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LIVESTOCK RESEARCH
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Samenvatting NL
Dit onderzoek is uitgevoerd om de invloed te bepalen van de hoogte (15-160 mg/kg voer) en duur (2-8 weken) van gebruik van een kopersupplement in het voer van gespeende biggen op de groeiprestaties, mestconsistentie en expressie van genen gerelateerd aan de koperabsorptie vanuit verschillende segmenten van de dunne darm.

Summary UK
This study was conducted to determine the influence of level (15-160 mg/kg voer)and period (2-8 weeks) of supply of a Cu-supplement on growth performance and expression of Cu absorption-related genes in different segments of the small intestine of weaned pigs.

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Foreword

This research was initiated and financed as a joint project of the Dutch Product Board Animal Feed (PDV) and the Dutch Ministry of Economic Affairs (EZ). Their financial contribution is gratefully acknowledged. The project was supervised by a working group of representatives of these two sponsors. The persons involved were E.R. Deckers (EZ), H. Boelrijk (LTO), M.C. Blok, A. Cazemier, F.A.J. Gort, M. Hessing, C.A. Makkink, J. Michels, and A. van Wesel (PDV). After the development of the PPO Feed4Foodure, supervision was conducted by the members of the MMM5 project team focussing on research into copper and zinc utilisation. We highly appreciated the stimulating discussions and valuable suggestions in order to successfully conduct this research project.

Paul Bikker

Summary

Introduction
Inclusion of pharmacological levels of copper (150 to 250 mg Cu/kg) in diets of weaned pigs generally increases feed intake and daily gain. The majority of this copper is excreted in the manure, thus contributing to accumulation in soil, ground and surface waters. Therefore, this study was conducted to determine the influence of level and duration of a growth-promoting Cu supplement within EC legal limits, with a maximum inclusion level of 170 mg/kg in diets for growing pigs as allowed until 12 weeks of age.

Experimental design
The experiment was conducted with newly weaned piglets from 4 to 12 weeks of age at the research farm Sterksel of Wageningen UR, the Netherlands. It comprised 8 treatments with 10 replicates (pens) per treatment and 10 piglets (with equal numbers of females and castrates) per pen. In the experimental period from Day 0-56 post weaning, pigs of Treatment 1 to 4 received diets supplemented with 15, 80, 120 and 160 mg Cu per kg from $CuSO_4$ during the experimental period of 8 weeks post-weaning to determine the dose effect of Cu inclusion. The trial was replicated over time. In the last four replicates, 2 pigs from each pen of treatments 1, 2 and 4 were sacrificed at Day 56 to harvest tissue of the liver and several parts of the small intestine. Pigs of Treatments 5 to 7 received a diet supplemented with 160 mg Cu/kg during a period of either 40, 28 or 14 days post-weaning, respectively, followed by a diet supplemented with 15 mg Cu/kg to determine the effect of duration of the high Cu supplement. Pigs of Treatment 8 received only the high Cu supplement during Day 0-14 and Day 40-56 post-weaning, after transfer to the growing-finishing unit. All experimental diets contained 500 FTU/kg of phytase and an addition of 45 mg zinc/kg. From Day 56 to slaughter, all pigs received the same commercial grower and finisher diet supplemented with 10-15 mg Cu/kg.

Observations
The following performance parameters were determined: body weight and feed intake per pen were determined in two-week periods to calculate the average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR). Faecal consistency was recorded per pen two times a week. Two pigs of the last four replicate pens of treatments 1, 2 and 4 (receiving 15, 80 and 160 mg Cu/kg, respectively) were sacrificed on Day 56 and plasma Cu level, liver Cu and Zn content. Furthermore, the mRNA abundance of genes involved in intestinal Cu and/or Zn absorption were determined along the small intestine by real-time quantitative PCR. Nutrient and mineral contents of diets, serum and body tissue were analysed according to standard procedures. Data were analysed with analysis of variance for eight individual treatments, whereas linear and quadratic effects of dietary copper level (i.e. Treatments 1-4) were additionally determined.

