. Zn concentrations were measured by atomic absorption spectrometry (NovAA 350; Analytik Jena AG) after microwave wet digestion (Ethos 1; MLS GmbH).

Analyses of cardiac concentrations of glutathione and tocopherols

Glutathione and tocopherols were assayed with the use of HPLC on a VWR International Hitachi LaChrom Elite system. Tissues were homogenised before extraction (25 and 50 mg fresh weight/sample for the extraction of glutathione and tocopherols, respectively) with the FastPrep system (MP Biomedicals).

Cardiac concentrations of reduced glutathione (GSH) and oxidised glutathione (GSSG) were analysed according to Asensi, et al. (10) with the use of a Spherisorb NH2 column (i.d., 4.6 mm; o.d., 250 mm; particle size, 5 mm) (Waters). External (L-glutathione–reduced and L-glutathione–oxidised) (Sigma-Aldrich) and internal (γ-L-glutamyl-L-glutamic acid (Acros Organics) standards were used for quantification.

Cardiac concentrations of α-tocopherol and γ-tocopherol were assayed according to Balz, et al. (11) with the use of a LiChrosorb silica 60 column (i.d., 4 mm; o.d., 250 mm; particle size, 5 mm) and a 4 * 4 silica 60 precolumn (Merck kGaA). External standards (α- and γ-tocopherol) (Sigma-Aldrich) were used for quantification.

Cardiac H_2O_2-detoxification activity

Protein concentration in homogenised tissues was determined as described previously (*Chapter 3, page 91*). The total protein concentration was used to normalise the H_2O_2-detoxification activity (HDA). Cardiac HDA was quantified with the use of the catalase activity assay from Beers and Sizer (12). Quartz cuvettes were applied with a total reaction volume of 3 mL that consisted of 100 mL diluted tissue homogenate, 1.9 mL ultrapure water (type 1), and 1 mL 30% H_2O_2 with 0.05 M K_2HPO_4 on the UV MC^2 system (SAFAS Scientific Instruments). The HDA was calculated as follows:

$$HDA\ (U/mg) = \frac{\Delta A^{240\ nm/min} * 1000}{43.6 * TP}$$

where $\Delta A^{240\ nm/min}$ is the absorption change per minute at 240 nm, 43.6 is the extinction coefficient of H_2O_2 at 240 nm, TP is the total protein (expressed as mg/mL) in the reaction mixture, and U/mg is the per-minute reduction of H_2O_2 normalised to the total protein concentration in the reaction mixture (1 U ~ reduction of 1 µmol H_2O_2). Catalases are not the only H_2O_2-detoxifying factors within mammal tissue. Therefore, we refer to this measure as HDA rather than catalase activity.

Gene expression analysis

Primer design, assay quality control, and chemical procedures (total RNA extraction, RT-qPCR) were carried out as presented in *Chapter 2, pages 54-57*. The quantity and purity of the extracts was measured on a NanoDrop 2000 (Thermo Scientific) (total RNA quantity: 900 ± 91.1 ng/mL; $OD^{260\ nm}$:$OD^{280\ nm}$ ratio: 2.13 ± 0.09; $OD^{260\ nm}$:$OD^{230\ nm}$ ratio: 2.17 ± 0.07). Total RNA integrity was assayed with the use of automated capillary gel electrophoresis (Experion; Biorad) (RNA quality index: 6.60 ± 0.32). Primer pairs (Eurofins Scientific) were designed for the potential reference transcripts glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-glucuronidase (GUSB), ribosomal protein subunit 18 (RPS18), and β-actin (ACTB) as well as the target transcripts metallothionein 1A (MT1A), metallothionein 2B (MT2B), superoxide dismutase (SOD) 1, SOD2, glutathione peroxidase 1 (GPX1), peroxiredoxin (PRDX) 1, PRDX3, PRDX4, glutathione reductase (GSR), catalase (CAT), tumour protein p53 (TP53), Fas cell-surface death receptor (FAS), B-cell lymphoma 2–associated X protein (BAX), etoposide-induced 2.4 (EI24), shisa family member 5 (SHISA5), insulin-like growth factor-binding protein 3 (IGFBP3), caspase (CASP) 3, CASP8, CASP9, cyclin-dependent kinase inhibitor 1A (CDKN1A), growth arrest and DNA damage–inducible α (GADD45A), and stratifin (SFN) (Supplementary Tables 1 and 2) with the use of published porcine sequence information (13). All oligonucleotides bind to homologous regions of respective transcripts to amplify potential variants. We identified GAPDH, TP53, and SHISA5 as suitable reference genes for data normalisation (14). The $2^{-\Delta\Delta Ct}$ method (15) was used to normalise the gene expression data because the determination of the amplification efficiency revealed comparable values between 95% and 100% of applied RT-qPCR assays (for details on the methodology see *Chapter 2, page 56-57*).

Statistical analyses

Data were analysed with the use of SAS 9.4 (SAS Institute Inc.). Linear and broken-line regression models were calculated based on independent group means relative to dietary Zn concentration ($n = 8$). The significant regression models with the highest coefficients of determination (R^2) were used for presenting and interpreting data. Furthermore, Pearson correlation coefficients (r) were estimated for all measured parameters and gene expression patterns relative to changes in cardiac Zn concentration. All $2^{-\Delta\Delta Ct}$ gene expression data were log-transformed (decadal logarithm) before statistical analysis and data presentation. $P \leq 0.05$ was considered to be significant.

Results

No piglets showed signs of clinical Zn deficiency (e.g., growth depression, anorexia, impaired organ development, tissue necrosis, etc.) (6) throughout the entire trial (*Chapter 2, page 65*).

Response of parameters of cardiac antioxidative capacity to changes in dietary zinc concentration

Linear and broken-line models were used to analyse the response of cardiac antioxidative capacity to declining dietary Zn concentration. Statistical measures of the respective regression curves are shown in Figure 1 and Table 1.

Cardiac GSH concentration did not change with a reduction in dietary Zn concentration from 88.0 mg Zn/kg to a significant breakpoint of 57.6 mg Zn/kg ($P < 0.0001$). Below this threshold, it linearly decreased with further declining dietary Zn ($P = 0.004$). Cardiac GSSG and the GSH:GSSG ratio did not change with a reduction in dietary Zn concentration ($P = 0.42$ and 0.25, respectively; data not shown).

Cardiac α-tocopherol concentration remained stable between 36.0 and 88.0 mg Zn/kg. A further reduction of the dietary Zn concentration below the significant threshold of 36.0 mg/kg ($P < 0.0001$) promoted a linear decrease of α-tocopherol concentration ($P = 0.03$). Cardiac γ-tocopherol concentrations fell below the lower limit of detection in all analysed samples (0.2 nmol/mg tissue dry matter). Cardiac HDA linearly declined with the reduction in dietary Zn concentration over the entire range of applied Zn doses ($P = 0.007$).

Response of cardiac proapoptotic and antioxidative gene expression to changes in dietary zinc concentration

Figure 2 presents the broken-line regression curves that highlight the response of cardiac proapoptotic and antioxidative gene expression to changes in dietary Zn concentration. The statistical measures of the respective regression curves are shown in Table 2.

Relative BAX gene expression remained stable with declining dietary Zn concentration until a significant threshold of 55.3 mg Zn/kg ($P = 0.001$), below which it linearly increased inversely to a further reduction in dietary Zn. However, this slope was not significant ($P = 0.1$). It is noteworthy that the linear regression analysis yielded a significant linear increase inversely to changes in dietary Zn concentration from 88.0 to 28.1 mg/kg (0.002 $Log_{10}(2^{-\Delta\Delta Ct})$/mg dietary Zn; $P \leq 0.05$; data not shown). Cardiac CASP9 gene expression remained unaffected between 33.8 and 88.0 mg Zn/kg. Below the significant breakpoint of 33.8 mg Zn/kg ($P < 0.0001$), CASP9 expression linearly increased inversely to a further reduction in dietary Zn concentration ($P = 0.009$).

Cardiac CAT gene expression remained stable in response to declining dietary Zn concentration until a significant breakpoint of 36.5 mg Zn/kg ($P < 0.0001$), below which a linear upregulation occurred inversely to further declining dietary Zn ($P = 0.04$).

Cardiac GSR gene expression exhibited a response pattern similar to that of cardiac CAT. The significant breakpoint of the regression curve was 41.3 mg Zn/kg ($P < 0.0001$). Below this threshold, expression linearly increased inversely to the declining dietary Zn concentration ($P = 0.007$).

The remaining cardiac genes involved in antioxidative defence and apoptosis (CASP3, CASP8, FAS, GADD45A, GPX1, IGFBP3, CDKN1A, EI24, PRDX1, PRDX3,

PRDX4, SOD1, SOD2) were not significantly affected by the dietary Zn concentration ($P > 0.8$ in all cases; data not shown).

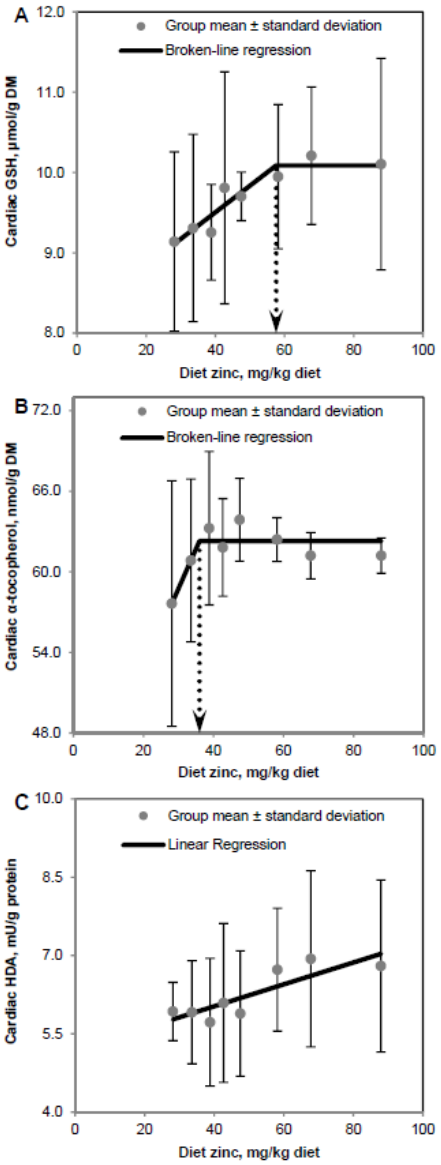

Figure 1. Response of cardiac GSH (A), α-tocopherol (B), and HDA (C) in weaned piglets fed diets with different zinc concentrations for 8 d (see Table 1 for detailed information on the statistical measures of the respective regression models). Notes: Values are arithmetic means ± SDs, $n = 6$. Diet zinc, dietary zinc; d, days; DM, dry matter; GSH, reduced glutathione; HDA, H_2O_2-detoxification activity (U ~ µmol H_2O_2) of per-minute activity change per milligram of total protein within the reaction mixture.

Table 1. Linear and broken-line regression analyses of the response of cardiac GSH, α-tocopherol and HDA in weaned piglets fed diets with different zinc concentrations for 8 d[1].

	Regression model	Parameter estimates	*P* values	R^2
GSH, µmol/g DM	$y = 8.20 + b_1x$ *for* $x \leq X_B$	X_B 57.6 ± 5.60	*< 0.0001*	*0.89*
	$y = Y_B$ *for* $x > X_B$	Y_B 10.1 ± 0.07	*< 0.0001*	
		b_1 0.03 ± 0.008	*0.004*	
α-Tocopherol, nmol/g DM	$y = 41.1 + b_1x$ *for* $x \leq X_B$	X_B 36.0 ± 2.30	*< 0.0001*	*0.76*
	$y = Y_B$ *for* $x > X_B$	Y_B 62.3 ± 0.30	*< 0.0001*	
		b_1 0.60 ±0.20	*0.03*	
HDA, mU/mg protein	$y = 5.20 + bx$	b 0.02 ± 0.005	*0.007*	*0.73*

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. Linear and 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 ≤ 0.05* was considered to be significant. b, slope of the parameter response of the linear regression curves; b_1, slope of the broken-line regression curves over dietary zinc doses ≤X_B; d, days; DM, dry matter; GSH, reduced glutathione; HDA, H_2O_2-detoxification activity (U ~ µmol H_2O_2) of per-minute activity change per milligram of total protein within the reaction mixture; X_B, X intercept of the breakpoint in parameter response; Y_B, Y intercept of the breakpoint in parameter response.

Response of measures of cardiac zinc status to changes in dietary zinc concentration

Figure 3 shows the broken-line and linear regression models that describe the response of measures of cardiac Zn status to changes in dietary Zn concentration. The statistical measures of the respective regression curves are shown in Table 3.

Declining dietary Zn concentration was directly associated with cardiac Zn concentration (*P = 0.006*) in piglets fed Zn doses above a significant breakpoint of 42.7 mg Zn/kg (*P < 0.0001*). Below this threshold, cardiac Zn concentration was inversely associated with the dietary Zn concentration (*P = 0.04*).

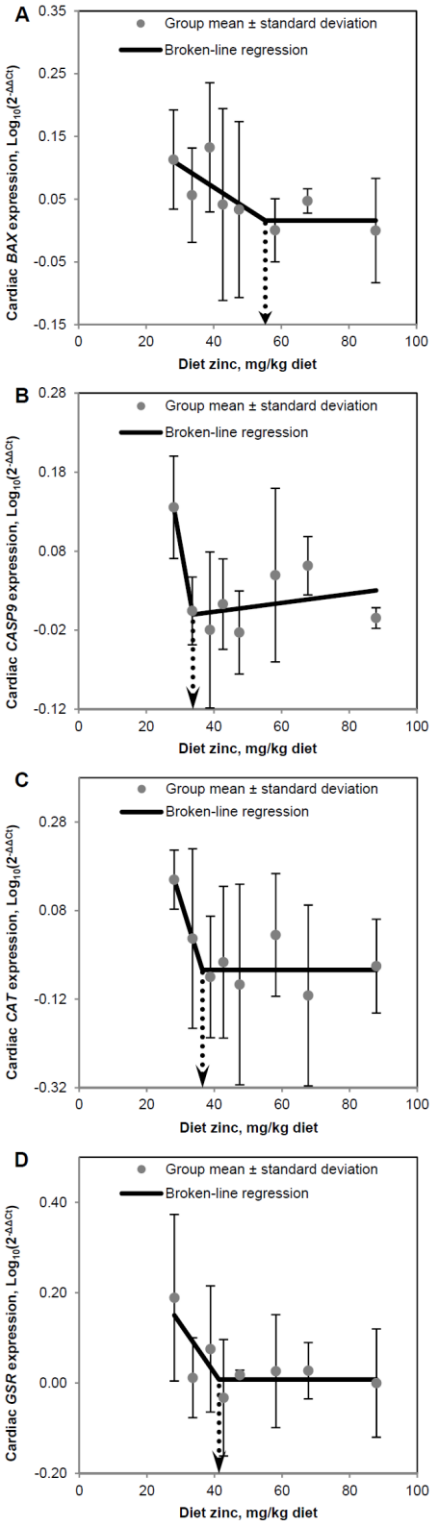

Figure 2. Response of relative cardiac expression of BAX (A), CASP9 (B), CAT (C), and GSR (D) in weaned piglets fed diets with different zinc concentrations for 8 d (see Table 2 for detailed information on the statistical analysis of the respective regression models). Notes: Values are arithmetic means ± SDs, *n* = *6*. BAX, B-cell lymphoma 2–associated X protein; CASP9, caspase 9; CAT, catalase; d, days; Diet zinc, dietary zinc; GSR, glutathione reductase; $Log_{10}(2^{-\Delta\Delta Ct})$, decadal logarithm of the relative gene expression value according to Livak and Schmittgen (15).

Table 2. Broken-line regression analyses of the relative cardiac expression response of BAX, CASP9, CAT, and GSR in weaned piglets fed diets with different zinc concentrations for 8 d[1].

	Regression models	Parameter estimates	*P* values	R^2
BAX, $Log_{10}(2^{-\Delta\Delta Ct})$	$y = 0.20 + b_1x$ for $x \leq X_B$	X_B 55.3 ± 5.00	*0.001*	*0.59*
	$y = Y_B$ for $x > X_B$	Y_B 0.02 ± 0.02	*0.37*	
		b_1 -0.004 ± 0.002	*0.10*	
CASP9, $Log_{10}(2^{-\Delta\Delta Ct})$	$y = 0.80 + b_1x$ for $x \leq X_B$	X_B 33.8 ± 1.40	*<0.0001*	*0.71*
	$y = -0.02 + b_2x$ for $x > X_B$	Y_B -0.0002 ± 0.02	*0.99*	
		b_1 -0.02 ± 0.007	*0.009*	
		b_2 0.0006 ± 0.0007	*0.42*	
CAT, $Log_{10}(2^{-\Delta\Delta Ct})$	$y = 0.80 + b_1x$ for $x \leq X_B$	X_B 36.5 ± 2.60	*<0.0001*	*0.73*
	$y = Y_B$ for $x > X_B$	Y_B -0.05 ± 0.02	*0.008*	
		b_1 -0.02 ± 0.01	*0.04*	
GSR, $Log_{10}(2^{-\Delta\Delta Ct})$	$y = 0.50 + b_1x$ for $x \leq X_B$	X_B 41.3 ± 4.60	*<0.0001*	*0.62*
	$y = Y_B$ for $x > X_B$	Y_B 0.008 ± 0.02	*0.66*	
		b_1 -0.01 ± 0.005	*0.007*	

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. Linear and 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 ≤ 0.05* was considered to be significant. BAX, B-cell lymphoma 2–associated X protein; 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; CASP9, caspase 9; CAT, catalase; d, days; GSR, glutathione reductase; $Log_{10}(2^{-\Delta\Delta Ct})$, decimal logarithm of the relative gene expression value according to Livak and Schmittgen (15); X_B, X intercept of the breakpoint in the parameter response; Y_B, Y intercept of the breakpoint in the parameter response.

Cardiac MT1A gene expression linearly decreased with the dietary Zn concentration between 28.1 and 88.0 mg Zn/kg diet (*P = 0.02*). Cardiac MT2B gene expression was not affected by dietary Zn (*P = 0.29*; data not shown). It is noteworthy that plasma Zn concentrations were directly associated with the dietary Zn concentration between 28.1 and 88.0 mg Zn/kg (*P < 0.0001*) (data presented and discussed in *Chapter 2, pages 65, 70-71*).

Response of heart antioxidative capacity and target gene expression to changes in the tissue zinc concentration

Cardiac FAS, EI24, and CDKN1A gene expression exhibited a significant positive correlation to cardiac Zn concentration in groups fed ≤42.7 mg Zn/kg (*r = 0.97*, *0.99*, and *0.99* and *P = 0.03*, *0.01*, and *0.007*, respectively, with 42.7 mg Zn/kg representing

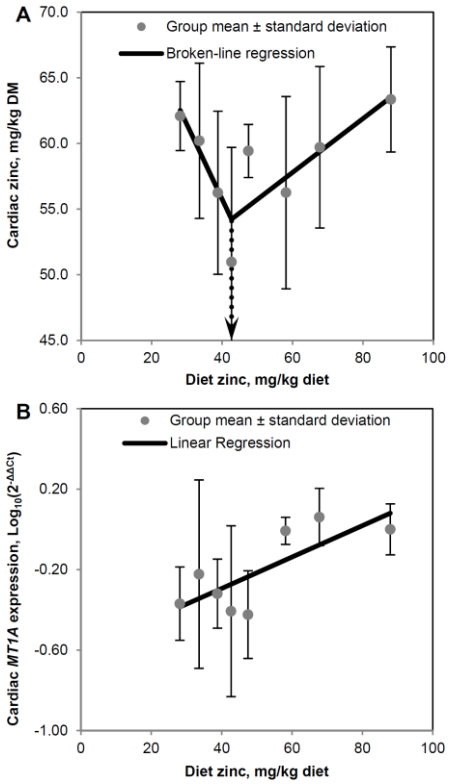

Figure 3. Response of cardiac zinc concentration (A) and relative cardiac expression of MT1A (B) in weaned piglets fed diets with different zinc concentrations for 8 d (see Table 3 for the statistical measures of the respective regression models). Notes: Values are arithmetic means ± SDs, *n* = 6. d, days; Diet zinc, dietary zinc; DM, dry matter; $Log_{10}(2^{-\Delta\Delta Ct})$, decadal logarithm of the relative gene expression value according to Livak and Schmittgen (15); MT1A, metallothionein 1a.

the approximate breakpoint in the response of cardiac Zn concentration to changes in the dietary Zn concentration). There was no significant correlation evident in groups fed >42.7 mg Zn/kg (*r* = *-0.44*, *-0.92*, and *-0.88* with *P* = *0.56*, *0.08*, and *0.12*, respectively) or when estimated over all feeding groups (*r* = *0.06*, *0.55*, and *0.45* with *P* = *0.46*, *0.16*, and *0.27*, respectively).

There were no significant associations between the expression of other cardiac genes (BAX, CASP3, CASP8, CASP9, GADD45A, GPX1, IGFBP3, PRDX1, PRDX3, PRDX4, SOD1, SOD2, CAT, GSR, MT1A, MT2B) or cardiac antioxidative capacity (GSH, GSSG, GSH:GSSG, HDA, α-tocopherol), respectively, and the cardiac Zn concentration (*P* > *0.8* in all cases; data not shown).

Table 3. Linear and broken-line regression analyses of the response of cardiac zinc concentration and relative cardiac expression of *MT1A* in weaned piglets fed diets with different zinc concentrations for 8 d[1].

	Regression models	Parameter estimates	*P* values	R^2
Cardiac zinc, mg/kg DM	$y = 78.5 + b_1x$ for $x \leq X_B$	X_B 42.7 ± 0.006	*<0.0001*	*0.72*
	$y = 45.5 + b_2x$ for $x > X_B$	Y_B 54.2 ± 1.2	*<0.0001*	
		b_1 -0.60 ± 0.2	*0.006*	
		b_2 0.20 ± 0.05	*0.004*	
MT1A, $Log_{10}(2^{-\Delta\Delta Ct})$	$y = -0.60 + bx$	b 0.008 ± 0.003	*0.02*	*0.60*

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. Linear and 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 ≤ 0.05* was considered to be significant. b, slope of the parameter response of the linear regression curve; b_1, slope of the broken-line regression curve over dietary zinc doses ≤XB; b_2, slope of the broken-line regression curve over dietary zinc doses >X_B; d, days; DM, dry matter; $Log_{10}(2^{-\Delta\Delta Ct})$, decimal logarithm of the relative gene expression value according to Livak and Schmittgen (15); MT1A, metallothionein 1A; X_B, X intercept of the breakpoint in the parameter response; Y_B, Y intercept of the breakpoint in the parameter response.

Discussion

We studied short-term differences in dietary Zn concentrations above and below the estimated gross Zn requirement for piglets (*Chapter 2, page 67*). Because the cardiac GSSG concentrations did not substantially change during this study, the reduction in cardiac GSH may have resulted from reduced synthesis or increased degradation rather than increased oxidation. Furthermore, the breakpoint in the response of GSH to dietary Zn corresponds to the point of the satisfied gross Zn requirement (58.0 mg Zn/kg). Therefore, we conclude that GSH was affected in a Zn-supply status–dependent manner. Considering the response patterns of cardiac HDA and α-tocopherol, our data suggests a decrease in the heart antioxidative capacity during short-term SZD.

However, decreasing tissue α-tocopherol concentrations may not necessarily reflect increased oxidation but also reduced vitamin E uptake, although the supplementation of RRR-α-tocopherol equivalents occurred equally and in adequate amounts to each experimental and control ration. Furthermore, the homogeneity of diets was excellent, as has been shown earlier (*Chapter 2, page 52*). Indeed, we previously recognised a decrease in apparent lipid digestibility, which affects fat-soluble vitamin absorption from the gastrointestinal tract. However, the significant breakpoint in the response of apparent lipid digestibility to the dietary Zn concentration was 47.0 mg Zn/kg (*Chapter 3, page 101*). Hence, if decreased vitamin E uptake was responsible for the cardiac α-tocopherol depletion, the breakpoint in the response of α-tocopherol should have been in the same range. Furthermore, the total differences in fat digestion were probably too low to promote substantial changes within the timeframe of this experiment. Therefore, we suggest that the reduction in cardiac α-tocopherol concentration was mainly caused by an increased α-tocopherol oxidation through lipid peroxides.

The changes in the redox metabolism parameters were associated with an increase in the expression of proapoptotic genes (BAX, CASP9). Oltvai, et al. (16) identified an association between higher BAX expression and increased apoptosis that seemed to have been due to a modulation of the permeability of the outer mitochondrial membrane by BAX, which promotes the efflux of cytochrome c into the cytosol (17). In response to this efflux, a signalling cascade during which the activator CASP9 initiates effector CASP3 facilitates apoptosis (18). The upregulation of BAX and CASP9 in this study indicates increased intrinsic apoptotic signalling, which seems plausible considering a potential increase in α-tocopherol oxidation by membrane lipid peroxides.

GSR is an enzyme involved in converting GSSG back to GSH to restore its ability to detoxify ROS. Furthermore, catalase activity detoxifies H_2O_2 (19). Previous studies have suggested that the expression of both genes (GSR and CAT) was upregulated during oxidative stress, which resulted in higher protein abundance and activity (20, 21). This is consistent with our findings of increased GSR and CAT expression to the simultaneous decrease in GSH and HDA. Indeed, GSH and HDA were reduced over a broader range of dietary Zn intake than we recognised for the response of GSR and CAT. This indicates that piglets fed ≤40 mg Zn/kg experienced higher concentrations of ROS in the heart tissue than all other groups.

The alterations in cardiac metabolism described previously raise the question about their functional relation to the dietary Zn supply. Our initial assumption was that a decrease in cardiac Zn status in response to declining dietary Zn intake promoted these adaptions. However, the curvilinear response of cardiac Zn does not support this hypothesis. Zn is an important cofactor of gene expression mechanisms (22). Therefore, the upregulation of cellular stress response may have fostered the increase in cardiac Zn concentration. This is supported by a positive correlation between the expression of FAS, EI24, and

CDKN1A and cardiac Zn concentration in groups fed 28.1–42.7 mg Zn/kg. The TP53-regulated gene FAS codes for a so-called "death receptor" that induces an extrinsic proapoptotic pathway after binding the FAS ligand (23). Increased expression of EI24 in response to TP53 has been linked to impaired cell growth and increased apoptosis (24, 25). As a potential B-cell lymphoma 2–binding protein, it may be involved in intrinsic proapoptotic signalling (26). The gene CDKN1A causes gap 1 cell-cycle arrest in response to TP53 (27). Taken together, the increase in cardiac Zn concentration seemed to represent a compensatory response to facilitate stress-associated gene expression in response to increased oxidative stress.

The decline in the concentration of MT1A mRNA may support this hypothesis. MT1A expression is regulated by the amount of cytosolic free Zn ions, which activate metal-regulatory transcription factor 1 (28). The reduction of MT1A gene expression inversely to the increase in cardiac Zn concentration might indicate a lower necessity for capturing free Zn ions in the heart muscles of piglets fed ≤42.7 mg Zn/kg. This may have been caused by a direct transfer of Zn to other Zn-binding peptides, especially stress-associated transcription factors such as TP53. Interestingly, MT1A gene expression exhibited a strong and significant correlation to HDA ($r = 0.91$; $P = 0.002$; data not shown). It has been shown that a reduction of metallothioneins in the course of Zn deficiency can promote oxidative stress (29, 30). Therefore, apart from other parameters such as GSH, a decrease in cardiac metallothioneins may have contributed to the observed reduction of HDA.

Although we recognised changes in the behaviour of TP53 target genes, TP53 expression was not affected by changes in dietary or cardiac Zn concentration. Indeed, TP53 is also regulated by other factors, e.g., its phosphorylation pattern (31). Some studies have reported an upregulation of TP53 under certain treatment conditions (e.g.,

(32)). Therefore, our experimental design does not seem to have induced a stimulus that was strong enough to promote TP53 transcription.

Conclusion

In conclusion, short-term SZD in weaned piglets was associated with decreased heart antioxidative capacity and increased cardiac proapoptotic and antioxidative gene expression. Contrary to our initial hypothesis, however, the cardiac Zn concentration increased in piglets fed less than the estimated gross Zn requirement. This correlated positively to the relative expression of further stress-responsive genes in cardiac tissue. We conclude that this represented a compensatory response in times of increased cardiac ROS concentrations as an attempt to maintain tissue integrity. Remarkably, these adaptions were evident after just 8 d of insufficient dietary Zn supply. To our knowledge, this is the first study to report experimental data on the relation between SZD and redox metabolism.

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4th Chapter

Supplementary material

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 °C
RPS18	NM_213940.1	TGTGGTGTTGAGGAAAGCAG	TCCCATCCTTCACATCCTTC	155→309	155	58.0
ACTB	XM_003357928.1	GACTCAGATCATGTTCGAGACCTT	CATGACAATGCCAGTGGTGC	449→551	103	62.0
GAPDH	NM_001206359.1	CACATGGCCTCCAAGGAGTAA	GGAGATGCTCGGTGTGTTGG	1082→1210	129	61.0
GUSB	XM_013995566.1	GCTGCTTACTACTTCAAGATGCT	GGCACCCCCAGGTCTTTTTC	1458→1564	107	57.0
MT1A	NM_001001266.2	TCTCACCTGCCTCCACTCAT	AGCAGCAGCTCTTCTTGCAG	33→153	121	57.0
MT2B	XM_003355808.2	GCAAATGCAAAGACTGCAAA	TACATCTGGGGCAGGGTCT	139→296	158	61.0
SOD1	NM_001190422.1	AGGCTGTACCAGTGCAGGTC	CCAATGATGGAATGGTCTCC	241→417	177	61.0
SOD2	NM_214127.2	ATTGCTGGAAGCCATCAAAC	GGTTAGAACAAGCGGCAATC	665→816	152	58.0
GPX1	NM_214201.1	CAAGAATGGGGAGATCCTGA	GATAAACTTGGGGTCGGTCA	276→465	190	61.0
PRDX1	XM_003128042.3	TGCTTCGCGTGTCTGCTTC	GACCATCTGGCATAACAGCAG	68→204	137	58.6
PRDX3	NM_001244531.1	GTGAAGGCGTTCCAGTTTGT	TCCCAACTGTGGCTCTTCTC	674→853	180	58.0
PRDX4	XM_005673496.1	ATTTCCAAGCCAGCACCTTA	GGCGATAATTTCAGTTGGACA	401→550	150	58.0
GSR	XM_003483635.3	TGTCGGATGTGTACCCAAAA	GTGTTCAGTCGGCTCACGTA	323→478	156	58.0

Notes: [1]Sequence information was provided by the National Center for Biotechnology Information (NCBI) RefSeq database; O´Leary et al. (13). [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; PCR, polymerase chain reaction.

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 °C
CAT	NM_214301.2	GCACGTTGGAAAGAGGACAC	GGCTGTGGATAAAGGATGGA	414→613	200	58.0
TP53	NM_213824.3	CCCTTTTGAAGTCCCTGGCA	GCCCCAGGCCAAGCATATAG	1320→1427	108	60.0
FAS	NM_213839.1	AGTGACTGACCCCGATTCTG	ATGTTTCCGTTTGCCAGGAG	81→207	127	61.3
BAX	XM_005664710.2	CCGAGAAGTCTTTTTCCGAGT	CGATCTCGAAGGAAGTCCAG	764→940	177	58.0
EI24	XM_003130045.3	TGTGGTCTCTTGCTCCACTG	GGCACTTGTCCAAAACTGGT	1772→1959	188	61.0
SHISA5	XM_013981660.1	ATGGGGTTTGGAACGACTGT	GTGGTGGTAGTCACGACTGG	409→548	140	58.0
IGFBP3	NM_001005156.1	GGGTGCCTGACTCCAAACTC	GAGGAGAAGTTCTGGGTGTCC	588→717	130	60.0
CASP3	NM_214131.1	GCCATGGTGAAGAAGGAAAA	GTCCGTCTCAATCCCACAGT	359→525	167	61.0
CASP8	NM_001031779.2	GCCTGGACTACATCCCACAT	TCCTCCTCATTGGTTTCCAG	165→342	178	58.0
CASP9	XM_003127618.3	GACCCCTTACCCTGCCTTAC	CTCTTTCTCCATCGCTGGTC	1413→1602	190	61.0
CDKN1A	XM_001929558.3	GGGTTCCCCAGTTCTACCTC	CCTCCTGGAAATGTCTGCTC	1511→1666	156	61.0
GADD45A	NM_001044599.1	ATCTTCCTGAACGGTGATGG	CATCTATCTTCGGGCTCCTG	771→950	180	61.0
SFN	XM_013988791.1	CTGAACTGTGTGGCAGAGACT	TTTCCCTCTCATCCTCGGTCT	1144→1259	116	64.0

Notes: [1]Sequence information was provided by the National Center for Biotechnology Information (NCBI) RefSeq database; O´Leary et al. (13). [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; PCR, polymerase chain reaction.

5th Chapter

The hierarchy of body zinc depletion and redistribution in weaned piglets challenged with subclinical zinc deficiency

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Abstract

This study investigated the effects of short-term subclinical zinc (Zn) deficiency (SZD) on the depletion and redistribution of body Zn in weaned piglets.

Forty-eight fully weaned piglets (hybrids of German Large White x Land Race x Piétrain; 50% female, 50% male-castrated; initial average body weight 8.5 ± 0.27 kg) were fed *ad libitum* a corn-soybean meal-based diet with sufficient Zn supply from $ZnSO_4$ * $7H_2O$ (88.0 mg Zn/kg diet) during a 2-week acclimatisation period. Subsequently, piglets were fed restrictively (450 g/d) the same diet but with varying Zn concentrations (28.1, 33.6, 38.8, 42.7, 47.5, 58.2, 67.8, 88.0 mg Zn/kg diet) for an experimental period of 8d. Analyses comprised Zn concentrations in jejunum, colon, kidney, skeletal muscle, mesenteric lymph follicles, thymus, spleen and epidermis. Statistical analyses included linear and broken-line regression models as well as average linkage cluster analysis by Euclidian distance.

No signs of clinical Zn deficiency were recognised during this study. Zn concentrations in jejunum and kidney expressed a significant direct linear regression to the dietary Zn concentration ($P < 0.05$). All other Zn pools responded in a non-linear fashion to changes in dietary Zn concentration, by declining (colon, epidermis, spleen) or increasing (mesenteric lymph follicles, thymus, skeletal muscle) below significant dietary thresholds of 63.6, 48.0, 47.5, 68.0, 43.0 and 53.1 mg Zn/kg diet, respectively ($P < 0.01$). Above respective breakpoints, Zn concentrations in epidermis, mesenteric lymph follicles and skeletal muscle plateaued whereas they exhibited a decrease in colon and thymus as well as an increase in spleen, in each case relative to the changes in the dietary Zn concentration. Average linkage cluster analysis by treatment groups indicated clusters of varying Zn supply status as well as pathophysiological status.

Clustering by biological matrices revealed a grouping by function (excretion media, storage media, transport media, Zn homeostatic regulation).

Taken together, our data highlights the hierarchy of tissue Zn redistribution during short-term SZD in weaned piglets. Furthermore, novel response patterns have been described, indicating compensation reactions in tissues that are essential for the acute survival of the developing organism (heart and skeletal muscle, immune tissues).

Introduction

Most experimental datasets on zinc (Zn) metabolism reflect studies that used severely Zn depleted animals expressing symptoms of clinical Zn deficiency. However, this condition appears to be rare in nature and especially under the terms of livestock rearing, since generous supplementation of Zn to feed is frequent practice (1-6). Furthermore, investigations in Zn metabolic functions or the estimation of measures reflecting the efficacy of Zn feeding interventions under pathophysiological conditions may be biased because of significant secondary metabolic events. A more likely scenario of Zn malnutrition is a subclinical deficiency (SZD), under which the animal expresses no visible symptoms of Zn depletion (for example growth depression, feed refusal, tissue necrosis etc., (7)) (*Chapter 2, page 65*) but significant shifts in physiological parameters (*Chapters 2, pages 65-67, Chapter 3, pages 95-96, 100-101, Chapter 4, pages 128-133*). This condition could arise through periods of fluctuating dietary Zn supply and/or increased demand (for example during inflammation, stress etc.). As SZD is not accompanied by severe secondary metabolic events, it may be a more appropriate model for studies on Zn metabolism.

An experimental approach has recently been published that promotes short-term SZD in fully weaned piglets. We were able to demonstrate that this condition is accompanied by a loss of digestive and antioxidative capacity (*Chapter 3, pages 95-96, 100-101, Chapter 4, pages 128-129*). Simultaneously, no symptoms of clinical Zn deficiency were evident (*Chapter 2, page 65*). Furthermore, we estimated the gross Zn requirement under given experimental conditions at 58 mg Zn/kg diet (*Chapter 2, page 67*).

To better understand the Zn homeostatic regulation behind these functional adaptions, this study investigated the global response of Zn concentrations in various biological matrices in response to finely-graded differences in alimentary Zn supply. This

represents to our knowledge the first comparative description of tissue Zn concentration patterns in growing individuals as affected by short-term SZD.

Material and Methods

This animal study was 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).

Animals and diets

This is the fourth publication based on an experiment which has been described in detail earlier (*Chapter 2, page 45*). In brief, 48 fully weaned piglets (hybrids of German Large White x Land Race x Piétrain; 50% female, 50% male-castrated; initial average body weight 8.5 ± 0.27 kg) from six litters were fed a Zn adequate (60 mg added Zn/kg as $ZnSO_4$ * $7H_2O$ yielding 88.0 mg analysed Zn/kg diet) corn-soybean meal-based diet *ad libitum,* during a 2-week acclimatisation phase. Subsequently, animals were subject to eight different treatment groups by a balanced distribution of litter mates, sex and body weight. These groups received restrictively (450 g/d) the same basal diet (28.1 mg native Zn / kg diet) as during acclimatisation but with finely graded differences in dietary Zn supply (0, 5, 10, 15, 20, 30, 40, 60 mg added Zn/kg diet from $ZnSO_4$ * $7H_2O$ yielding 28.1, 33.6, 38.8, 42.7, 47.5, 58.2, 67.8, 88.0 mg total Zn/kg diet) for a total experimental period of eight days. Tables 1 and 2 within *Chapter 2, page 52*, present detailed information on the composition and ingredients of the basal diet as well as the analysed dietary Zn concentrations as affected by varying Zn supplementation to the experimental diets.

Sampling conditions

After precisely eight experimental days, all animals were killed without fasting by exsanguination under anaesthesia (azaperone and ketamine). Sampling procedures for

feed, faeces, blood plasma, bone and soft tissue have been already described earlier (*Chapter 2, page 53*). All samples were stored at -20°C until Zn analyses.

Chemical analyses

All samples have been subject to dry matter and total Zn analysis as described in *Chapter 2, pages 53-54*. Quantification of total Zn occurred by atomic absorption spectrophotometry (AAS) (NovAA 350, Analytik Jena AG) using a certified external AAS Zn standard (Merck 109953, Merck Millipore) after microwave wet digestion (Ethos 1, MLS GmbH).

Statistical analyses

All statistical procedures were performed with SAS 9.4 (SAS Institute Inc.). Linear broken-line regression models ($y = a + bx$; $y = a + bx + cx$) were calculated for tissue Zn concentrations in response to changes in dietary Zn concentration (procedure NLMIXED) using individual group means ($n = 8$). This approach represents an iterative procedure to estimate a potential dietary threshold (breakpoint) within non-linear datasets above and below which, respectively, a significant difference in the response behaviour of a certain parameter to the dietary treatment is evident (8). If no significant breakpoint in parameter response could be estimated from respective datasets, a linear regression was calculated instead ($y = a + bx$) (procedure REG). Only significant regression models were applied for data presentation and interpretation. A threshold of $P \leq 0.05$ was considered as indicator of significance. Additionally, hierarchical average linkage cluster analyses of mean Zn concentrations by Euclidian distance between dietary Zn concentrations and type of biological matrix, respectively, were performed by applying the procedure CLUSTER.

Results

All animals developed equally during the whole trial period and expressed no signs of impaired health and wellbeing (no signs of feed refusal, growth depression etc., (7)) (*Chapter 2, page 65*).

Figure 1 highlights the response of tissue Zn concentration in jejunum, colon, mesenteric lymph follicles, thymus, spleen, skeletal muscle, kidney and epidermis in weaned piglets fed diets with varying dietary Zn concentration for 8 d. The statistical measures of the respective regression curves are shown in Table 1.