Influence of level of copper supplementation
The Cu supplement linearly enhanced the daily gain in each of the 2-week periods, in the 40-day nursery period and in the 56-day period until 12 weeks of age, being the legal limit to the period of inclusion in the EU. A linear increase was also found in feed intake until Day 40 and a linear improvement in FCR until Day 28. Overall, in the nursery period, approximately 75% of the effect on growth rate could be explained by an increase in feed intake. Compared to T1 (15 mg Cu/kg), the highest Cu supplementation (160 mg Cu/kg) increased the BW at the end of the nursery period with 2.8 kg and at the end of the treatment period (Day 56) with 3.4 kg. The increase in Cu supplementation reduced the percentage of piglets with soft faeces from 10-15% to approximately 5% This effect was most pronounced during Day 0-28 post-weaning.

Influence of duration of high copper supplementation
Each early withdrawal of the high Cu supplement in the nursery period resulted in a reduction in growth rate. This effect was most drastic, a transient loss of 20% in ADG, after withdrawal on Day 14 post-weaning. The effect was relatively small and of short duration after withdrawal on Day 28. Early

withdrawal also enhanced the percentage of pigs with soft faeces, with most dramatic effects after withdrawal on Day 14 in the subsequent 2-week period. Inclusion of the high Cu supplement in the first 2 weeks of the grower period (Day 40-56) increased pig performance in this period and numerically increased the growth rate in the entire grower period from Day 40-70.

Discussion and conclusions

Overall, the highest performance and lowest percentage of growing pigs with soft faeces was realised when 160 mg Cu/kg was supplied in the entire experimental period (T4), indicating that any reduction in level or duration of Cu supplementation causes a small, but significant, reduction in both animal performance and faecal consistency.

The reduction in level or duration of the Cu supplementation did not significantly affect the mortality and required medical treatments. Nonetheless, the number of medicated piglets was numerically higher at some of the lower levels and periods of Cu supplementation, thus a possible effect cannot be fully excluded and should be taken into consideration.

Compared to T4, relatively good results were realised when growing pigs were fed 120 mg Cu/kg from Day 0-56 (T3) and in pigs fed the 160 mg Cu/kg until Day 28 with subsequent adaptation to a low Cu diet in the nursery period (T6). Because of the large reduction in usage and excretion of Cu, this would reward further evaluation.

Most Cu- and Zn-related absorption genes are more abundantly expressed in the upper part of the small intestine, indicating that this part is the major site for uptake of Cu and Zn.
In line with this, dietary Cu supplementation increased the level of duodenal metallothioneine (MT1A) mRNA and pigs receiving low Cu content displayed more SLC11A2/DMT1 and SLC30A5 mRNA in the duodenum and proximal jejunum, respectively, probably to increase the capacity of intestinal metal transport. Moreover, SLC31A1/Ctr1, SLC11A2/DMT1 and SLC36A1 were more abundantly expressed in the upper small intestine in male growing pigs, which may contribute to the lower feed conversion rate in male growing pigs comparted to females after the growing fase.

It should be emphasised that in this experiment, no alternative health supporting products (feed additives) were included in the diet when the Cu content was reduced to compensate for the effect of the Cu-withdrawal. Hence, this study does not advice whether such products might alleviate the effect of a reduction in level or duration of Cu supplementation.

1 Introduction

Copper (Cu) is an essential trace element for pigs, mainly as a component of a large number of metabolic enzymes. As suggested by Suttle (2010), the Cu requirement of growing pigs is relatively low, 4-6 mg/kg, and may be met by the Cu in commonly used feed ingredients in pig diets. Bikker et al. (2011) did not observe any effect on growth performance of weaned pigs when 0-18 mg dietary Cu was added to a basal diet containing 7 mg Cu from the ingredients, thus confirming the conclusion of Suttle (2010). Bikker and Jongbloed (2014) recommend a dietary Cu content of 12 mg/kg for growing pigs. Nonetheless, a number of studies have shown that a Cu supplementation at pharmacological levels (125-250 mg/kg) drastically improves growth performance and faecal consistency in weaned pigs (Cromwell et al., 1989; Jongbloed et al., 2011; Bikker et al., 2011). Because of the low body retention of Cu of approximately 1.1 mg/kg body weight (BW) (Corpen, 2003), the majority is excreted into the manure. After manure spreading, this amount of Cu substantially contributes to the accumulation of Cu in the soil and in ground and surface water. The general aim of this project was to study possibilities to reduce the inclusion of Cu in diets for weaned piglets without loss in performance and health of the animals.