Zn concentrations in jejunum and kidney followed a straight linear response with significant positive slopes over the full range of applied dietary Zn concentrations ($P = 0.04, 0.004$, respectively). The remaining tissue Zn concentrations exhibited a non-linear behaviour in response to changes in dietary Zn concentration. Breakpoints were evident based on significant estimates for X and Y intercepts of respective thresholds (X-intercept (mg Zn/kg diet) and Y-intercept (mg Zn/kg tissue DM): 63.6 and 94.4, 68.0 and 59.2, 43.0 and 56.9, 47.5 and 60.5, 53.1 and 52.7 and 48.0 and 40.8 for colon, mesenteric lymph follicles, thymus, spleen, skeletal muscle and epidermis, respecitively; $P < 0.001$ in all cases).

Above these breakpoints, the response of total Zn concentration in mesenteric lymph follicles, skeletal muscle and epidermis plateaued in response to changes in dietary Zn concentration, whereas the Zn concentrations in colon, thymus and spleen correlated positively or negatively, respectively, to the dietary Zn concentration ($P = 0.001, 0.006, 0.24$, respectively).

Below the respective breakpoints, the Zn concentrations in colon, spleen, and epidermis exhibited a direct relationship ($P < 0.0001$. $P = 0.22, P = 0.14$) whereas mesenteric lymph follicles, thymus and skeletal muscle followed an inverse response to further

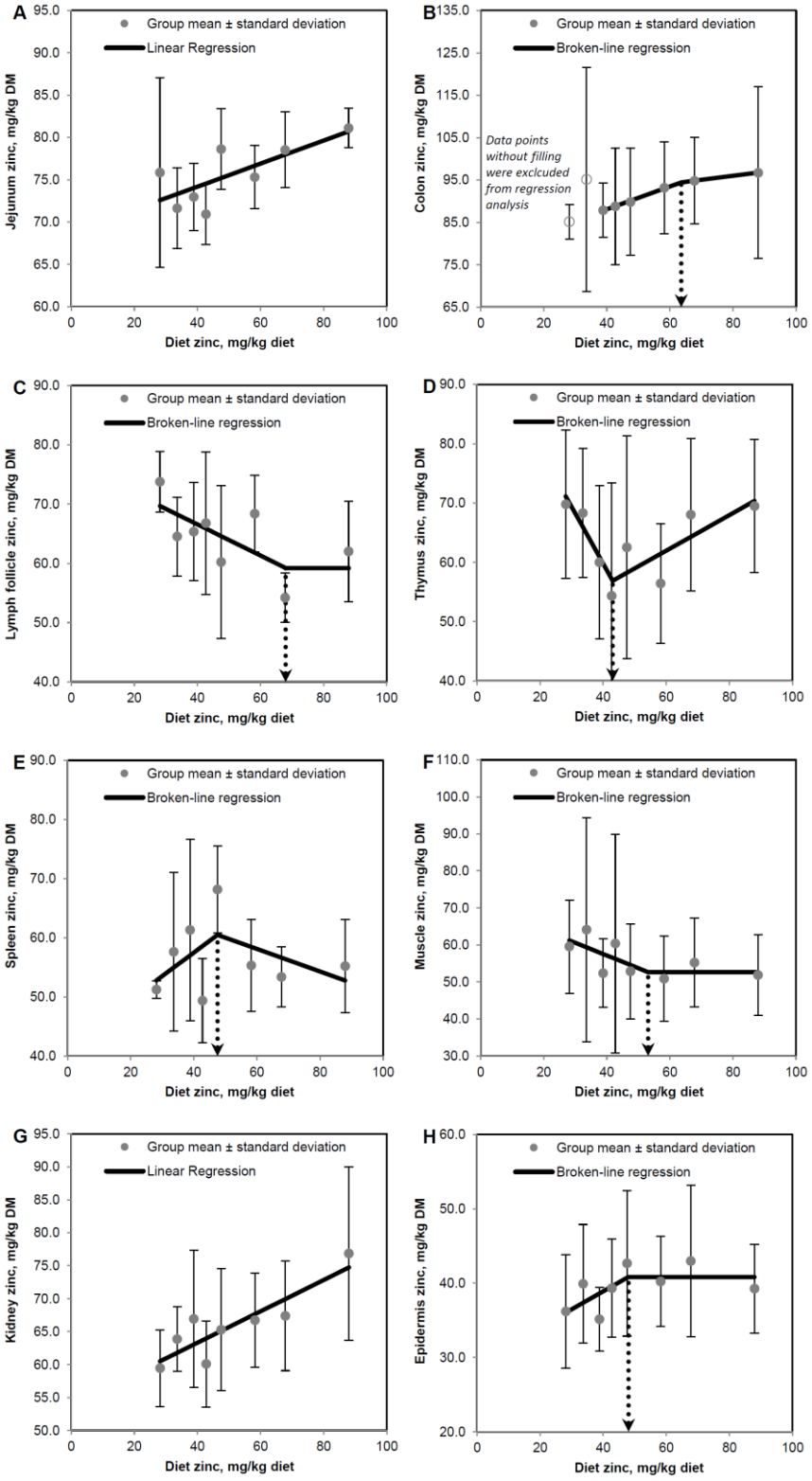

Figure 1. Response of zinc concentrations in jejunum (A), colon (B), mesenteric lymph follicles (C), thymus (D), spleen (E), skeletal muscle (F), kidney (G) and epidermis (H) in weaned piglets fed diets with different zinc concentrations for 8d (see Table 1 for detailed information on the statistical measures of the respective regression curves). Notes: Values are arithmetic means ± SDs, *n* = 6. d, days; DM, dry matter.

Table 1. Linear and broken-line regression analysis of tissue zinc concentrations (mg/kg DM) of jejunum, kidney, colon, epidermis, skeletal muscle, mesenteric lymph follicle, thymus and spleen in weaned piglets fed diets with different zinc concentrations for 8d[1].

	Regression model	Parameter estimates	*P values*	R^2
Jejunum	$y = 68.7 + bx$	b 0.14 ± 0.05	*0.04*	*0.55*
Colon	$y = 77.4 + b_1x$ *for* $x \leq X_B$	X_B 63.6 ± 2.43	*< 0.0001*	*0.99*
	$y = 88.4 + b_2x$ *for* $x > X_B$	Y_B 94.4 ± 0.49	*< 0.0001*	
		b_1 0.27 ± 0.02	*< 0.0001*	
		b_2 0.10 ± 0.02	*0.001*	
Mesenteric lymph follicle	$y = 77.1 + b_1x$ *for* $x \leq X_B$	X_B 68.0 ± 0.02	*< 0.0001*	*0.47*
	$y = Y_B$ *for* $x > X_B$	Y_B 59.2 ± 2.41	*< 0.0001*	
		b_1 -0.26 ± 0.10	*0.03*	
Thymus	$y = 98.1 + b_1x$ *for* $x \leq X_B$	X_B 43.0 ± 0.008	*< 0.0001*	*0.73*
	$y = 44.0 + b_2x$ *for* $x > X_B$	Y_B 56.9 ± 1.82	*< 0.0001*	
		b_1 -0.96 ± 0.23	*0.003*	
		b_2 0.30 ± 0.08	*0.006*	
Spleen	$y = 41.6 + b_1x$ *for* $x \leq X_B$	X_B 47.5 ± 0.01	*< 0.0001*	*0.22*
	$y = 69.5 + b_2x$ *for* $x > X_B$	Y_B 60.5 ± 3.24	*< 0.0001*	
		b_1 0.40 ± 0.30	*0.22*	
		b_2 -0.19 ± 0.15	*0.24*	
Skeletal Muscle	$y = 70.9 + b_1x$ *for* $x \leq X_B$	X_B 53.1 ± 11.4	*0.002*	*0.49*
	$y = Y_B$ *for* $x > X_B$	Y_B 52.7 ± 1.87	*< 0.0001*	
		b_1 -0.34 ± 0.21	*0.13*	
Kidney	$y = 53.8 + bx$	b 0.24 ± 0.05	*0.004*	*0.77*
Epidermis	$y = 29.2 + b_1x$ *for* $x \leq X_B$	X_B 48.0 ± 8.96	*0.0007*	*0.43*
	$y = Y_B$ *for* $x > X_B$	Y_B 40.8 ± 1.19	*< 0.0001*	
		b_1 0.24 ± 0.15	*0.14*	

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. Linear and broken-line regression models were estimated based on independent arithmetic group means relative to dietary Zn concentration ($n = 8$; except for colon where the two lowest supplied groups were excluded from the analyses → $n = 6$). Parameter estimates are presented as means ± SEs to indicate the precision of estimation. $P \leq 0.05$ was considered to be significant. d, days; DM, dry matter; b, slope of the parameter response of the linear regression curves; b_1, slope of the broken-line regression curves over dietary Zn doses $\leq X_B$; b_2, slope of the broken-line regression curves over dietary Zn doses $> X_B$; DM, dry matter; X_B, X intercept of the breakpoint in parameter response; Y_B, Y intercept of the breakpoint in parameter response.

reduction in dietary Zn concentration ($P = 0.03, 0.003, 0.13$, respectively). In case of colon Zn, the datapoints of the groups receiving 28.1 and 33.6 mg Zn/kg diet were excluded from the estimation of the broken-line regression model because they seemed to exhibit a distinct status of Zn homeostatic regulation. This aspect has been further

addressed within the Discussion section of this chapter (*pages 162-164*). The response of Zn concentrations in faeces, femur, blood plasma, liver, pancreas and heart muscle has been already presented and analysed earlier. These response patterns are presented in *Chapter 2, pages 65-67* for femur, blood plasma, faeces and liver as well as *Chapter 3, pages 95-96* for pancreas and *Chapter 4, pages 132-133,* for the heart muscle.

Figure 2 presents an average linkage cluster analysis of Zn concentration patterns in various biological matrices (soft tissue, bone, blood plasma, faeces) in weaned piglets fed diets with different Zn concentrations for 8 d.

Clustering the tissue Zn concentrations according to dietary Zn concentrations revealed two main clusters, separating the three highest Zn supplied groups (≥58.2 mg Zn/kg diet) from the five lowest supplied groups (<58.2 mg Zn/kg diet). Both main clusters are further separated into a total of four subclusters, which group animals in numerical order according to the dietary Zn concentrations they received during the 8d experimental period ((a) 28.1+33.6, (b) 38.8+42.7+47.5, (c) 58.1+67.8 and (d) 88.0 mg Zn/diet, respectively).

Clustering the tissue Zn concentrations between biological matrices separated the (e) faeces Zn, (f) femur Zn and (g) blood plasma Zn from each other and the (h) soft tissues, respectively. The soft tissues themselves were clustered into two main groups, separating liver+colon and jejunum+pancreas from kidney+thymus+mesenteric lymph follicles, heart muscle+skeletal muscle+spleen and epidermis, respectively.

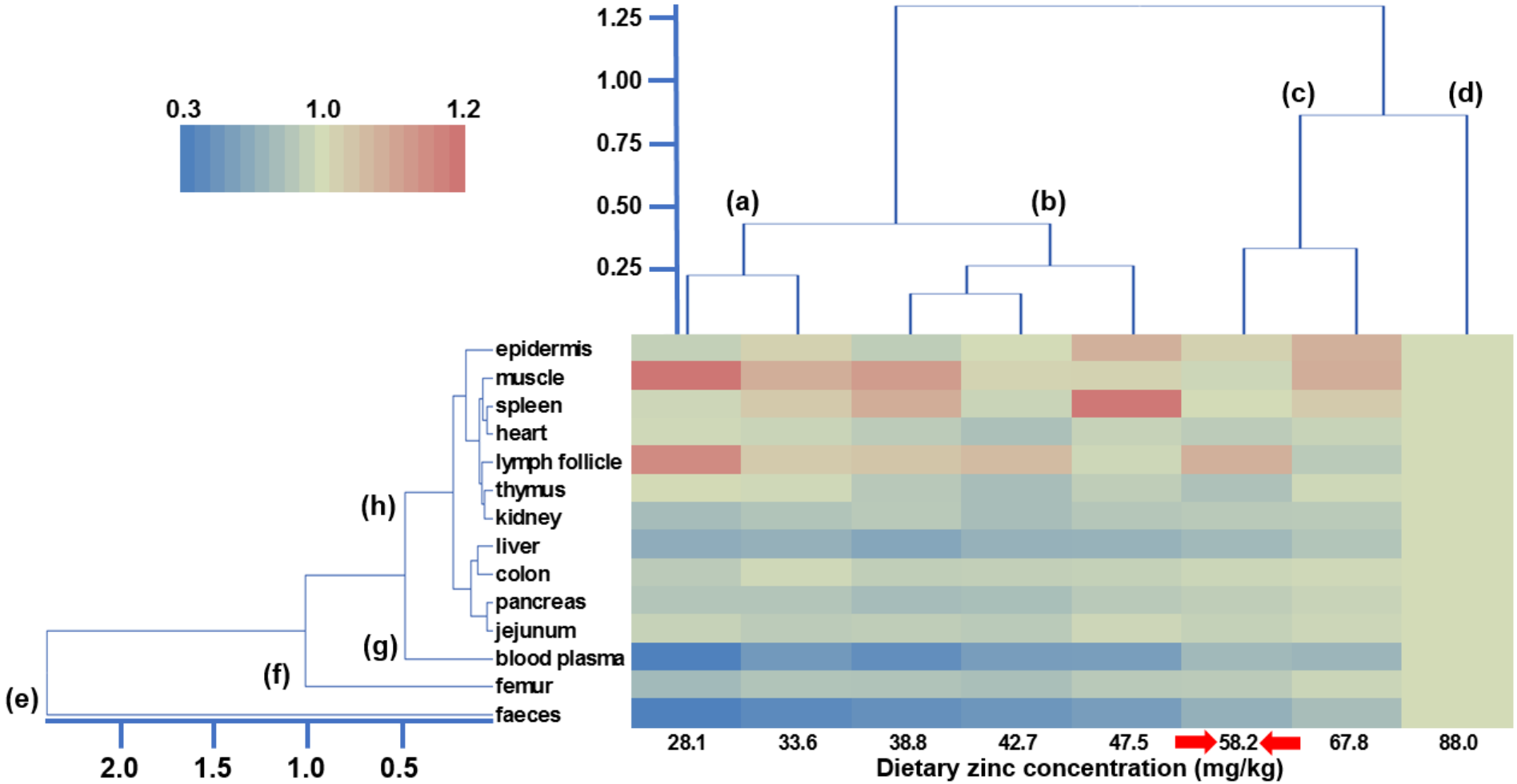

Figure 2: Hierarchical average linkage cluster analysis of Zn concentration patterns in distinct types of biological matrices of weaned piglets fed diets with varying Zn concentration for 8d. Notes: Clustering of experimental groups: (a) 28.1 and 33.6 mg Zn/kg diet, (b) 38.8, 42.7 and 47.5 mg Zn/kg diet, (c) 58.2 and 67.8 mg Zn/kg diet, (d) 88.0 mg Zn/kg diet. Clustering of biological matrices: (e) faeces Zn, (f) femur Zn, (g) blood plasma, (h) soft tissues. Clustering occurred by Euclidian distance. Numerical scales indicating the colour assigned to each fold change are shown in the upper left corner; 0.3fold represents the lowest reduction and 1.2fold the highest increase within the dataset, in each case relative to a level of 1.0 within the control group (88 mg Zn/kg diet). Red arrows highlight the gross Zn requirement threshold under the present experimental conditions.

Discussion

The Zn concentrations within the biological matrices examined during the present study showed either a linear or non-linear pattern in response to changes in dietary Zn concentration. There was no tissue fraction that was not affected by the dietary treatment, which is in sharp contrast to earlier studies using models of clinical Zn deficiency. For example, it has been reported earlier that total Zn concentrations in heart, spleen and thymus were not affected by changes in alimentary Zn supply (9-11).

Muscle tissues (heart, skeletal) as well as immune tissues (mesenteric lymph follicles, thymus) exhibited a response pattern to varying dietary Zn supply which to our knowledge has been reported earlier only for the total Zn pool (but not the Zn concentration) within the skeletal muscle of clinical Zn deficient growing rats (9). These tissues either replenished their Zn concentrations after an initial depletion phase (heart, thymus) or even exceeded the control level (muscle, mesenteric lymph follicles) under the terms of deficient alimentary supply. This was to some extent also the case in pancreatic tissue, which has been demonstrated in *Chapter 3* (*pages 95-96*). For the example of the heart, the repletion of tissue Zn concentrations in deficiently Zn supplied groups correlated to higher proapoptotic and antioxidative gene expression, which has been concluded to compensate for a significant loss in the tissue's ability to detoxify reactive oxygen species (*Chapter 4, pages 128-133*). Indeed, Zn is essential for basic cellular processes, like the regulation of gene transcription or the maintenance of DNA integrity (12). Therefore, we conclude that the response of Zn concentrations in muscle and immune tissues of Zn depleted animals may also represent a compensation reaction. It is interesting that this kind of behaviour was mainly evident in organs which are supposed to be essential for the acute survival of the organism. Obviously, these tissues acted as Zn acceptors at the expense of other tissues, which either released parts of their

total Zn load to the periphery and/or reduced the Zn uptake from the circulation (for example bone, kidney, liver, epidermis).

The non-linear response of spleen Zn concentration in the present study might have reflected an increased degradation of white blood cells and associated recycling of Zn released from degraded immune cells. This would be in context to Hosea, et al. (13) who recognised a decrease in T-cells and B-cells within the spleen of Zn deficient growing rats. However, this hypothesis has yet to be tested. Furthermore, an earlier study on the time-course of SZD in rats recognised a stepwise decrease in spleen Zn concentration (14). However, other groups did not recognise any changes (10, 11). Future experiments must reproduce the spleen Zn response from the present study and investigate its mode-of-action.

In some earlier studies, the Zn concentrations within kidney and urine did not significantly respond to changes in dietary Zn supply (11, 15, 16). Therefore, the relevance of the kidney for the regulation of whole-body Zn homeostasis was considered to be less important. However, a stable Zn load in times of fluctuating demands and/or supply levels, respectively, indicates a strict control of renal Zn fluxes. Given the time-scale of earlier studies (~2 weeks of insufficient dietary Zn supply), a lack in responsiveness of kidney/urinary Zn may indicate an endpoint in homeostatic adaption to clinical Zn deficiency. Hence, under such conditions urinary Zn may represent inevitable endogenous losses (for example from sloughed nephric endothelial cells). However, the response of kidney Zn concentrations indicates a direct functional relationship to the status of bone Zn stores under the present experimental conditions. This decrease in Zn concentration may have happened in favour of other tissues (heart, skeletal muscle, thymus, mesenteric lymph follicles, pancreas) to compensate short-term Zn losses. This supports the data of Canton and Cremin (14), who recognised a decrease

of the concentrations of renal Zn in parallel to bone Zn in the course of SZD in the rat. Own data on the response of certain Zn transporters within the kidney indicated a controlled upregulation of Zn recycling from the primary urine and, at the same time, an increase of Zn transport towards the circulation (*Chapter 6, pages 190-191*). Therefore, it can be concluded that the kidney plays a key role within the network of whole-body Zn homeostasis.

The present study highlighted obvious differences in the response of tissue Zn concentrations between the small and the large intestine. Jejunal Zn responded in a direct linear fashion to changes in dietary Zn concentration, whereas colonic Zn expressed a broken-line response. Interestingly, studying Zn transporter gene expression (namely the response of porcine solute carrier family (SLC) 39 member A4 (ZIP4)) indicated a significant non-linear response to the dietary Zn supply within the colon but no correlation within the jejunum under the present experimental conditions (*Chapter 6, pages 184-187*). In a former study, Pfaffl and Windisch (17) speculated about a potential shift of the main Zn absorption site from the small to the large intestine during the early stages of Zn deficiency. The authors based their assumption on peaks in the colonic expression of certain Zn responsive genes. Other groups demonstrated that the large intestine contributes to Zn absorption in times of impaired absorption from the small intestine (18, 19). Indeed, certain digestive enzymes, like carboxypeptidases A and B, need Zn as catalytic cofactor (20-22). Therefore, in light of earlier data regarding the loss in digestive capacity in response to SZD (*Chapter 3, pages 95-96, 100-101*), we hypothesise the organism shifted its main absorption site of Zn to the lower intestinal segments as an attempt to maintain the already impaired digestive capacity.