Several authors have studied the influence of the dosage of Cu supplementation in growing pigs. From these studies, Jongbloed et al. (2011) determined an optimal supplementation of 146 and 150 mg/kg for daily gain and ADFI. This study suggested that a reduction of Cu supplementation from 160 mg/kg, as commonly applied within the EU, to 100 mg/kg would reduce the growth rate by only 6 g/d. This potentially large reduction in Cu usage at the expense of a relatively small reduction in performance would reward further validation of the effect of this reduction. Furthermore, the consequences of this reduction in dietary Cu content for faecal consistency of pigs were not reported and thus require further study. We are not aware of studies have been published in which the duration of high Cu supplementation in the nursery period has been studied. Nonetheless, because of the rapid increase in feed intake of growing pigs in the period from 4 to 12 weeks of age, a shorter period of Cu supplementation would substantially reduce the total Cu consumption and excretion. Therefore, in this project the influence of both the level and duration of Cu supplementation was investigated, in combination with the effect of Cu withdrawal from the diet on the performance of the growing pigs.

Despite the consistent effect of a high Cu supplement in diets of weaned pigs, limited insight is available in the mechanisms involved (NRC, 2012). Suggested mechanisms include the anti-microbial effect of Cu (Fuller et al., 1960), a systemic action of absorbed Cu (Zhou et al., 1994), an effect on hormone production in the intermediary metabolism (Li et al., 2008), an effect on appetite regulating genes in the hypothalamus (Yang et al., 2011), an effect of ghrelin production in the stomach (Yang et al. (2012) and an effect on lipase and phospholipase A activity and fat digestibility (Luo and Dove, 1996). Limited information is available on the regulation of Cu absorption from the small intestine in relation to high Cu supply, and the interaction with zinc (Zn) absorption. Therefore, we also determined the expression levels of genes related to the transport of Cu and Zn across the mucosa of the small intestine in a selection of pigs in this study.

1.1 Objectives

The objectives of this study were to:

- determine the effect of level and duration of a pharmacological Cu supplement on performance and health of newly weaned pigs;
- determine the effect of the withdrawal of the Cu supplement on performance and health of the pigs;
- determine the effect of the supplement on Cu level in plasma and liver;
- determine the expression of Cu- and Zn absorption-related genes in various segments of the small intestine.

2 Material and Methods

The protocol of this study was approved by the ethical committee (DEC) of Wageningen UR Livestock Research (DRS code 2013001).

2.1 Experimental animals

This study was conducted with weaned pigs, equal numbers of entire male and female animals of Tempo x (Dutch Landrace x Great Yorkshire) breed from the closed herd of the research farm Sterksel of Wageningen UR. Healthy animals with a weaning weight of 6-10 kg at about 27 days of age were selected for this experiment. Piglets showing signs of ill-health, injury or being in poor condition were excluded from the selection process. The total number of animals was 832 piglets, selected from three weaning batches (periods). The piglets were uniquely identified by plastic ear-tags.

2.2 Experimental treatments and design

The experiment comprised 8 treatments with 10 replicates (pens) per treatment and 10 piglets per pen. The experiment was replicated over time in three successive periods, with 3, 3, and 4 replicates, respectively. At the day of weaning (Day 0) piglets were blocked on the basis of sex, weaning weight and litter of origin and randomly allocated to pens within replicate such that pens had similar mean body weight. The eight pens of a replicate were housed in one room in which pens were randomly allocated to the eight treatments. The dietary treatments lasted 8 weeks, from 0 to 56 days post-weaning. Thereafter all pigs received the same grower and finisher diets with 15 mg Cu/kg.

The experimental treatments (T1 to T8) have been summarized in Table 1. T1 and T4 were negative and positive control treatment with 15 and 160 mg/kg Cu added to the diets during the entire 8-week period, respectively. The latter represents the period up to 12 weeks of age in which the growth-promoting dose of Cu is allowed in the EU. T1 to T4 represent a dose-response study with incremental Cu supplements of 15, 80, 120 and 160 mg Cu/kg for 8 weeks, respectively. T5 to T8 represent a shorter duration of the Cu supplementation for only 6, 4 or 2 weeks, respectively. T8 received the Cu supplement during the first 2 weeks of the nursery period and the first 2 weeks of the grower period in which the pigs were housed in the grow-finish unit.