The response of colon Zn concentration in the group receiving 33.6 mg Zn/kg diet was completely different from the response over the six highest supplied groups (38.8-88.0

mg Zn/kg diet) and in the lowest supplied group (28.1 mg Zn/kg diet), respectively. For this reason, the two lowest supplied groups were excluded from the regression analysis of the colon data. This peak may have indicated an increased Zn uptake in this group, which presumably indicates a distinct status of homeostatic regulation. Interestingly, colonic metallothionein gene expression (MT1A and MT2B) also peaked in animals fed 33.6 mg Zn/kg diet (data not shown). Therefore, we conclude this finding was not due to analytical bias. Although, the reason for this high Zn load remains yet unclear. Based on earlier data, the apparent feed Zn digestion in this group was slightly negative, indicating these animals excreted more Zn with the faeces than they ingested with the diet (*Chapter 2, page 66-67*). Indeed, Windisch and Kirchgessner (15) demonstrated that dietary phytate concentrations of 9 g/kg (which have been applied in the present study (*Chapter 2, page 52*)) completely eliminate true absorption of native dietary Zn from the gastrointestinal tract. Therefore, we conclude the observed Zn uptake within the colonic mucosa of animals fed 33.6 mg Zn/kg diet occurred from the basolateral rather than the apical side. Gene expression data obtained from the same tissue samples indicated a numerical upregulation of SLC39A14 (ZIP14) (data not shown) with decreasing dietary Zn supply below the gross Zn requirement threshold (58 mg Zn/kg diet). This transporter is associated to the basolateral side of plasma membranes, where it pumps Zn from the circulation towards the cytosol (23, 24). Interestingly, Guthrie, et al. (24) demonstrated that the ZIP14 may be involved in the maintenance of intestinal barrier function, by demonstrating an increase in mucosal permeability in ZIP14$^{-/-}$ (knockout) mice. Other studies demonstrated clear interconnections between the Zn supply and the status of gut permeability (25-27). Therefore, the observed peak in colonic Zn concentration in animals fed 33.6 mg Zn/kg diet may reflect a compensation reaction to stabilise gut permeability. However, we did not see such a peak in the lowest supplied group (28.1. mg Zn/kg diet). This may indicate differences in the homeostatic

status after 8d of varying Zn supply. The colon of the strongest Zn depleted group (28.1. mg Zn/kg diet) may have been forced to "donate" Zn (or reduce uptake from the circulation) in favour of other tissues that may be more important regarding the acute survival of the organism (for example the heart muscle or immune tissues). An experimental period >8d might have shifted the peak in colonic Zn concentration closer to the groups meeting the gross Zn requirement threshold. Further studies must address the gastrointestinal permeability along all segments of the gut at different timepoints in the development of Zn deficiency to support this hypothesis.

To our knowledge, this is the first time the Zn supply status in response to varying dietary Zn supply was mapped by cluster analysis of body Zn pools. Interestingly, the sufficiently supplied groups were further separated into two subclusters (58.2+67.8 and 88.0 mg Zn/kg diet). This is in good context to the specific response patterns of certain matrices, which pointed towards a different response of animals receiving 58.2 and 67.8 mg Zn/kg diet relative to control (88.0 mg Zn/kg diet) (for example jejunum and kidney). This could be explained by earlier data on the kinetics of Zn homeostatic regulation over time. As has been demonstrated earlier, a full adaption of whole-body Zn homeostasis to changes in dietary Zn supply (as expressed by an upregulation of absorptive capacity together with a decrease in endogenous losses) happens within ~3-5 d (11, 28-33). This means every decrease in dietary Zn supply, even within ranges that would still be sufficient on a long-term scale, induces a short-term lack of absorbed dietary Zn, which is compensated mainly by Zn mobilisation from the skeleton. This aspect has been addressed in more detail earlier (*Chapter 2, pages 70-71*). Furthermore, the two subclusters of the deficiently supplied groups (28.1+33.6 and 38.8+42.7+47.5 mg Zn/kg diet) are in good context to our earlier data on the response of cardiac redox metabolism (*Chapter 4, pages 128-132*). Therefore, these subclusters seem to

discriminate between animals expressing a different physiological status. The lowest Zn supplied groups (28.1-33.6 mg Zn/diet) already showed first signs of pathophysiological adaption (loss in cardiac antioxidative capacity, upregulation of stress-responsive genes), whereas between 38.8-47.5 mg Zn/kg diet animals still expressed a basal physiological status (*Chapter 4, pages 128-131)*.

Clustering our data over biological matrices illustrated the hierarchy of certain Zn reservoirs within the complex network of whole-body Zn homeostasis. On the one hand, the excretion, storage and distribution media (faeces, bone, plasma) were separated from each other and soft tissues, respectively. The soft tissues were further separated into main subgroups, obviously representing tissues directly involved in Zn uptake, excretion and distribution (liver, pancreas, jejunum, colon) and those that were probably affected by these tissues (kidney, heart muscle, skeletal muscle, lymph follicles, thymus, spleen, epidermis). Interestingly, liver and colon were grouped together. Considering the hypothesis of a shift of the main side of Zn absorption to lower intestinal segments, this may further indicate a transfer of luminal Zn via the colon to the liver as the predominant route of Zn uptake under the terms of subclinical Zn deficiency.

Conclusion

Based on the present dataset, certain tissues can be grouped into Zn donors and Zn acceptors during episodes of short-term SZD. The group of Zn accepting tissues seems to be represented by organs which are important for the acute survival of the developing organism (immune tissues, heart muscle, skeletal muscle). These tissues not only replenished their Zn concentrations during Zn deficiency (heart muscle, thymus) but in some cases (mesenteric lymph follicles, skeletal muscle) even exceeded them above the control level. Based on earlier findings, this may highlight compensation reactions to maintain tissue integrity and anabolism. Using cluster analysis allowed a separation of sufficiently and deficiently Zn supplied groups as well as a discrimination of groups that have been earlier shown to express compensative stress responsive cellular programs. Clustering groups of biological matrices discriminated between excretion, storage and transport media (faeces, femur, blood plasma) as well as soft tissues. The latter were further grouped in tissues involved in Zn homeostatic regulation and others. Taken together, the present study highlights for the first time the hierarchy of body Zn depletion and redistribution during short-term SZD in growing individuals.

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6th Chapter

Comparative analysis of zinc transporter gene expression in jejunum, colon, liver and kidney of weaned piglets challenged with subclinical zinc deficiency

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Abstract

This study compared the relative mRNA expression patterns of the currently known mammal zinc (Zn) transporters (solute carrier families 30 (ZnT) and 39 (ZIP)) in selected tissues of weaned piglets challenged with short-term subclinical Zn deficiency (SZD).

Forty-eight fully weaned piglets (50% female, 50% male-castrated) were fed *ad libitum* a corn and soybean meal-based diet with adequate dietary Zn supply (+60 mg Zn/kg diet from $ZnSO_4$ * $7H_2O$ yielding 88 mg total Zn/kg diet) during two weeks of acclimatisation. Subsequently, animals were assigned to eight dietary treatment groups, which were fed restrictively (450 g/d) the same basal diet but with varying dietary Zn concentrations (+0, +5, +10, +15, +20, +30, +40, +60 mg Zn/kg diet from $ZnSO_4$ * $7H_2O$ yielding 28.1, 33.6, 38.8, 42.7, 47.5, 58.2, 67.8, 88.0 mg total Zn/kg diet) for a total experimental period of 8 d. Analyses included quantitative PCR gene expression profiling of the currently known ZnT and ZIP mammal Zn transporter genes within jejunum, colon, liver and kidney. Data analyses comprised broken-line regression models.

Many gene expression patterns exhibited highly significant breakpoints in response to changes in dietary Zn supply that lay either at ~40 or ~60 mg Zn/kg diet ($P < 0.0001$). However, only certain genes within the tissues of interest showed also a significant dose-response behaviour. This indicates these genes to be directly involved in the adaption of Zn fluxes during body Zn depletion and redistribution. Furthermore, ZIP4 in the colon but not the jejunum was significantly affected by the dietary Zn supply, yielding a high correlation to the apparently-digested amount of Zn. This may point towards a shift of the main side of Zn absorption towards lower parts of the digestive tract.

In summary, this study presents the first comparative analysis of Zn transporter gene expression in several tissues of weaned piglets challenged with SZD.

Introduction

Basic cellular processes are dependent of zinc (Zn) as a structural and catalytical cofactor of peptide function (for example transcription and replication of DNA, maintenance of DNA integrity). In fact, 10% of the genes within the human genome are coding for Zn peptides, highlighting its ubiquitous importance for the mammal organism (1). In contrast, Zn has a strong toxic potential if its content 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.

Mammal Zn homeostasis is maintained by a complex molecular network, within which the expression of specific Zn transport peptides is modulated to maintain metabolic function and avoid Zn intoxication. So far, up to 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 in mammals (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. The ZnT and ZIP transporters express differences regarding their tissue specificity, subcellular localisation 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 (4-6).

Currently, there is a considerable lack of data regarding Zn transporter expression and function in mammal organisms other than rodents and humans. Even less information is given on the response of Zn transporters regarding whole body Zn homeostasis. Therefore, this study aimed in investigating the abundance and response of ZnT and ZIP transporter gene expression in jejunum, colon, liver and kidney of weaned piglets challenged with short-term subclinical Zn deficiency (SZD). This study presents to our knowledge for the first time, gene expression patterns of ZnT and ZIP transporters in a large mammal under the impact of deficient to mild oversupply with dietary Zn.

Material and Methods

This animal study was 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).

Animals and diets

The experimental approach has been described in detail in *Chapter 2, pages 50-52*. In brief, a total of forty-eight fully weaned piglets from six litters (8 animals per litter, 50% male-castrated, 50% female, initial average body weight 8.5 ±0.27 kg, 28 d of age) were individually housed and fed *ad libitum* a Zn-adequate diet (+60 mg Zn/kg added by addition of $ZnSO_4$ * $7H_2O$ (analytical grade; Merck 108883; Merck KGaA), analysed total dietary Zn concentration of 88.0 mg Zn/kg) based on corn and soybean meal for a total acclimatisation period of 2 weeks, in order to ensure full body Zn stores at day one of the experimental period. Subsequently, all animals were assigned to eight dietary treatment groups by a blocking according to life weight, litter mates and sex. The treatment groups were fed restrictively (450 g/d) the same basal diet as during the acclimatisation period but with varying dietary Zn concentrations, as modulated by varying supplementation of $ZnSO_4$ * $7H_2O$ (+0, +5, +10, +15, +20, +30, +40, +60 mg Zn/kg diet; analysed 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 was considered to serve as control, because it represented the feeding situation during the acclimatisation 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 (7). *Chapter 2, page 52*, presents detailed information on the composition and ingredients of the basal diet as

well as the analysed Zn concentrations as affected by varying Zn supplementation to the experimental diets.

Sampling conditions

Diet samples were collected and processed as described previously (*Chapter 2, page 53*). At experimental day 8, all animals were killed by exsanguination under anaesthesia (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 overnight and subsequently stored at -80°C until further usage in gene expression studies. Tissue samples for the analyses of total Zn contents were taken and processed as described previously (*Chapter 2, page 53*).

Analyses of dry matter and total zinc concentration in diets and soft tissue

Chapter 2, pages 53-54, presents details on the dry matter and Zn analyses in diets and soft tissue. Zn concentrations were measured by atomic absorption spectrometry (NovAA 350; Analytik Jena AG) after microwave wet digestion (Ethos 1; MLS GmbH).

Gene expression analysis

Primer design, assay quality control and chemical procedures (total RNA extraction, reverse transcription, quantitative PCR (qPCR)) were performed as described earlier (*Chapter 2, pages 54-56*). 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 using state-of-the-art qPCR methodology (8, 9). Primer pairs (Eurofins Scientific) were designed 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; *pages 180-181*). All oligonucleotides bind to homologous regions of respective transcripts to amplify potential 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 using appropriate statistical algorithms (10). The $2^{-\Delta\Delta Ct}$ method (11) was used to normalise the gene expression data because the determination of the amplification efficiency revealed comparable values between 95% and 100% of applied RT-qPCR assays (*Chapter 2, page 56-57*).

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 (porcine brain was not available).

Statistical analyses

Data analysis was performed with SAS 9.4 (SAS Institute Inc.). Linear broken-line regression models ($y = a + bx$, $y = a + bx + cx$) were calculated based on independent group means relative to dietary Zn concentration ($n = 8$). Thereby, the dietary threshold was estimated iteratively above and below which, respectively, the response pattern of certain parameters to the dietary Zn supply changes significantly. The significant regression models with the highest coefficients of determination (R^2) were used for

presenting and interpreting data. All $2^{-\Delta\Delta Ct}$ gene expression data is presented as xfold differences compared to a relative mRNA abundance of 1.0 within the control group (88.0 mg Zn/kg diet). $P \leq 0.05$ was considered to be significant.

Results

All animals remained in good health throughout the whole trial. There were no signs of clinical Zn deficiency (for example growth retardation, anorexia (12)) evident at any time (*Chapter 2, page 65*).

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

Table 1 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 Normfinder and Genorm algorithms (10)).

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 recognised 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

Table 1: 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 (10). N/A, transcript not expressed 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.

colon tissue as well as the candidate genes that served as reference genes for data normalisation within respective tissues. Recognised 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, hence, yielded only low coefficients of determination (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 ZnT and ZIP family member gene expression as affected by varying dietary Zn supply. Table 2 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 ≤ 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 ZnT and ZIP family member gene expression as affected by varying dietary Zn supply. Table 3 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

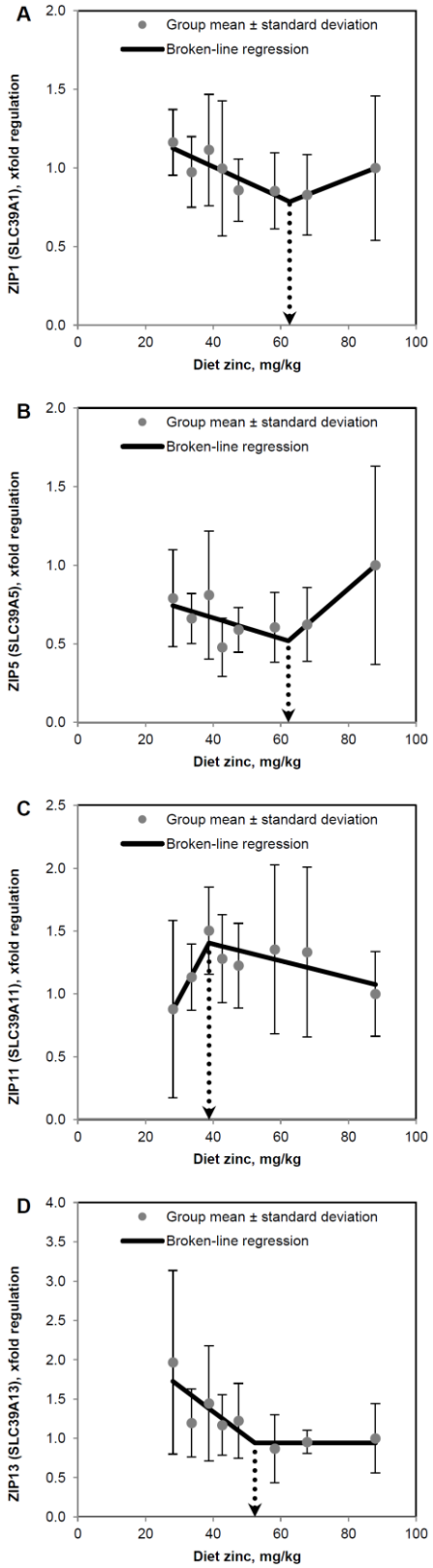

Figure 1. Response of relative jejunal gene expression of ZIP1 (A), ZIP5 (B), ZIP11 (C) and ZIP13 (D) in weaned piglets fed diets with different zinc concentrations for 8 d (see Table 2 for detailed information on the statistical measures of the respective regression models). Notes: Values are arithmetic means ± SDs, *n* = 6. Diet zinc, dietary zinc; xfold, difference in the gene expression value according to Livak and Schmittgen (11) 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.

Table 2. 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 (11) 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.

in dietary Zn. On the contrary, these genes significantly increased their relative expression levels in response to further reduction in dietary Zn below these breakpoints ($P \leq 0.001$, ≤ 0.001 and ≤ 0.05, respectively). On the contrary, colonic ZnT4 and ZIP11 significantly increased ($P \leq 0.05$ and ≤ 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 ≤ 0.05, respectively) whereas ZnT9 and ZIP13 did not change significantly.

Figure 3 presents the broken-line response of hepatic ZnT and ZIP family member gene expression as affected by varying dietary Zn supply. Table 4 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

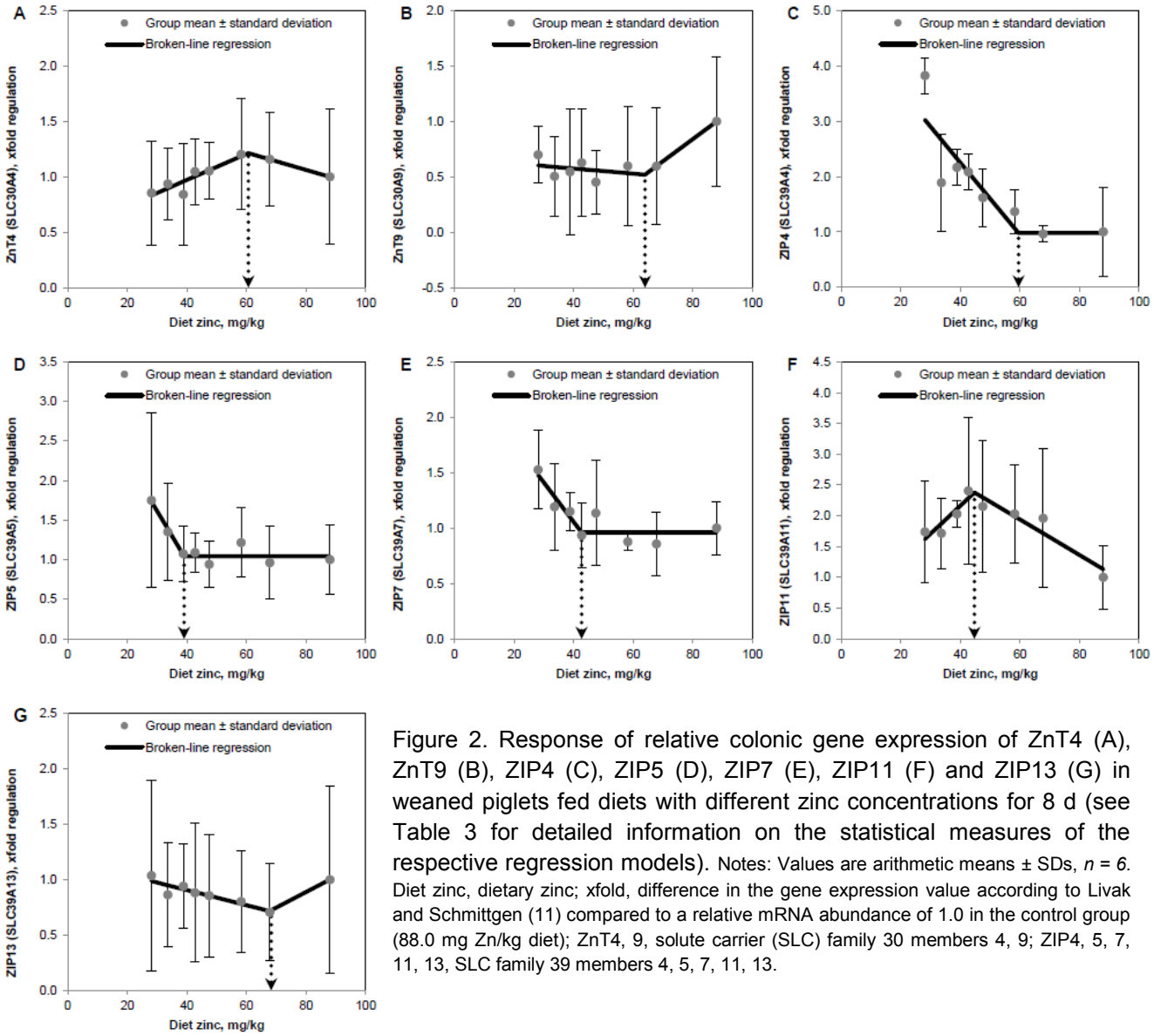

Figure 2. Response of relative colonic gene expression of ZnT4 (A), ZnT9 (B), ZIP4 (C), ZIP5 (D), ZIP7 (E), ZIP11 (F) and ZIP13 (G) in weaned piglets fed diets with different zinc concentrations for 8 d (see Table 3 for detailed information on the statistical measures of the respective regression models). Notes: Values are arithmetic means ± SDs, *n* = *6*. Diet zinc, dietary zinc; xfold, difference in the gene expression value according to Livak and Schmittgen (11) 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.

Table 3. 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 (11) 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.

varying dietary Zn supply at breakpoints of 48.4, 38.8, 57.3, 47.5 and 42.7 mg Zn/kg diet, respectively ($P \leq 0.0001$, respectively). 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 ≤ 0.05, respectively) whereas ZnT8, ZIP1 and ZIP14 significantly increased ($P \leq 0.05$, ≤ 0.05 and ≤ 0.0001,

respectively) with reduction in dietary Zn concentration below the respective dietary thresholds.

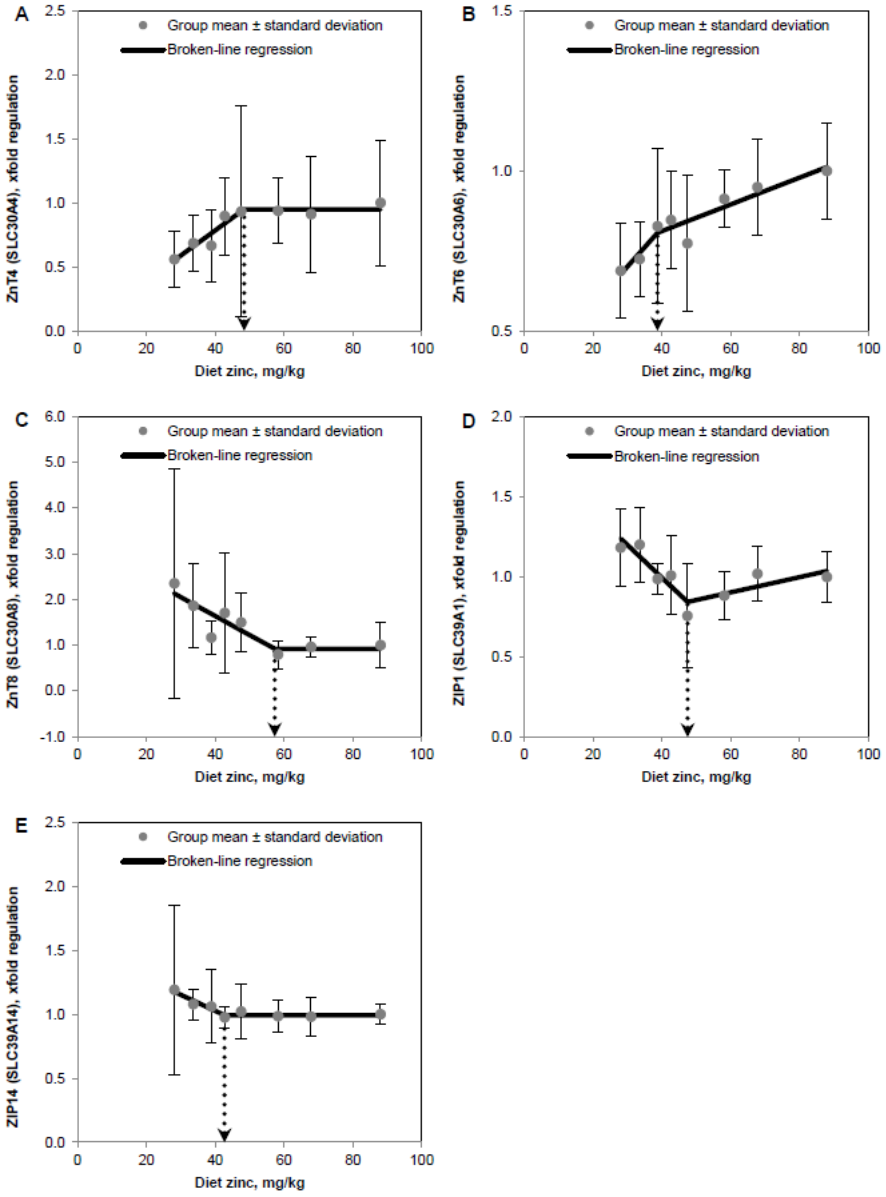

Figure 3. Response of relative hepatic gene expression of ZnT4 (A), ZnT6 (B), ZnT8 (C), ZIP1 (D) and ZIP14 (E) in weaned piglets fed diets with different zinc concentrations for 8 d (see Table 4 for detailed information on the statistical measures of the respective regression models). Notes: Values are arithmetic means ± SDs, *n* = *6*. Diet zinc, dietary zinc; xfold, difference in the gene expression value according to Livak and Schmittgen (11) 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.

Table 4. 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 (11) 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.

Figure 4 presents the broken-line response of nephric ZnT and ZIP family member gene expression as affected by varying dietary Zn supply. Table 5 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 ≤ 0.05, respectively) as well as a significant decrease of ZnT1 gene expression ($P \leq 0.05$).

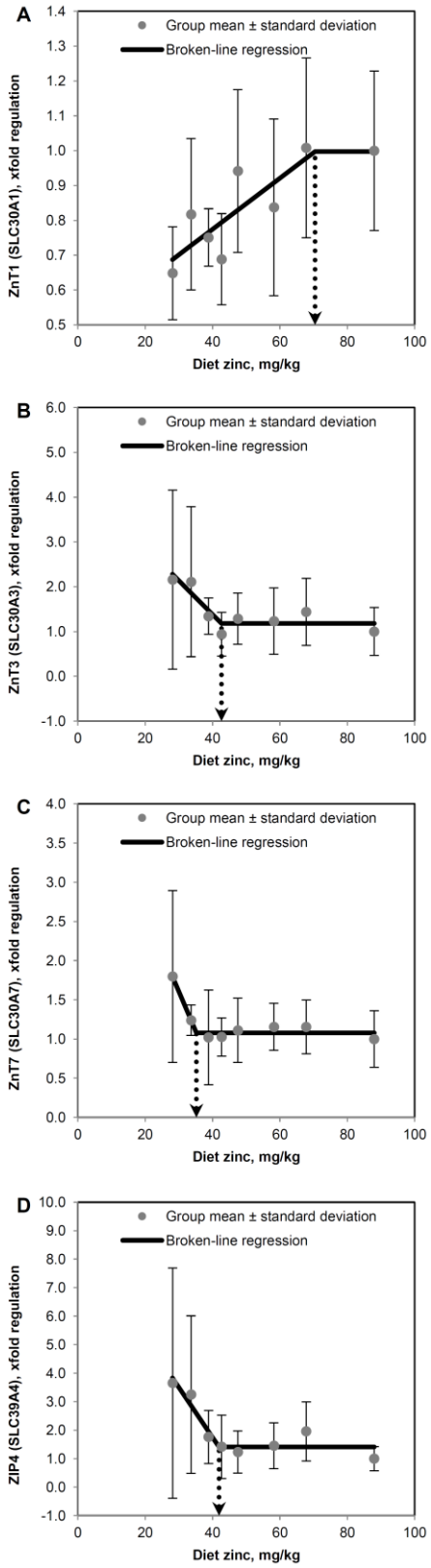

Figure 4. Response of relative nephric gene expression of ZnT1 (A), ZnT3 (B), ZnT7 (C) and ZIP4 (D) in weaned piglets fed diets with different zinc concentrations for 8 d (see Table 5 for detailed information on the statistical measures of the respective regression models). Notes: Values are arithmetic means ± SDs, *n* = *6*. Diet zinc, dietary zinc; xfold, difference in the gene expression value according to Livak and Schmittgen (11) 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.

Table 5. 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 (11) 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.

Discussion

We investigated the gene expression response of the currently known members of the ZnT (SLC30) and ZIP (SLC39) family of Zn transporters in weaned piglets, challenged with finely graded differences in dietary Zn concentration (ranging from 28.1 to 88.0 mg Zn/kg diet). We chose jejunum, colon, liver and kidney as target tissues for the analyses, as they represent important hubs in the complex network of body Zn acquisition, (re)distribution and excretion (13). Pancreatic gene expression could not be investigated in the present study due to technical issues.

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

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

The ZIP10 and ZIP12 gene expression assays were both designed to amplify mRNA regions which were supposed to be highly conserved in mammalian species (14). As they worked properly in cDNA from murine brain and liver, this may indicate these genes have no function within the porcine jejunum, colon, liver and kidney.

So far, the ZnT3 has been recognised in several organs including brain, adipose tissue, pancreatic beta-cells, epithelial cells, testis, prostate and retina (15). Until know, the majority of studies focused on its role in transporting cytosolic Zn into synaptic vesicles (16). Interestingly, this also involved enteric neurons within the gastrointestinal tract of pigs (17, 18). 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 predominantly represented the mucosal layer. So far, no study has been

examined ZnT3 gene expression within liver and kidney. Therefore, this is to our knowledge the first report that claims the absence of ZnT3 gene expression in liver and its detection within nephric tissue of growing pigs, respectively.

Response of ZnT and ZIP family member gene expression to changes in dietary zinc concentration

Many of the analysed ZnT and ZIP gene expression patterns revealed highly significant breakpoints in response to changes in dietary Zn concentration. However, although several broken-line models included a significant statistical breakpoint, this was not always accompanied by significant slopes in response to changes in dietary Zn concentration. We hypothesise that only genes expressing a significant dose-response behaviour to the dietary Zn concentration were regulated directly by changes in the Zn supply status. On the contrary, all other Zn transporter genes may have just indirectly adapted to the changes in Zn fluxes initiated by these key transporters. So far, some mammalian Zn transporter mRNAs have been demonstrated to be directly affected by deficient dietary Zn supply, including ZnT1, ZnT2, ZnT4, ZnT5, ZnT6, ZIP4 and ZIP10 (19-22)). 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. 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 have not been described earlier.

In a former publication associated with this project, the gross Zn requirement under given experimental conditions was estimated to lie at ~60 mg Zn/kg diet (*Chapter 2, page 67*). Therefore, it may be hypothesised that ZnT and ZIP genes which changed their expression level in response to decreasing dietary Zn concentrations <~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. This is in line with earlier published work that identified the intestinal ZIP4 as the major transport route for luminal Zn into the enterocytes of mammals (21, 23, 24). Our ZIP4 gene expression data further corresponds to *Chapter 2, pages 66-67*, regarding the response of apparent Zn digestion under the present experimental conditions. In fact, colonic ZIP4 gene expression and the apparently-digested amount of feed Zn exhibited a direct inverse relationship to each other ($r = -0,91$; $P = 0.002$; data not shown). This demonstrates the role of this gene within the homeostatic network that controls the body Zn level and especially the Zn absorption in swine. Interestingly, we did not identify any significant reaction of the ZIP4 mRNA abundance in jejunal tissue relative to changes in dietary Zn supply. Although the expression levels tended to be numerically increased in deficiently supplied groups (data not shown), the dose-response

relationship was not consistent enough to allow precise estimation through regression analysis. This is in contrast to former studies that claim the jejunum to be the main side of Zn absorption (25), which highlights again strong 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 apparently-digested feed Zn, we hypothesise that this indicates a shift of the main absorption site from the jejunum to the large intestine during the initial stages of Zn deficiency. This has been already proposed by other authors, which recognised peaks in colonic ZnT1 gene expression as an indicator of increased cytosolic Zn^{2+}, under the terms of mild Zn deficiency in adult rats (26, 27). Furthermore, there are reports of a significant contribution of caecal and colonic Zn absorption in times of reduced Zn acquisition from the small intestine (28, 29). We demonstrated earlier a decrease in digestive capacity under the present experimental conditions (*Chapter 3, pages 95-96, 100-101*). Therefore, we conclude that a shift of the main side of Zn absorption to the large intestine may have happened in favour of Zn-dependent digestive enzymes (like carboxypeptidases A and B, (30)) to stabilise the already impaired protein digestion in the small intestine. However, this must be further investigated in appropriate follow-up studies.

We further postulate that certain Zn transporters may play a key role in the compensation of increased cellular stress and inflammation. We suppose this involves all genes which expression patterns exhibited significant breakpoints at ~40 mg Zn/kg diet. Indeed, it has been demonstrated in *Chapter 4, pages 132-133,* that under the present experimental conditions the heart muscle of piglets increased its total Zn concentration. This was probably due to an increase in Zn transport from the circulation. It seemed to occur in response to increased cardiac oxidative stress during SZD, in

groups fed <~43 mg Zn/kg diet (*Chapter 4, pages 128-131*). Therefore, breakpoints of Zn transporter genes around ~40 mg Zn/kg diet may indicate similar events in other tissues. However, this has yet to be proven. 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 (31, 32). In the present study, its expression significantly increased in piglets fed ≤42.7 mg Zn/kg diet. This may indicate that SZD in weaned piglets induces systemic inflammation in response to a loss in antioxidative capacity. Earlier published data supports this hypothesis, by demonstrating an inverse correlation between systemic inflammatory activity and the Zn supply status in the elderly (33).

It is noteworthy that the threshold of ~40 mg Zn/kg diet does not represent a minimum dietary Zn concentration above which no adverse effects in terms of redox metabolism occur. It is 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% (*Chapter 2, page 65*). This represents depletion of the mobilizable skeletal Zn fraction, which has been demonstrated earlier in rats (34-36). A continuation of the study >8 d and associated further emptying of 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.

Indeed, also Zn transporter genes other than the ones discussed above, expressed a significant dose-response behaviour to changes in the dietary Zn concentration. 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. This included jejunal ZnT5 and 6, colonic ZIP1 and 7, hepatic ZIP7 and 13 as well as nephric ZnT4, 5 and 6. Therefore, they served as reference genes for data normalisation in the present study. Future studies should further investigate under which conditions the transcription of these genes changes. This is crucial in order to understand their role in whole-body Zn homeostasis and associated metabolic pathways.

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 either lay close to ~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.

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6th Chapter

Supplementary material

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 et al. (3). [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 et al. (3). [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.

7th Chapter

General discussion

This doctoral project aimed to develop an experimental model of short-term subclinical zinc (Zn) deficiency (SZD) in weaned piglets (*Chapter 2, page 45*). The approach applied an acclimatisation phase during which all piglets were fed a practical corn-soybean meal-based diet with sufficient Zn supply. Thereby, it was ensured that all animals had full body Zn stores at day one of the experiment. During the subsequent experimental phase, piglets were assigned to dietary treatment groups in a completely randomised block design, by which an evenly distribution of sex, initial life weight and littermates over treatment groups has been achieved. These groups were challenged with finely-graded differences in alimentary Zn doses, ranging from deficient supply levels to mild oversupply based on the available information on the gross Zn requirements of growing piglets (28.1, 33.6, 38.8, 42.7, 47.5, 58.2, 67.8, 88.0 mg Zn/kg diet) (1-8).

The total experimental period was 8 d. This has been decided on base of already published literature. On the one hand, it has been shown that the Zn homeostatic regulation needs ~3-5 d to fully adapt to changing dietary supply levels (9, 10). On the other hand, Windisch (11) demonstrated that piglets fed a corn-soybean meal-based diet without Zn supplementation develop first symptoms of clinical Zn deficiency (anorexia) already after ~10-12 d. Therefore, to ensure full adaption of the Zn homeostatic regulation and, at the same time, avoid clinical Zn deficiency, it has been concluded that the optimal experimental period for the promotion of SZD in weaned piglets should range between 5-10 d at given dietary conditions.

An important cornerstone of this experimental approach was a high precision in the production of diets. This is a fundamental prerequisite for dose-response studies that allows, together with animal individual housing, a monitoring of the precise amounts of Zn consumed per individual and day. Hence, the analytical recovery of supplemented Zn as well as the accuracy of the determination of the native dietary Zn concentration

must be as high as possible. To achieve these goals, a precise algorithm for our inhouse feed mixing facility has been applied. It was shown that the analytical recovery rate was virtually 100%. Hence, the final feed mixtures exhibited a high degree of homogeneity. This was evident by a slope close to 1.0 of the total analysed dietary Zn concentration in response to varying supplementation of Zn from $ZnSO_4 * 7H_2O$ (+0, +5, +10, +15, +20, +30, +40, +60 mg Zn/kg diet) (Figure 1).

Using this experimental approach allowed the creation of a comprehensive tissue bank, to investigate the physiological adaption to short-term and finely-graded differences in Zn supply status of weaned piglets. Special interest was related to the adaption of digestive capacity, cardiac redox metabolism, redistribution of body Zn as well as Zn transporter gene expression.

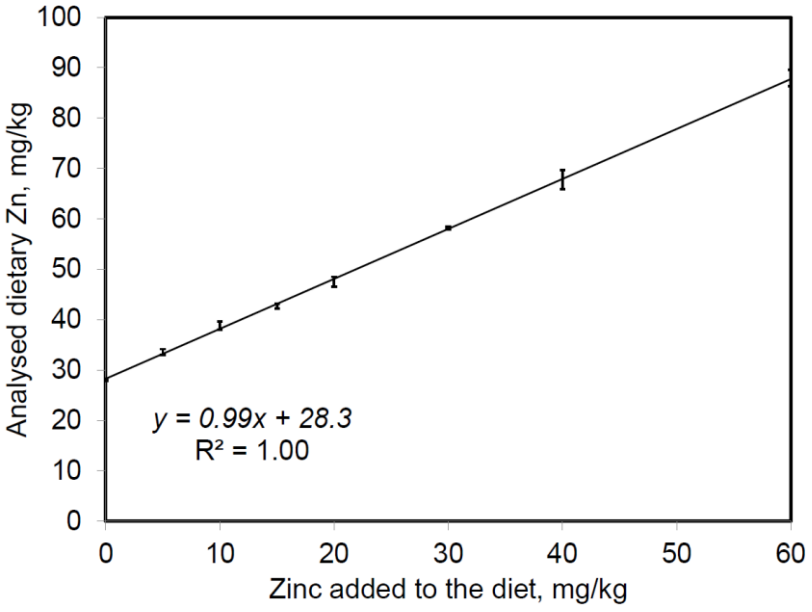

Figure 1: Response of analysed dietary zinc to varying supplementation of zinc from $ZnSO_4 * 7H_2O$ to weaned piglet feed.
Notes: Values represent means of a triplicate analysis of three independent samples per diet (*n = 9*; *N = 72*) ± standard deviation; this figure shows data of Table 2 from *Chapter 2, page 52*; Zn, zinc.

The efficacy of the experimental approach to induce short-term subclinical zinc deficiency in weaned piglets

The overall purpose for the development of an experimental model of SZD was to provide an approach that mimics the potentially predominant Zn malnutrition phenotype within livestock production systems (12). This condition may occur due to fluctuations in the dietary composition (for example by inaccurate mixing of dietary components), reduced feed intake or other stressful events, which may temporarily increase the Zn demand during the production cycle. In contrast to a clinically manifest Zn deficiency, this condition is *per definitionem* characterised by a complete absence of clinical symptoms of Zn deficiency (for example growth retardation, feed refusal, necrosis, (13)) but, at the same time, a Zn supply status dependent adaption of metabolic pathways.

Throughout the present study, all animals remained in good health and developed equally. Simultaneously, Zn status parameters adapted either directly to total bone Zn (e.g. plasma parameters) or in response to the status of Zn homeostatic regulation (significant change between ~50-60 mg Zn/kg diet in the response of apparently-digested feed Zn, liver Zn and hepatic metallothionein 1A (MT1A) and MT2B gene expression (*Chapter 2, pages 65-67*). Taken together, it was concluded that the state of Zn deficiency was of subclinical nature. Indeed, follow-up measurements of parameters of digestive and antioxidative capacity pointed towards first signs of pathophysiological adaption (*Chapter 3, pages 95-96, 100-101*, *Chapter 4, pages 128-131*). This may be interpreted as first clinical symptoms. However, these observations were only recognised after an in-deep analysis of enzyme activities, redox metabolites and transcriptomic adaption post mortem. Nevertheless, no animal expressed any signs of impaired health or productivity throughout the study. Hence, the primary goal of modelling SZD in weaned piglets was successful.