Tabel 1

Dietary treatments (T1 to T8) with added Cu content in 2-week periods[1)]

Added Cu, mg/kg	T1	T2	T3	T4	T5	T6	T7	T8
Day 0-14	15	80	120	160	160	160	160	160
Day 14-28	15	80	120	160	160	160	15	15
Day 28-40	15	80	120	160	160	15	15	15
Day 40-56	15	80	120	160	15	15	15	160
Day 56-slaughter	15	15	15	15	15	15	15	15

[1)]All diets contained 5-8 mg/kg Cu from the ingredients

In order to determine possible carry-over effects of the treatments in the experimental nursery period on performance and health in the subsequent grower-finisher period, the performance of the pigs of all treatments was determined from day 56 post weaning to slaughter. For this purpose, the pigs of each pen of these treatments in the nursery phase were placed together in one pen in the grow-finish unit. Thus, the experimental unit was the same in the nursery phase and in the growing-finishing phase. In each of the two periods, the pigs in a replicate were housed together in the same room.

2.3 Experimental diets and feeding

The experiment was conducted in a typical house for weaned piglets in climate-controlled rooms with a central corridor and 4 pens on each side. The pens had a surface area of 4.8 m^2 (0.6 m^2 per piglet based on 8 piglets/pen) with plastic coated mesh floors, plastic fences and feeders of aluminium or stainless steel. Environmental conditions during the trial (temperature and ventilation rate) were computer-controlled and appropriate for the age of the piglets. Standard management and husbandry practices were applied throughout the experiment under the control of experienced personnel. The piglets were monitored daily for any abnormalities, such as abnormal behaviour and clinical signs of sickness. All deviations from normal and required medical treatments were recorded. Piglets were individually medicated only if necessary in case of poor health. Any piglet removed from the trial because of poor health or death was weighed and the date and reason for removal recorded, based on judgement of the farm staff.

2.4 Housing and management

The experiment was conducted in a typical house for weaned piglets in climate-controlled rooms with a central corridor and 4 pens on each side. The pens had a surface area of 4.8 m^2 (0.6 m^2 per piglet based on 8 piglets/pen) with plastic coated mesh floors, plastic fences and feeders of aluminium or stainless steel. Environmental conditions during the trial (temperature and ventilation rate) were computer-controlled and appropriate for the age of the piglets. Standard management and husbandry practices were applied throughout the experiment under the control of experienced personnel. The piglets were monitored daily for any abnormalities, such as abnormal behaviour and clinical signs of sickness. All deviations from normal and required medical treatments were recorded. Piglets were individually medicated only if necessary in case of poor health. Any piglet removed from the trial because of poor health or death was weighed and the date and reason for removal recorded, based on judgement of the farm staff.

2.5 Observations during the study

2.5.1 Performance and health

Individual body weight of the piglets was recorded at Day 0 (weaning, i.e. start of the trial period), Day 14 (diet change-over from pre-starter to starter diets), Day 28, Day 40 (change-over from nursery to grow-finish unit), Day 56 (end of dietary treatment period), Day 70 (change-over from grower to finisher diets) and prior to slaughter. Feed residuals were recorded on these days as well. From these data average daily feed intake (ADFI), average daily gain (ADG), and feed conversion ratio (FCR) were calculated for the two-week periods Day 0-14, Day 15-28, Day 28-40, and Day 40-56, the complete experimental period Day 0-56 and the subsequent periods Day 56-70 and Day 56 to slaughter. Faecal consistency was recorded twice each week. In each pen, the pigs were categorised in one of three categories: normal faeces, soft faeces and watery diarrhoea. The three observations per week were combined per pen to obtain the mean observation per week. Furthermore, signs of poor health, biting of ears, tails and other body parts, and required medical treatments were registered daily.