Usability of the model to estimate measures of the gross zinc requirement under given experimental conditions

The question of the efficacy of feeding interventions in livestock rearing is of considerable importance. In case of Zn and essential trace elements in general, this relates directly to the question of bioavailability. The necessary amount of Zn in the diet (gross Zn requirement) is a product of solubility kinetics of native and supplemented Zn species, respectively, as affected by their interaction with other dietary components as well as endogenous secretions within the digestive tract. Using a chemical Zn species with a higher bioavailability compared to a reference species should yield a decrease in the gross Zn requirement. Hence, the animals' Zn requirements could be met with an xfold lower total feed Zn concentration. This can be measured under the terms of a dose-response study, which provides finely-graded differences in total Zn concentrations, ranging from deficient to sufficient supply levels (below and above the putative gross Zn requirement threshold, respectively). Using appropriate status parameters, which significantly change their behaviour in response to deficient and sufficient Zn supply, respectively, the gross Zn requirement threshold can be estimated as statistical breakpoint by applying appropriate regression models (14). Furthermore, relative differences in bioavailability of different supplemented Zn species can be assessed using the slopes in response over deficiently supplied treatment groups as well as the estimated breakpoints (Figure 2).

The experimental approach presented within this thesis has been shown to be appropriate to measure the gross Zn requirement threshold. In this context, the apparently-digested feed Zn revealed to be a robust parameter for the estimation within a linear one-slope broken-line model (*Chapter 2, page 67*). Feeding dietary Zn concentrations ranging from 28.1 (native Zn content) to 88.0 mg Zn/kg diet (+60 mg Zn/kg diet, from $ZnSO_4 * 7H_2O$), linearly increased the apparently digested amount of

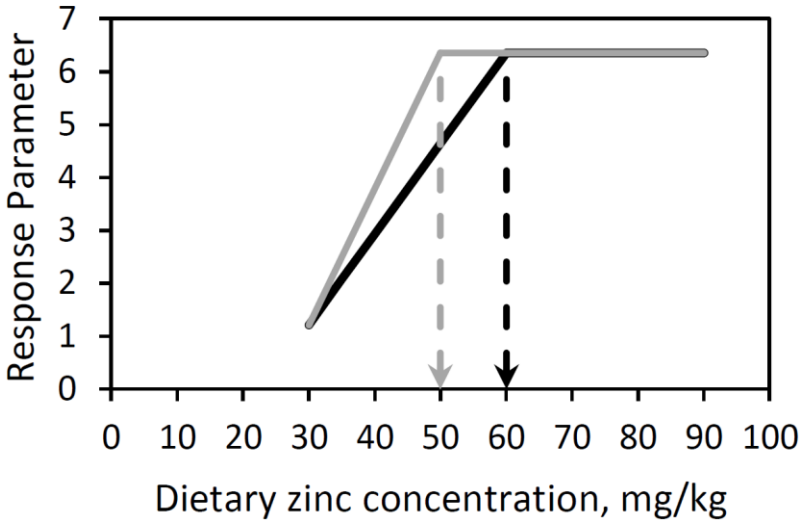

Figure 2: Theoretical competitive broken-line response of a random zinc status parameter to changes in dietary zinc concentration from two different supplemental Zn species. Notes: In this example, the parameter (for example apparently-digested feed zinc) exhibits a plateau in response above dietary thresholds of 60 and 50 mg Zn/kg diet when feeding zinc species A (black) and B (grey), respectively. Below the respective threshold, parameter response to changes in dietary zinc concentration from species A and B decreases/increases by 0.17 and 0.26/mg reduction/rise in dietary zinc, respectively. In conclusion, feeding zinc species B results in a decreased gross zinc requirement (-10 mg zinc/kg diet). Based on the slope comparison, feed zinc species B provided a 1.5-fold higher feed zinc utilisation compared to species A.

feed Zn from -2.69 to +4.38 mg Zn/kg feed intake, over the six lowest supplied groups (28.1-58.2 mg Zn/kg diet). At dietary doses of ≥58.2 mg Zn/kg, the response of apparently-digested feed Zn remained at the average plateau of +4.38 mg Zn/kg feed intake. Indeed, this gross Zn requirement threshold (~58 mg Zn/kg diet) is numerically higher than the average value obtained in earlier studies (~50 mg Zn/kg diet) (1-8). On the one hand, this could account for the fact that the present experimental diet was designed to induce as much pressure as possible on the piglet Zn homeostasis under practical feeding conditions (high amounts of dietary phytate; negligible dietary phytase activity; see *Chapter 2, page 52*). Windisch and Kirchgessner (15) have demonstrated that the true absorption of Zn in ^{65}Zn labelled adult rats tended to zero when applying comparable dietary phytate contents. The data of this doctoral thesis is in good agreement with these findings. Here, piglets which received just the native Zn content (28.1 mg Zn/ kg diet) lost an average of ~1.2 mg Zn/d more than they ingested with the

feed (~12.6 mg Zn intake/d). Hence, the luminal Zn pool within the gastrointestinal tract of these animals seemed to be completely associated to the insoluble fraction, rendering the Zn bioavailability virtually to zero. This strong dietary stimulus might have accounted for the necessity to increase total dietary Zn above 50 mg/kg in order to meet the daily metabolic requirements.

Another plausible explanation relates to the fact that the current dataset regarding the estimation of Zn requirements of piglets is out of date (47-61 years old, (1-8)). With an ongoing selection for higher growth rates and especially higher lean mass of fattening pigs, the potential for Zn retention may have been also increased. The majority of Zn in the body is associated to proteins, with ~10% of the mammal genes coding for Zn peptides (16)). Therefore, the present data highlights the necessity to revaluate the Zn requirement thresholds of modern swine genotypes under practical dietary and physiological conditions.

Modelling SZD compared to clinical Zn deficiency has some advantages for the estimation of gross Zn requirements and feed Zn bioavailability. First, an impairment of health and wellbeing of experimental animals is avoided, which is beneficial regarding ethical concerns related to trials with vertebrate species. Thereby, it eases the legal hurdles when it comes to applications for animal experiments. Secondly, by avoiding a clinical manifestation of Zn deficiency, the degree of secondary metabolic imbalance is drastically reduced. Hence, the resolution of analytical measurements increases due to a reduction of background noise. This may also yield the potential to reduce the necessary number of experimental animals. In fact, using the data from *Chapter 2, page 65-66,* in a statistical power analysis, suggested that the sample size could be reduced from six to four animals per treatment group without losing information (*N =32* piglets; data not shown).

However, the most important advantage arising from working under basal physiological conditions is the practicability of estimations. Data obtained in clinical Zn deficiency models may be significantly biased. In fact, a stepwise increase in body Zn depletion is directly correlated to an increase in absorption efficiency (9, 17). Clinically Zn-deficient animals must not only replenish their stressed Zn stores. They also need surplus Zn to maintain repair mechanisms. Hence, these animals probably express a higher daily Zn demand and associated higher Zn absorption efficiency at the gut barrier than under non-pathological conditions. Therefore, estimations of the Zn feeding efficacy obtained under clinical conditions are unlikely to reflect the situation under normal rearing conditions.

Taken together, the gross Zn requirement of piglets could be measured under practical feeding conditions using the experimental approach presented in *Chapter 2, page 45*. Indeed, every experiment must be reproduced to prove its conclusions. It is noteworthy that this model has been recently applied to test the effect of a specific feed supplement on the gross Zn requirement in weaned piglets. In brief, 96 weaned piglets were challenged with finely graded differences in dietary Zn concentration through varying supplementation with $ZnSO_4$ * H_2O, to a basal diet with the same ingredients as highlighted in *Chapter 2, page 52*. Same-sex litter mates were used as counterparts, with one receiving the supplement and the other being the respective control at given Zn supply status. Thereby, half of the animals received the test substance and the other half did not. A balanced distribution of sex, life weight and litter mates over treatment groups was applied throughout the experiment. Data collection comprised apparently-digested feed Zn, liver Zn as well as hepatic metallothionein gene expression. It was concluded that the results from *Chapter 2* (*pages 65-67*) could have been successfully reproduced and comparable gross Zn requirement thresholds were recognised by

broken-line regression analysis. Furthermore, the response of parameters over deficiently Zn supplied treatment groups has been compared in the presence and without the tested feed supplement, respectively. In summary, it was evident that this test substance reduced the necessary total concentration of dietary Zn from ~60 to ~52 mg/kg, to meet the metabolic requirements of growing piglets under the present experimental conditions. Unfortunately, this data is still unpublished due to ongoing EFSA approval procedures and could, therefore, not be used in detail for this thesis.

Physiological adaption of weaned piglets to short-term subclinical zinc deficiency

Zinc homeostatic regulation

The present knowledge on the physiological adaption to Zn deficiency is mostly based on experiments applying models of clinical Zn deficiency. The data of this doctoral thesis illustrates this condition differs significantly from SZD. Apart from the absence of visible symptoms, the adaptions on the subcellular level appeared to be quite distinct, highlighting patterns of regulation that to our knowledge have not been reported so far.

Comparative screening of ZnT (solute carrier family 30) and ZIP (solute carrier family 39) transporter gene expression in jejunal, colonic, hepatic and nephric tissue indicated significant breakpoints for many transcripts in response to the dietary Zn concentration (*Chapter 6, pages 184-191*). Interestingly, these breakpoints were either in a range of ~60 or ~40 mg Zn/kg diet. The first threshold indicates a relationship to the Zn homeostatic regulation under basal conditions, as it reflects the point of satisfied gross Zn requirement under the terms of the present study (*Chapter 2, page 67*). The second breakpoint relates to thresholds that have been recognised during the investigation of cardiac redox metabolism in response to dietary Zn supply. In fact, cardiac antioxidative capacity declined whereas proapoptotic signalling was upregulated with further decline in dietary Zn concentration below 40 mg Zn/kg diet (*Chapter 4, pages 128-131*). Therefore, it can be concluded that some ZnT and ZIP peptides are not directly responding to the Zn supply status of the organism, but to a stimulation of cellular stress arising during Zn depletion. It further highlights the necessity to include the redox metabolism into any future studies related to the search for diagnostic parameters of an individuals' Zn supply status.

It is important to keep in mind that the threshold of ~40 mg Zn/kg diet does not represent a minimum dietary Zn concentration above which no adverse effects in terms of redox metabolism occur. It is related to the maximum tolerable bone Zn depletion during our experiment. In fact, animals receiving ~40 mg Zn/kg diet and below, showed a reduction in bone Zn concentration between ~20-25% (*Chapter 2, page 65*). This represented depletion of the mobilizable skeletal Zn fraction as has been demonstrated earlier in rats (18-20). A continuation of the study >8d and related further emptying of 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.

Clustering total Zn concentrations of various biological matrices (faeces, blood plasma, bone tissue, soft tissues) by dietary treatment groups (*Chapter 5, page 159*) supported the observations of Zn transporter gene expression (*Chapter 6, pages 184-191*). In fact, the clusters clearly separated sufficiently supplied treatment groups (≥58 mg Zn / kg diet) from those which were deficiently supplied (<58 mg Zn/kg diet). The latter were further separated into groups fed above and below ~43 mg Zn/kg diet, indicating differential physiological adaption after 8 d of dietary treatment. This is in line with the already mentioned data on cardiac antioxidative capacity and proapoptotic signalling (*Chapter 4, pages 128-131*). Interestingly, also the sufficiently supplied treatment groups (≥58.0 mg Zn/kg diet) were further separated in subclusters. Indeed, plasma parameters and certain tissue Zn pools (e.g. bone, pancreas, heart, thymus, liver, kidney, jejunum) indicated a dose-dependent decline with stepwise reduction in dietary Zn supply from 88.0 to 58.0 mg Zn/kg diet (*Chapter 2, page 65, Chapter 3, pages 95-96, Chapter 4, pages 132-133, Chapter 5, pages 156-157)*. Hence all groups fed <88.0 mg Zn/kg diet seemed to have been affected by short-term body Zn depletion, even if their supply level would have delivered sufficient amounts of dietary Zn on a long-term

basis. This phenomenon could be explained by the time-kinetics of Zn homeostatic regulation. The adaption of Zn absorptive processes as well as secretory processes from and into the gut, respectively, under the terms of deficient dietary Zn supply needs ~3-5 d to fully adapt to changing feeding conditions (9-11, 19, 21-23). This means the decrease of body Zn losses to an inevitable amount together with the optimisation of luminal Zn recycling has a lag-time. Hence, every reduction in dietary Zn supply irrespective of its magnitude, has the potential to promote SZD by temporal depletion of body Zn.

Clustering of tissue Zn concentrations by respective biological matrices separated soft tissues involved into Zn homeostatic regulation (jejunum, colon, liver, pancreas) and such that are affected by Zn metabolism. Interestingly, liver and colon were further separated from jejunum and pancreas. In consideration of the ZIP4 gene expression being significantly responding over dietary treatment groups in the colon but not the jejunum (including its correlation to the apparently-digested feed Zn), this may support the hypothesis for a shift of the main absorption site form the upper to the lower intestinal compartments under the terms of SZD. Furthermore, the clustering of colon together with liver might indicate this to be the major route of luminal Zn transfer via the portal vein into the organism under the present experimental conditions. Further studies must support this hypothesis, including a comparative quantification of Zn fluxes over apical membranes in jejunum and colon during SZD.

Data on the relationship between cardiac Zn homeostasis and redox metabolism (*Chapter 4, pages 128-133*) in context to the results on the adaption of body Zn pools (*Chapter 5, pages 156-157, 159*) indicate that other tissues should be further considered when looking for shifts in antioxidative capacity during SZD. Immune tissues (mesenteric lymph nodes, thymus), skeletal muscle and pancreas also exhibited a

stepwise increase in tissue Zn concentration over deficiently supplied groups. Therefore, these tissues may have been also affected by increased cellular stress. This appears plausible, as such organs have a quite high metabolic turnover especially in growing individuals (24). Hence, increased stress levels in these tissues during SZD may have been compensated by restoring their total Zn loads or even accumulation of surplus Zn. This may occur in favour of stress responsive enzyme activity and gene expression. Obviously, any increase in tissue Zn concentrations under Zn deficient conditions is achieved at the expense of other tissues that are triggered to reroute Zn fluxes to the circulation. In the present study, this mainly included bone as the major store of mobilizable body Zn (18-20) as well as pancreas, liver and kidney as important regulators of Zn redistribution and excretion.

Adaption of digestive function and redox metabolism to changes in zinc homeostatic regulation

The overall adaption of Zn homeostatic regulation to insufficient dietary Zn supply appears to have triggered shifts in certain metabolic pathways, including digestion and redox metabolism. A significant adaption of pancreatic digestive enzyme synthesis, apparent faecal dry matter and crude nutrient digestion (*Chapter 3, pages 95-96, 100-101*) as well as cardiac antioxidative capacity and oxidative stress-related gene expression (*Chapter 4, pages 128-131*) has been recognised in response to changes in the Zn supply status. This illustrates that even short periods (~8 d) of insufficient Zn supply must be avoided to maintain metabolic function.

It has been shown earlier, that clinical Zn deficiency is associated with impaired growth. These studies also recognised anorexia and concluded this to be the initiator of growth retardation (17, 25). However, the conclusions of other authors (26, 27) indicate that the growth depression may take place prior to the feed refusal, which has been suggested

indirectly also in the course of this doctoral project. Indeed, a decline in digestive capacity was recognised without any signs of feed refusal or growth depression. This adaption was at least partially correlated to a decrease in the pancreatic Zn status. Unfortunately, technical issues hindered a deeper analysis of pancreas physiology in response to dietary Zn supply. However, earlier published data highlights a close interaction between Zn homeostasis and digestive capacity (26, 28-34). Moreover, it supports the hypothesis that Zn deficiency-associated anorexia may at least in part be related to an enrichment of undigested nutrients within the gastrointestinal tract.

A growing body of evidence suggests a direct relationship between Zn deficiency or excess, respectively, and oxidative stress (35, 36). Zn excess seems to be related to increased metabolism for the synthesis of Zn-binding peptides to quickly excrete the surplus Zn and avoid toxification. For example, Pieper, Martin (37) observed increased cellular stress as a result of higher syntheses of Zn-dependent digestive enzymes and metallothionein during pancreatic Zn accumulation in weaned piglets. On the other hand, Zn deficiency seems to trigger oxidative stress through various modes-of-action (35). This has been demonstrated *in vitro* as well as *in vivo* (38-41). In the present study, we recognised a significant reduction in cardiac glutathione concentration in response to a decrease in the bodies' Zn supply status (*Chapter 4, pages 128-129*). This led to a decrease in cardiac antioxidative capacity as well as an increase in antioxidative and proapoptotic gene expression. The spectrum of significantly upregulated apoptosis genes (B-cell lymphoma 2 associated X protein (BAX), caspase 9) indicates an activation of intrinsic apoptosis mechanisms (42-44), which corresponds to the depletion of α-tocopherol as major detoxification agent of membrane-lipid peroxides (45, 46). It has yet to be studied, what precisely promoted this loss in mitochondrial integrity.

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8th Chapter

Conclusions and outlook

One of the biggest future challenges of feeding zinc (Zn) to monogastric livestock is the increase in precision. On the one hand, a sufficient Zn supply to high-yielding farm animals must be maintained at all stages within the production cycle. On the other hand, too excessive supplementation of Zn must be avoided to obviate negative effects on the environment as well as animal and consumer health (1, 2). Most recently, the European authorities acknowledged this by decreasing the allowed upper limits for Zn in complete feed (3). However, the currently authorised 150 mg Zn/kg piglet feed still equal the ~3fold gross Zn requirements (4, 5). This indicates considerable uncertainty regarding the feed Zn utilisation under varying rearing conditions.

To further reduce the amounts of Zn in feedstuffs for pigs and poultry without impairments of animal health and productivity, the following research questions must be addressed:

1) How does the gross Zn requirement of modern genotypes change under varying dietary and environmental conditions?
2) What are the chemical interactions of Zn with dietary components and endogenous secretions that determine its availability within the gastrointestinal tract (GIT)?
3) What are the molecular parameters and pathways involved in transmitting and receiving information of the bodies Zn supply status?
4) What are suitable diagnostic markers for the evaluation of an individuals' Zn status in the field?

The basic prerequisite to address these tasks are appropriate experimental setups. Therefore, the aim of this doctoral thesis was to develop an experimental model of short-term subclinical Zn deficiency (SZD) in weaned piglets. Further interest was related to the physiological adaption under the terms of this condition.

The measurement of gross Zn requirements implies the necessity to discriminate between deficiently and sufficiently supplied animals under experimental conditions. This can be achieved by following the response of suitable status parameters to varying dietary Zn supply. Such measures must change their response behaviour over Zn doses as soon as the requirements are met. The available literature dictates that under practical feeding conditions of weaned piglets, the satiation of gross Zn requirements could be expected at ~50 mg Zn/kg diet (6-13). In the present doctoral project, a few parameters showed such a response behaviour, including the apparently-digested feed Zn, liver Zn, hepatic metallothionein gene expression, the percentage faecal digestibility of dry matter and crude ash as well as a few soft tissue Zn concentrations and Zn transporter gene expression patterns in jejunum, colon, liver and kidney. However, metabolic parameters for the estimation of Zn requirements should predominantly be affected by the status of whole-body Zn homeostasis and not by secondary metabolic response to Zn depletion or excess. Based on earlier literature, factors which reflect the status of Zn absorption efficiency from the GIT appear to be most suitable. For example, Weigand and Kirchgessner (14) as well as Windisch and Kirchgessner (15) recognised a non-linear response of Zn absorption efficiency from the GIT of rats, which plateaued in sufficiently Zn supplied animals. This demonstrated how the organism is increasing its absorptive capacity in times of deficiency and, on the contrary, the down-regulation of active absorption under the terms of full body Zn stores. These studies also demonstrated that the apparently-digested amount of feed Zn correlates directly and significantly to the true Zn absorption. Therefore, it can be concluded that this parameter and further measures which express a direct functional interaction and correlation to it, are suitable biomarkers for the estimation of gross Zn requirements. This hypothesis is supported by the present dataset, which yielded a gross Zn requirement of ~60 mg Zn/kg diet based on its estimation using the response of

apparently-digested feed Zn (*Chapter 2, page 67*) as well as colonic ZIP4 (solute carrier family 39 member A4) gene expression (*Chapter 6, page 186-187*). Such parameters can also be used to compare the efficacy of Zn feeding interventions (for example differences in bioavailability between Zn sources), which has been shown in a yet unpublished follow-up study (data not shown).

During this project, no signs of clinical Zn deficiency were recognised (for example growth depression, anorexia, tissue necrosis etc., (16)). At the same time, finely graded adaptions of Zn homeostatic parameters as well as secondary metabolic pathways (digestion, redox metabolism) were evident (*Chapter 2, pages 65-67, Chapter 3, pages 95-96, 100-101, Chapter 4, pages 128-133, Chapter 5, pages 156-157, 159, Chapter 6, pages 184-191*). A few of these response patterns have not been reported so far, including Zn accumulation in immune tissues or the response of certain Zn transporters to the dietary Zn supply. This indicates, that the adaption within this early stage of Zn deficiency is not comparable to clinical Zn deficiency. The latter marks the endpoint in response to a completely emptying of mobilizable body Zn stores. This must be considered when planning studies on the estimation of gross Zn requirements or Zn metabolism. The severity of the depletion indicates how intense the homeostatic reaction (especially in terms of Zn absorptive capacity from the gut lumen) as well as the response of secondary metabolic pathways is (decline in functional parameters like digestion and stress response). Hence, the situation after depletion periods >2 weeks and clinical deficiency arising thereof, are not comparable to the situation under basal or subclinical conditions, respectively. Therefore, clinically Zn deficient animals should not be used for the evaluation of feeding regimes for healthy livestock herds. In this context, a reassessment of the currently defined Zn requirements for modern genotypes should be considered because former estimations comprised feeding groups of clinically

deficient animals (6-13). Furthermore, these studies were conducted between 1956 and 1970. Hence, their datasets do not necessarily reflect the metabolic requirements of modern genotypes.

One interesting finding of the present thesis was the observation that certain parameters exhibited a breakpoint in response to dietary Zn supply at ~40 mg Zn/kg diet. Moreover, these response patterns seemed to be related to compensation reactions, as has been demonstrated for the response of cardiac redox metabolism and stress responsive gene expression. Also, several Zn transporter genes in jejunum, colon, liver and kidney of SZD piglets changed their expression levels with further decline in dietary Zn supply below this threshold. This indicates the close connection between Zn homeostasis and redox metabolism. This interaction should be considered regarding the identification of biomarkers for the diagnosis of Zn deficiency in the field.

The identification of diagnostic markers of Zn status further presuppose a deeper understanding of the homeostatic regulation on level of the whole-organism. The tissue-specific response of certain Zn transporter gene expression patterns of the present study obviously reflected a finely-orchestrated regulative hierarchy of whole-body Zn homeostasis. Such a combined response of all body compartments is only possible, if the status of body Zn stores is communicated to respective tissues. The chemical nature of this signal(s) is yet unclear, including the molecules responsible for their transmission and reception. The identification of this signalling pathways represents an important future milestone of Zn biological research.

Another critical issue relates to the chemical interactions of Zn within the GIT. Indeed, a lot of progress has been made in understanding the interaction of phytate with various minerals (17). However, there is still a considerable lack of knowledge of the mode-of-action behind the effects of certain Zn sources within the GIT. This cannot be answered

solely by their solubility kinetics and affinity to transfer Zn^{2+} to phytic acid. Within the last decades, considerable progress has been made in the field of chemical speciation analysis. By combining high-throughput proteomic and metabolomic screening with the research field of "metallomics", it is possible to identify the qualitative and quantitative spectrum of metal species within complex matrices (18). Thereby, the metal loads in molecular fractions and single molecules are determined using liquid chromatography (LC) (and other separation methods) coupled to inductively coupled plasma mass spectrometry (ICP-MS). This data can be used to map the underlying Zn molecules using LC coupled to tandem mass spectrometry for the identification and characterisation of molecular masses. Indeed, this is a complicated and yet cost intensive technology. However, it is currently the only way to identify the complex network of chemical interactions of Zn and other elements along the GIT. In the future, obtaining such data could foster the development of regression models, which may estimate the gross Zn requirements under varying dietary conditions with high precision.

The most impressive observation during this doctoral project was the short-time frame during which compensation reactions were initiated. This led to significant impairments of basic biological mechanisms (digestion, redox metabolism) after only 8 d of insufficient dietary Zn supply. Hence, it appears plausible that temporal phases of fluctuations in feed intake and metabolic demands induce SZD on a regular basis under practical rearing conditions. Especially the first days post-weaning of piglets may be critical in this regard, because this situation is often associated with reduced feed intake and even anorexia. The potential of a temporal Zn deficiency has been already proposed by other authors (19), and may contribute to the pathophysiological adaption during the first two weeks post-weaning (20). The importance and frequency of temporal phases of

SZD for animal production systems might have been underestimated in the past. This issue should be considered during future reassessments of Zn feeding strategies for high-yielding livestock.

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Summary

This doctoral project aimed in the experimental modelling of short-term subclinical zinc (Zn) deficiency (SZD) in weaned piglets, which represents the most widespread phenotype of Zn malnutrition in livestock species. The approach should provide a useful tool for the estimation of reliable measures of gross Zn requirements of monogastric livestock under practical feeding conditions. Further research interest was related to physiological adaption under the terms of SZD; namely effects on digestive function, redox metabolism, body Zn redistribution and Zn transporter gene expression.

Chapter 2 presents the development of the experimental approach and the identification of suitable biomarkers for the Zn supply status under experimental conditions. For this purpose, 48 weaned piglets were fed a practical diet with insufficient native Zn contents that was supplemented with finely-graded levels of additional Zn from $ZnSO_4 * 7H_2O$. Final feed mixtures showed variations in total Zn concentrations, ranging from deficient to sufficient supply levels (28.1, 33.6, 38.8, 42.7, 47.5, 58.2, 67.8, 88.0 mg Zn/kg diet). Exposure to these diets lasted for only eight days to stay within the physiological capability of Zn metabolism to adapt to changes in dietary Zn supply. A total absence of clinical Zn deficiency symptoms (for example growth depression, feed refusal, necrosis of the skin and organs) and, at the same time, finely graded differences in the response of certain Zn status parameters proved the successful induction of SZD in animals fed <58 mg Zn/kg diet. Furthermore, the response of apparently-digested feed Zn, liver Zn concentration as well as relative hepatic metallothionein gene expression exhibited a Zn status dependent pattern, which allowed a differentiation between deficiently and sufficiently supplied animals. This was evident by breakpoints in response of all these parameters close to ~60 mg Zn/kg diet. In conclusion, it was possible to develop an experimental approach that allows studying basal Zn metabolism at high resolution.

The effects of SZD on the digestive capacity of weaned piglets are discussed in *Chapter 3*. By monitoring the activity of activated zymogens and α-amylase within pancreatic tissue homogenates, a direct connection between the Zn supply status and digestive function became evident. This appeared to be partly connected to a decrease in total pancreatic Zn concentration. Furthermore, the decrease in digestive enzymes significantly reduced the coefficients of apparent dry matter and crude nutrient digestion. In conclusion, only eight days of insufficient dietary Zn intake already promoted a decline in digestive capacity of weaned piglets.

Chapter 4 highlights the response of cardiac redox metabolism to SZD in weaned piglets. A reduced Zn supply status promoted a decrease in total cardiac glutathione and associated decline in antioxidative capacity, which was indicated by an impaired ability of the heart muscle to detoxify H_2O_2. This was accompanied by cardiac α-tocopherol depletion and the promotion of antioxidative and proapoptotic gene expression. Monitoring the cardiac Zn concentration in response to the dietary Zn supply indicated a stepwise repletion of cardiac Zn in animals fed <42.7 mg Zn/kg diet and associated upregulation of further stress-responsive genes. In conclusion, the heart muscle as a model for tissues with a high metabolic turnover, already experienced elevated stress levels after just eight days of insufficient dietary Zn supply.

Patterns of body Zn redistribution during short-term SZD in weaned piglets were analysed in *Chapter 5*. This data illustrated a clear hierarchy of Zn redistribution between tissues in response to homeostatic adjustments to an overall shortage of dietary Zn. Within this hierarchy, Zn donors (for example bone, liver, kidney) contrasted against Zn acceptors (for example heart muscle, skeletal muscle, immune tissue). The Zn accepting tissues replenished their Zn concentrations during SZD or even increased them above the average level assessed within control animals (receiving 88.0 mg Zn/kg

diet). This hierarchy was further confirmed using hierarchical cluster analysis of tissue Zn concentrations between biological matrices. Moreover, a cluster analysis of tissue Zn concentrations between treatment groups allowed a discrimination between deficiently (<58.0 mg Zn/kg diet) and sufficiently Zn-supplied animals (≥58.0 mg Zn/kg diet). This represents to our knowledge, the first report of a successful estimation of the Zn supply status according to clusters of tissue Zn concentrations. In summary, the data demonstrates the organism's attempt to maintain the integrity of certain organs for the disadvantage of others during temporal dietary Zn deficiency.

Chapter 6 presents a comparative analysis of gene expression of all known members of the solute carrier families 30 (ZnT) and 39 (ZIP) of Zn transporters in jejunum, colon, liver and kidney during SZD in weaned piglets. The qualitative and quantitative expression patterns were analysed in and between tissues. It was recognised that many of the investigated gene expression patterns exhibited breakpoints in response to changes in dietary Zn supply. These thresholds either lay at ~40 or ~60 mg Zn/kg diet. This indicated clear differences in the respective stimuli to which these genes responded. A breakpoint close to ~60 mg Zn/kg diet (~gross Zn requirement under given experimental conditions) highlighted a role of certain genes in the regulation of Zn fluxes to meet the basal requirements. In contrast, a subset of the investigated Zn transporter genes seemed to be involved in the regulation of Zn fluxes for the compensation of stress and inflammatory processes. This was evident by breakpoints close to ~40 mg Zn/kg diet, which have been reported earlier to also apply to parameters of cardiac antioxidative capacity under these experimental conditions. Taken together, this dataset highlights the molecular adaption of Zn homeostatic regulation, which presumably promoted the aforementioned adaptions on the metabolic level. This demonstrates the efforts of the organism to compensate the negative effects of

inadequate dietary Zn supply. To our knowledge, this represents the first description of a tissue-dependent expression of all known Zn transporter genes in weaned piglets.

Although no visible symptoms were evident, a short-term SZD of weaned piglets was accompanied with significant adaptions on the molecular and metabolic level. This underlines the importance of a constant and sufficient dietary Zn supply especially for growing individuals. Under consideration of the time-scale of the observed adaptions, it seems plausible that such events may occur frequently during piglet production. In this context, the first days post-weaning may be interesting because they are often associated with reduced feed intake. The significance of SZD for animal production may have been underestimated so far and should be considered regarding future reassessments of Zn supplementation strategies.

Zusammenfassung

Diese Dissertation beschäftigte sich mit der experimentellen Modellierung eines kurzfristigen, subklinischen Zinkmangels (SZM) in Absetzferkeln. Dieser Phänotyp stellt die am weitesten verbreitete Form einer Fehlernährung mit Zink (Zn) in Mensch und Tier dar. Damit sollte die methodische Grundlage für verlässliche Schätzungen des Bruttozinkbedarfs von Monogastriden unter praktischen Fütterungsbedingungen geschaffen werden. Weitere Forschungsschwerpunkte lagen auf der Beobachtung der physiologischen Anpassung unter den Bedingungen eines SZM. Dabei standen insbesondere die Verdauungsfunktion, der Redox-Stoffwechsel, die Umverteilung der Körperzinkreservoirs sowie die Expression von Zinktransportergenen im Mittelpunkt.

Kapitel 2 stellt die Entwicklung des experimentellen Modells und die Identifizierung geeigneter Biomarker zur Beurteilung des Zinkversorgungsstatus dar. Dabei wurden 48 Absetzferkeln praxisrelevante Diäten vorgelegt, die feinabgestufte Unterschiede in den Gesamtzinkgehalten infolge einer variierenden Supplementierung mit $ZnSO_4$ * $7H_2O$ aufwiesen. Die verabreichten Zinkdosen deckten den Bereich der defizitären zur bedarfsdeckenden Versorgung ab (28,1, 33,6, 38,8, 42,7, 47,5, 58,2, 67,8, 88,0 mg Zn/kg Futter). Die Fütterung dieser Diäten erfolgte für lediglich acht Tage. Auf diese Weise wurde sichergestellt, die physiologischen Kapazitäten des Organismus, hinsichtlich der Adaption an eine veränderte Zinkversorgung, nicht zu überschreiten. Im Verlauf des Experimentes kam es zu keinerlei offensichtlichen Symptomen eines klinischen Zinkmangels (z.B. Wachstumsdepression, Futterverweigerung, Gewebenekrosen). Gleichzeitig zeigten die untersuchten Zinkstatusparameter eine feinabgestufte Reaktion auf die Futterzinkversorgung. Daraus ließ sich die erfolgreiche Erzeugung eines SZM in Behandlungsgruppen ableiten, die Futterzinkgehalte im Bereich von <58 mg/kg vorgelegt bekamen. Insbesondere die Parameter scheinbar

verdautes Futterzink, Leberzink sowie die Expression von Metallothionein-Genen in der Leber, zeigten ein Regulationsmuster in Abhängigkeit zum Zinkversorgungsstatus. Dadurch war eine Unterscheidung zwischen defizitär und bedarfsdeckend versorgten Behandlungsgruppen möglich. Dies wurde durch statistische Umschlagpunkte in den jeweiligen Reaktionsmustern nahe ~60 mg Zn/kg Futter offenbar.

Kapitel 3 widmet sich den Effekten eines SZM auf die Verdauungskapazität von Absetzferkeln. Die Untersuchung der Aktivitäten der pankreatischen Zymogene und α-Amylase in entsprechenden Gewebehomogenaten, legte einen direkten Zusammenhang zwischen dem Zinkversorgungsstatus und Verdauungsfunktionen nahe. Dieser Zusammenhang schien zumindest teilweise mit einem reduzierten Zinkstatus der Bauchspeicheldrüse in Zusammenhang zu stehen. Des Weiteren konnte nachgewiesen werden, dass der Rückgang an pankreatischen Verdauungsenzymen die scheinbaren Verdauungskoeffizienten der Futtertrockenmasse und Rohnährstoffe beeinträchtigte. Zusammenfassend lässt sich festhalten, dass nach nur acht Tagen einer Zinkmangelernährung negative Auswirkungen auf die Verdauungskapazität von Absetzferkeln nachgewiesen werden konnten.

Kapitel 4 stellt die Anpassung des Redox-Stoffwechsels im Herzmuskel von Absetzferkeln an SZM dar. Die Konzentration von Glutathion im Gewebe nahm, in Abhängigkeit des reduzierten Zinkversorgungsstatus, ab. Dies mündete in einer reduzierten antioxidativen Kapazität des Herzmuskels, was sich an einer deutlich verringerten Fähigkeit zur Detoxifizierung von H_2O_2 äußerte. Diese Befunde wurden durch Beobachtungen einer α-Tocopherol-Depletion im Gewebe und einer erhöhten Expression stressabhängiger Gene begleitet. Die Untersuchung des Herzzinkgehaltes in Abhängigkeit von der Futterzinkversorgung, wies auf eine schrittweise Repletion der Zinkkonzentrationen in Behandlungsgruppen hin, die <42,7 mg Zn/kg Futter erhalten

haben. Dadurch kam es zu einer weiteren Förderung der Expression stress-abhängiger Gene. Zusammenfassend lässt sich festhalten, dass der Herzmuskel als Modell für Gewebe mit hohem Stoffwechselumsatz, nach lediglich acht Tagen defizitärer Futterzinkversorgung, erhöhtem oxidativem Stress ausgesetzt war.

In *Kapitel 5* ist die Umverteilung des Körperzinks in Abhängigkeit von SZM im Absetzferkel dargestellt. Demnach zeigte sich eine deutliche Hierarchie der Zinkumverteilung zwischen Geweben, infolge der homöostatischen Anpassung an eine unzureichende Futterzinkversorgung. Innerhalb dieser Hierarchie konnte zwischen solchen Geweben unterschieden werden, die im Verlauf des SZM Zink aufnahmen (z.B. Herzmuskel, Skelettmuskel, Immungewebe) und solchen, die ihre Zinkversorgung zugunsten dieser Gewebe einschränkten (z.B. Knochen, Leber, Niere). Gewebe die Zink während der Mangelversorgung aufnahmen repletierten damit entweder ihre vormals eingeschränkten Zinkgehalte oder akkumulierten Zink sogar über das durchschnittliche Niveau der Kontrollgruppe (die 88,0 mg Zn/kg Futter erhielt) hinaus. Diese Hierarchie konnte mittels einer hierarchischen Clusteranalyse der Zinkgehalte zwischen den einzelnen Gewebearten bestätigt werden. Darüber hinaus erlaubte eine weitere Clusteranalyse der Gewebezinkgehalte zwischen Behandlungsgruppen, eine Unterscheidung zwischen defizitär (<58,0 mg Zn/kg Futter) und bedarfsdeckend versorgten Tieren (≥58,0 mg Zn/kg Futter). Dies ist nach unserem Kenntnisstand, der erste Bericht über die erfolgreiche Schätzung des Zinkversorgungsstatus auf Basis von Gewebezinkclustern. Zusammenfassend stellen diese Befunde eindrücklich dar, wie der Organismus in Zeiten eines temporären, alimentären Zinkmangels versucht, die Integrität bestimmter Organe zulasten anderer zu gewährleisten.

Kapitel 6 präsentiert eine vergleichende Untersuchung der Genexpression sämtlicher bisher bekannter Vertreter von Zinktransportern der Solute Carrier Familien 30 (ZnT)

und 39 (ZIP), in Jejunum, Colon, Leber und Niere von Absetzferkeln während eines SZM. Dabei wurden die qualitativen und quantitativen Expressionsmuster innerhalb und zwischen Geweben analysiert. Viele der untersuchten Genexpressionsprofile wiesen statistische Umschlagpunkte in Abhängigkeit zur Futterzinkversorgung auf. Diese Schwellenwerte lagen entweder im Bereich von ~40 oder ~60 mg Zn/kg Futter. Daraus lassen sich klare Unterschiede ableiten, hinsichtlich der regulatorischen Stimuli auf die bestimmte Gene reagiert haben. Ein Umschlagpunkt nahe ~60 mg Zn/kg Futter (~Bruttozinkbedarf unter den gegebenen experimentellen Bedingungen) legt eine Funktion in der Regulation der Zinkströme, für die Sättigung des grundlegenden (basalen) Zinkbedarfs, nahe. Im Gegensatz dazu scheinen andere Zinktransporter für die Regulation von Zinkflüssen, zur Kompensation von erhöhtem Zellstress und inflammatorischen Prozessen, beteiligt zu sein. Dies offenbarte sich durch Umschlagpunkte nahe ~40 mg Zn/kg Futter und ist in klarem Kontext zu den Anpassungsreaktionen, von Parametern der antioxidativen Kapazität, im Herzmuskel zu sehen. Zusammenfassend zeigen diese Befunde die Adaption der Zinkhomöostase auf molekularer Ebene auf, die vermutlich in Zusammenhang mit den bereits erwähnten Veränderungen auf Stoffwechselebene steht. Dadurch offenbarten sich recht eindrücklich, die Bestrebungen des Organismus negative Auswirkungen einer unzureichenden Futterzinkversorgung zu kompensieren. Dies stellt nach unserer Kenntnis, die erste Beschreibung der gewebeabhängigen Expression, aller bekannten Zinktransportergene, im Absetzferkel dar.

Obschon in den vorliegenden Untersuchungen keinerlei sichtbare Symptome eines Zinkmangels auftraten, war der kurzfristige SZM von signifikanten Anpassungsreaktionen auf Ebene der molekularen Regulation und des Stoffwechsels begleitet. Dies unterstreicht die Notwendigkeit einer konstanten und ausreichenden

alimentären Zinkversorgung, insbesondere von wachsenden Individuen. Unter Berücksichtigung des Zeitrahmens dieser Beobachtungen erscheint es durchaus plausibel, dass derartige Ereignisse im Verlauf der Ferkelproduktion regelmäßig auftreten können. Dies betrifft insbesondere die ersten Tage nach dem Absetzen und die damit assoziierte, verringerte Futteraufnahme. Die Bedeutung des SZM für die Tierproduktion wurde in der Vergangenheit möglicherweise unterschätzt und sollte künftig, im Rahmen von Neubewertungen der derzeitigen Zinkfütterungsstrategien, berücksichtigt werden.

Appendices

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Author: Daniel Brugger, Marzell Buffler, Wilhelm Windisch

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PERSONAL DATA

Name	Daniel Brugger (M.Sc. (TUM); Dipl. Ing. (FH))
Date of birth	30.07.1981
Birthplace	Munich (Germany)
Web	http://lte.wzw.tum.de/Daniel-Brugger-M-Sc.100.0.html
E-mail	danbrugger@aol.com
	daniel.brugger@wzw.tum.de

EDUCATION

2011 – 2018 Technical University of Munich (TUM) – **Dr. agr.**
Doctoral thesis in animal nutrition on
"Experimental modeling of subclinical zinc deficiency in weaned piglets"
Faculty: "TUM School of Life Sciences Weihenstephan"
Department: Animal Science
Chair: Animal Nutrition
This PhD. project was generously funded by the Bayerische Arbeitsgemeinschaft Tierernährung (BAT) e.V.
Reference: Prof. Dr. Wilhelm Windisch
Tel.: +49 (0) 8161 71 3552
wilhelm.windisch@wzw.tum.de

2009 – 2011 Technical University of Munich (TUM) – **M.Sc. (TUM)**
Master of Science in agricultural sciences with focus on animal biosciences
Faculty: "Center of Life and Food Sciences Weihenstephan"
Master thesis (Chair of Animal Breeding):
"Optimierung der Extraktion von Gesamt-RNA aus bovinen Haarfollikeln zur Quantifizierung der Expression mit Fellpigmentierung assoziierter Gene"
(engl. „Optimization of total RNA extraction from bovine hair follicles to quantify the expression of genes associated with fur pigmentation")
Reference: Prof. Dr. Hans-Rudolf Fries
Tel.: +49 (0) 8161 71 3228
ruedi.fries@tierzucht.tum.de

2003 - 2008 University of Applied Sciences (UAS) Weihenstephan-Triesdorf - **Dipl. Ing. (FH)**
Agricultural engineering with focus on animal production
Diploma thesis (Department of Animal Nutrition):
"Einsatz eines angesäuerten Sammelkolostrums in der Kälberaufzucht"
(engl. „Use of acidified pooled colostrum in calve rearing")
Reference: Prof. Dr. Gerhard Bellof
Tel.: +49 (0) 8161 71 4329

gerhard.bellof@hswt.de

1999 - 2001 Graduation from the specialized secondary school – Fachhochschulreife

1997 - 1999 School of chemistry Dr. Erwin Elhardt

1993 - 1997 Graduation from secondary school – Mittlere Reife

1991 - 1993 Volksschule Planegg

1987 - 1991 Grundschule an der Limesstraße

Military / Community Service

2002 Bavarian Red Cross

Employments

2016 – 2018 Staff scientist at WZP GmbH, responsible for the conduction of research at the Chair of Animal Nutrition, Technical University of Munich **(pending)**
Research activities: Studies on potential health promoting measures of aquatic and terrestrial plant raw materials, including in vitro and in vivo models.
References: Jakob Pröpster
jakob.proepster@wzp-gmbh.com

Prof. Dr. Wilhelm Windisch
Tel.: +49 (0) 8161 71 3552
wilhelm.windisch@wzw.tum.de

2015 – 2018 Staff scientist at the Bavarian State Research Center for Agriculture (LfL), LVFZ Kitzingen **(pending)**
Research project: "The effect of germination on the feed value of grains and legumes"
This project is a collaboration between LfL and TUM and is generously supported by the Bavarian Ministry of Food, Agriculture and Forestry.
Reference: Dr. Klaus Damme
Tel.: +49 (0) 9321 39008-0
klaus.damme@lfl.bayern.de

2011 – 2014 Staff scientist at Technical University of Munich, TUM School of Life Sciences, Chair of Animal Nutrition
Research areas:
In vivo studies on livestock physiology: → trace elements
→ feed additives
→ anti-nutritive factors
→ nutrient requirements
In vitro studies on feed science: → New protein sources

→ Anti-nutritive factors

including: Development of experimental models, planning and conduction of feeding trials, diet design and quality control, feed analysis (nutrients, elements, anti-nutritive factors), gene expression studies, peptide detection and quantification, enzyme activity assays, analysis of oxidative cell damage, statistical analysis and mathematical modeling of biological data.

Reference: Prof. Dr. Wilhelm Windisch
Tel.: +49 (0) 8161 71 3552
wilhelm.windisch@wzw.tum.de

2010 Temporary employment at the Tech Data GmbH & Co. OHG
Collaboration in the IT Service Desk and Data Management.
Reference: Mark Becker
Tel.: +49 (0) 89 4700 0
marc.becker@techdata.de

2009 Temporary employment at the University of Applied Sciences Weihenstephan
Conduction and evaluation of feeding trials.
Reference: Prof. Dr. Gerhard Bellof
Tel.: +49 (0) 8161 71 4329
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2008 Temporary employment at the University of Applied Sciences Weihenstephan.
Evaluation of feeding trial data sets and results documentation.
Reference: Prof. Dr. Gerhard Bellof
Tel.: +49 (0) 8161 71 4329
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2008 Member of the research staff of the Landesanstalt für Landwirtschaft, Forsten und Gartenbau Saxony-Anhalt.
Planning, conduction and statistical evaluation of several feeding experiments in lactating ewes with subsequent results documentation.
Location: Research facility Rohrbeck
Zentrum für Technik und Tierhaltung (ZTT) Iden (Saxony-Anhalt)
Project: "Leistungsgerechte Fütterung der Mutterschafe unter den Bedingungen der Stallhaltung"
Reference: Dr. Gerd Heckenberger, ZTT Iden
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2008 Temporary employment at the Tech Data GmbH & Co. OHG
Collaboration in the IT Service Desk.
Reference: Mark Becker, Tech Data Germany
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marc.becker@techdata.de

2008 Temporary employment at the University of Applied Sciences Weihenstephan

Preparation of feed-, feces- and milk samples.
Reference: Prof. Dr. Gerhard Bellof
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2007 Temporary employment at the University of Applied Sciences Weihenstephan
Preparation of feed-, feces- and milk samples.
Reference: Prof. Dr. Gerhard Bellof
Tel.: +49 (0) 8161 71 4329
gerhard.bellof@hswt.de

Memberships

- Federal Institute for Risk Assessement (BfR) (**Member of the BfR-Commission for Animal Feed and Animal Nutrition 2018-2021**)
- Association of German Agricultural Analytic and Research Institutes (VDLUFA) (**Particicpation in work groups V and VI since 2017**)
- German Society of Minerals and Trace Elements ("Gesellschaft für Mineralstoffe und Spurenelemente", GMS e.V.) (**Member of the Scientific Advisory Board since 2016**)
- Federation of European Societies on Trace Elements and Minerals (FESTEM)
- German Life Sciences Association (VBio)
- Alliance for Life Sciences, Food, Veterinary Expertise and Agriculture (ALVA)
- European Federation of Animal Science (EAAP)

Referee and Associate Editor for peer reviewed journals

- Journal of Nutrition
- British Journal of Nutrition
- Animal Feed Science and Technology
- Archives of Animal Nutrition
- Livestock Science
- Journal of Trace Elements in Medicine and Biology
- Proteome Science

➔ Animal Nutrition (**Member of the Editorial Board since 2017**)

➔ Journal of Zhejiang University-Science B (**Associate Editor since 2017**)

Awards

2012 Poster-Prize on occasion of the annual conference of the German Society of Minerals and Trace Elements (GMS e.V.)
Title: "Using piglets as an animal model: Impact of short-term marginal zinc supply on oxidative stress and cell fate dependent gene expression in the heart muscle"

Trainings

Two-week Livestock husbandry course

Two-week agricultural technology course

ICP-MS Training

BfR-Academy Traning School: Transfer of substances along the food chain

Languages

German:	native language
English:	fluent
French:	basic skills

Capabilities

<u>Animal science:</u>	
Independent planning and conduction of feeding trials	(Advanced Skills)
Design and production of high-precision diets for various animal models (livestock, laboratory)	(Advanced Skills)
Feed quality monitoring	(Advanced Skills)
<u>Experimental/Statistical Skills:</u>	
Experiment design, especially feeding trials	(Advanced Skills)
Statistical analysis of biological data	(Advanced Skills)
Mathematical modeling of biological data	(Advanced Skills)

Laboratory techniques:

Isolation and handling of nucleic acids (gDNA, total RNA)	(Advanced Skills)
Electrophoresis	(Advanced Skills)
Standard and real-time PCR techniques	(Advanced Skills)
Isolation and handling of peptides	(Advanced Skills)
Peptide detection and quantification	(Advanced Skills)
Nutrient and element analysis ((Crude) nutrients, AAS, ICP-MS, HPLC, GC)	(Advanced Skills)
Detection and quantification of anti-nutritive factors	(Advanced Skills)
Measurement of endpoints of oxidative cell damage	(Advanced Skills)
Cell cultures	(Basic Skills)
Histological techniques	(Basic Skills)
Microarrays/DNA chips	(Basic Skills)
Nucleic acid sequencing	(Basic Skills)
Genotyping	(Basic Skills)

Computer Skills:

Microsoft Windows and Office	(Advanced Skills)
Analysis of nucleic acid and peptide sequences	(Advanced Skills)
Online Tools for oligonucleotide design and optimization	(Advanced Skills)
Statistical Software: R, SAS	(Advanced Skills)
Feed Optimization Systems: ZIFOwin, Single Mix	(Advanced Skills)
Programming: HTML, Java Script, C++	(Basic Skills)

Others:

First Aid Training

Driving Licenses: M, A1, B, BE, C, C1, CE, C1E, D1, L, T

Publications list (until March 26th, 2018)

Publications in peer reviewed journals

Brugger D, Windisch W (2018): Tissue-dependent interaction of essential trace-metal concentrations in subclinically zinc deficient weaned piglets. Brit. J. Nutr. (under review)

Brugger D, Hanauer M, Windisch W (2018): Comparative analysis of zinc transporter gene expression in jejunum, colon, liver and kidney of weaned piglets challenged with subclinical zinc deficiency. Brit. J. Nutr. (under review)

Brugger D, Windisch W (2018): The hierarchy of body zinc depletion and redistribution in weaned piglets challenged with subclinical zinc deficiency. Brit. J. Nutr. (under review)

Brugger D, Windisch W (2018): Zinc metabolism of monogastric species and implications for the efficacy of zinc feeding interventions. JZUS-B. (under review)

Brugger D, Schlattl M, Buffler M, Windisch W (2018). Short-term kinetics of tissue zinc exchange in ^{65}Zn-labelled adult rats. Brit. J. Nutr.. (under review)

Brugger D, Schusser B, Matthes J, Meyer K, Shi L, Zhao L, Windisch W (2018): Masson Pine pollen (Pinus massoniana) activated HD11 chicken macrophages in a lipopolysaccharide-like manner in vitro. Brit. J. Nutr.. (under review)

Brugger D, Schlattl M, Buffler M, Bolduan C, Becker C, Windisch W (2018): Effects of Laminaria saccharina supplementation on zootechnical performance and nutrient digestibility in weaned piglets. J. Anim. Sci.. (under review)

Brugger D, Inhuber V, Buffler M, Bolduan C, Becker C, Pinna C, Biaci, G, Windisch W (2018): Effects of laminaria saccharina supplementation on immune gene expression in various tissues of weaning piglets. J. Anim. Sci.. (under review)

Hoffmann D, Brugger D, Windisch W, Thurner S (2017): Calibration model for a near infrared spectroscopy (NIRS) system to control feed quality of soy cake based on feed value assessments in-vitro. Chemical Engineering Transactions. 58. DOI: 10.3303/CET1758064.

Brugger D, Windisch W (2017): Strategies and challenges to increase the precision in feeding zinc to monogastric livestock in light of current feeding practices. Animal Nutrition. doi: 10.16/j.aninu.2017.03.002..

Brugger D, Windisch W (2017): Short-term subclinical zinc deficiency in weaned piglets affects cardiac redox metabolism and zinc concentration. J. Nutr. 147(4): 521-527.

Brugger D, Nadler C, Windisch W, Bolduan C (2016): Feed protein value of acidic precipitates obtained from press juices of three types of green forage leaves. Anim. Feed Sci. Technol. 222: 236-241. doi:10.1016/j.anifeedsci.2016.10.017

Brugger D, Windisch W (2016): Subclinical zinc deficiency impairs pancreatic digestive enzyme activity and digestive capacity of weaned piglets - CORRIGENDUM. Brit. J. Nutr. 116(5): 950-951

Brugger D, Windisch W (2016): Subclinical zinc deficiency impairs pancreatic digestive enzyme activity and digestive capacity of weaned piglets. Brit. J. Nutr. 116(3): 425-433

Brugger D, Windisch W (2015): Environmental responsibilities of livestock feeding using trace mineral supplements. Animal Nutrition. doi:10.1016/j.aninu.2015.08.005

Loibl P, Brugger D, Schedle K, Windisch W, Fahn C (2015): In silico and in vitro evaluation of the potential of maize kernels to inhibit trypsin activity. Anim. Feed Sci. Technol. 207: 289-294.

Brugger D, Hanauer M, Windisch W (2014): Using piglets as an animal model: Preliminary results on the impact of short-term marginal zinc deficiency on zinc acquisition and storage dependent gene expression in jejunal and colonic tissue. Perspectives in Science. 3: 30-31.

Brugger D, Buffler M, Windisch W (2014): Development of an experimental model to assess the bioavailability of zinc in practical piglet diets - CORRIGENDUM. Archives of Animal Nutrition 68(5): 423-424

Brugger, D., Buffler, M., Windisch, W. (2014): Development of an experimental model to assess the bioavailability of zinc in practical piglet diets. Archives of Animal Nutrition 68(2): 73–92.

Windisch, W., Fahn, C., Brugger, D., Deml, M., Buffler, M. (2013): Strategies for sustainable animal nutrition. Züchtungskunde 85(1): 40–53, ISSN 0044-5401.

Abstracts and articles in conference proceedings

Brugger D, Windisch W (2018): Cluster analyses on the adaption of zinc reservoirs in weaned piglets challenged with short-term finely-graded reduction in dietary zinc supply. Proc. Soc. Nutr. Physiol. (27) (in press).

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

Brugger D, Schusser B, Schmidt E, Voss L, Shi L, Zhao L, Windisch W (2018): Effects of Masson Pine pollen (Pinus massoniana) on cytokine gene expression in HD11 chicken macrophages in vitro. Proc. Soc. Nutr. Physiol. (27) (in press).

Brugger D, Buffler M, Urban P, Windisch W, Bolduan C (2018): Zur Korrelation zwischen der hämagglutinierenden Aktivität und dem Phasingehalt von Stangenbohnen (Phaseolus vulgaris). In: Verband deutscher landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA). VDLUFA-Schriftenreihe 74/2017, VDLUFA-Verlag, Darmstadt (in press).

Schlattl M, Windisch W, Brugger D (2017): Experimental modelling of subclinical zinc deficiency in ruminants. In: Luksch C (edt.): 3rd HEZagrar Ph.D. Symposium. Freising (Germany), April 25, 2017, pp. 67-69.

Brugger D, Hanauer M, Ortner J, Windisch W (2017): Regulation der Zinkhomöostase im Absetzferkel in Verlauf eines kurzfristigen, subklinischen Zinkmangels. In: Hans Eisenmann-Zentrum (edt.): Herausforderung Klimawandel. 8. Agrarwissenschaftliches Symposium. September 21 2017, p 65-68.

Brugger D, Inhuber V, Obermeier S, Windisch W, Damme K (2017): Interaktionen zwischen Kohlenhydrat- und Proteinstoffwechsel von Getreiden und Leguminosen im Verlauf der Keimung in vitro. In: Hans Eisenmann-Zentrum (edt.): Herausforderung Klimawandel. 8. Agrarwissenschaftliches Symposium. September 21 2017, p 61-64.

Brugger D, Ettle T, Mangert S, Windisch W (2017): Eine Absenkung des Verhältnisses von neutraler Detergentienfaser zu leichtlöslichen Kohlenhydraten induziert Kompensationsreaktionen im Pansenepithel wachsender Bullen der Rasse Bayerisches Fleckvieh. In: Hans Eisenmann-Zentrum (edt.): Herausforderung Klimawandel. 8. Agrarwissenschaftliches Symposium. September 21 2017, p 69-71.

Puntigam R, Brugger D, Schedle K, Schwarz C, Hechenberger P, Eipper J, Gierus M (2017): Einfluss einer druckhydrothermischen Futtermittelbehandlung der Einzelkomponente Mais auf die ileale Proteinverwertung von Broilern in der Angfangsmast. In: Buffler M, Windisch W (Hrsg.) (2017): Phosphor – Bedarf decken, Überschüsse vermeiden. 55. Jahrestagung der Bayerischen Arbeitsgemeinschaft Tierernährung e.V., ISBN 978-3-9816116-4-9: 211-215.

Brugger D, Stäbler R, Thamm C, Riedl A, Obermeier S, Windisch W, Damme K (2017): Zum Einfluss der Keimung auf die Eiweißwertigkeit von Getreiden und Leguminosen. In: Arbeitsgemeinschaft für Lebensmittel-, Veterinär- und Agrarwesen (ALVA) e.V. (2017). Tagungsbericht 2017. ISSN 1606-612X. pp. 247-249

Brugger D, Mayer K, Eberdorfer D, Windisch W, Bolduan C (2017): Untersuchungen zur Korrelation zwischen Rohproteingehalt und Trypsininhibitor-Aktivität von Maiskörnern (Zea mays) bei unterschiedlichem Fraßdruck auf dem Feld. In: Verband deutscher landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA). VDLUFA-Schriftenreihe 73/2016, VDLUFA-Verlag, Darmstadt, pp. 508-515. ISBN: 978-3-941273-23-8.

Brugger D, Buffler M, Windisch W, Bolduan C (2017): Untersuchungen zum antinutritiven Potential von Gartenbohnen (Phaseolus vulgaris). In: Verband deutscher landwirtschaftlicher Untersuchungs- und Forschungsanstalten

(VDLUFA). VDLUFA-Schriftenreihe 73/2016, VDLUFA-Verlag, Darmstadt, pp. 500-507. ISBN: 978-3-941273-23-8.

Brugger D, Ettle T, Windisch W, Bolduan C (2017): Effect of varying dietary supply with neutral detergent fiber, starch and sugar to fattening bulls on post-mortem endpoints of ruminal fermentation and glutathione metabolites within rumen papillae. - In: Proc. Soc. Nutr. Physiol. (26), Göttingen, 14.-16.03.2017. Ed.: Gesellschaft für Ernährungsphysiologie. Frankfurt: DLG-Verlags-GmbH, p. 42. ISBN: 978-3-7690-4109-5.

Paulicks B, Eisen E, Becker C, Brugger D, Windisch W (2017): Growth performance and digestive capacity of mealworms (Tenebrio molitor L.) fed with low or high fiber diets. - In: Proc. Soc. Nutr. Physiol. (26), Göttingen, 14.-16.03.2017. Ed.: Gesellschaft für Ernährungsphysiologie. Frankfurt: DLG-Verlags-GmbH, p. 75. ISBN: 978-3-7690-4109-5.

Windisch W, Buffler M. Brugger G (2017): Spurenelementbedarf beim Schwein. In: Tagungsband „Forum angewandte Forschung in der Rinder- und Schweinefütterung“ 21.-22.03.2017, VLK, Bonn, pp. 125-132.

Windisch W, Brugger D, Buffler M (2016): Genetische Modulation der Ernährungsphysiologie. In: 26. Hülsenberger Gespräche 2016 – Die postgenomische Ära: Die Renaissance des Phänotyps, Heigener Europrint GmbH, Hamburg, pp. 103-110.

Brugger D (2016): Physiological adaption to subclinical zinc deficiency in the weaned piglet model. In: Luksch C (edt.): 2nd HEZagrar PhD symposium, Freising (Germany), April 26 2016, p 11-12.

Brugger D, Stäbler R, Obermeier S, Windisch W, Damme K (2016): Untersuchungen zur Veränderung des Futterwerts von Getreide und Leguminosen im Verlauf der Keimung. - In: Arbeitsgemeinschaft für Lebensmittel-, Veterinär- und Agrarwesen - „Eiweißpflanzen - Strategien und Chancen für Landwirtschaft und Industrie”; ALVA-Jahrestagung 2016, Klagenfurt, 30.-31. Mai 2016; p 182-184. ISSN: 1606-612X.

Brugger D, Ettle T, Feser S, Windisch W, Bolduan C (2016): Post mortem endpoints of ruminal fermentation and anion/proton transporter gene expression as affected by variations in the amounts of physically effective neutral detergent fibre in the diets of growing German Fleckvieh bulls. - In: Proc. Soc. Nutr. Physiol. (25), Hannover, 08.-10.03.2016. Ed.: Gesellschaft für Ernährungsphysiologie. Frankfurt: DLG-Verlags-GmbH, p. 17. ISBN: 978-3-7690-4109-5.

Brugger D (2015): Short-term experimental modelling of zinc status in weaned piglets. In: Luksch C (edt.): 1st HEZagrar PhD Symposium, Freising (Germany), April 21 2015, p 45-46.

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