2.5.2 Blood and body tissues

On Day 56, a selection of 2 pigs with representative body weight pigs (one male and female) per pen in the 4 replicate pens of Treatments 1, 2 and 4 (15, 80 and 160 mg added Cu/kg) in period 3 was sacrificed to collect samples of blood, bile, and tissue of liver, kidney, adrenal glands, duodenum, proximal and distal jejunum, and ileum as described hereafter.

The growing pigs were killed by an intra-venal injection of euthasol® (pentobarbital sodium and phenytoin sodium Solution, dose 1.2 mL/10 kg body weight) and exsanguinated via the arteria axillaris. Blood samples were obtained by vena puncture of the vena jugularis in a serum and PAX gene tube. After leaving the blood for 30-45 minutes at room temperature, serum was obtained by centrifugation

and stored at -20°C until analysis of Cu. The thoracic and abdominal cavity was opened, and bile was collected with a syringe and stored in plastic 15 mL tubes at -20°C.
After removal of the gallbladder, liver, kidneys, adrenal glands, and small intestinal tract were taken out. The liver was divided in two halves and stored at -20°C for further analysis. Furthermore, tissue samples (5-10 g) of liver, kidney cortex and the complete adrenal glands were collected in micronic tubes, immediately snap frozen in liquid nitrogen and stored at -80°C until further analysis.

The small intestinal tract was dissected as follows: the duodenum (at 1/2 of length), proximal jejunum (at 1/3 of length), distal jejunum (at 2/3 of length), ileum (at 1/2 of length) were isolated and longitudinally cut open. Luminal contents of the proximal and distal jejunum and colon were collected in 50 mL falcon tubes and frozen at -20°C. Subsequently, intestinal sections were cleaned from its (residual) luminal contents with tap water and mucosa of duodenum, proximal and distal jejunum and ileum were gently removed with a sterile microscope slide and individually collected in micronic tubes, rapidly snapfrozen in liquid nitrogen and stored at -80°C until gene expression analysis. From the same segments, an intact piece of approximately 2x2 cm of the gut was harvested, pinned on cork, fixated in a buffered formalin solution and stored in 70% ethanol at 4 °C degrees.

As a reference group, on Day 0 (i.e. at the start of replicate 3) an additional 8 representative weaned piglets, with equal numbers of males and females, were selected and sacrificed to harvest the same samples as described above.

2.6 Description of analytical methods

Dry matter, copper and zinc content were analysed in all experimental diets. The proximate composition was determined in the basal pre-starter, starter and grower diets without added copper. After grinding, diets were analysed using official methods described to determine moisture (dry matter), nitrogen (crude protein), ether extract, ash, crude fibre, starch, Cu, and Zn. Briefly, dry matter was determined by drying to a constant weight at 103°C (ISO, 6496). Crude protein was calculated as N * 6.25 after N was measured using the Kjeldahl method with $CuSO_4$ as catalyst (ISO 5983). Ether extract (crude fat) was determined gravimetrically after hydrolysis with HCl and extraction with light petroleum (boiling point 40-60°C) (ISO, 6492). Crude ash was determined by combustion to a constant weight at 550°C (ISO, 5984). Crude fibre was gravimetrically determined as the remaining insoluble organic fraction after acid and alkaline digestion of the sample (ISO, 6865). Starch was enzymatically determined according to ISO 15914. Samples were first extracted with 40% ethanol to remove sugars, followed by a two steps hydrolysis with DMSO at 100°C and concentrated HCl at 60°C. The dissolvent was quantitatively converted into glucose by amyloglucosidase and spectrometrically measured at 340 nm using the hexokinase method. Cu and Zn were determined after combustion of a 5 g sample at 550°C for 4 hours, dissolving the ash residue in HCl and subsequent analyses with ICP AES (Perkin Elmer Optima 7300 DV).

For serum Cu analysis, serum (0.5 mL) was diluted with 4.5 mL diluent (0.1% Triton X-100 in 0.05 % HNO_3) and measured against standards with ICP-OES (Inductively Coupled Plasma Atomic Emission Spectroscopy) (Perkin Elmer 3300DV).

For hepatic Cu and Zn analysis, one half of the liver was defrosted, then ground (Robocoupe R3.3000 3.7 L) and subsampled for further analysis. A subsample of approximately 5 g was dried at 103°C for four hours and the moisture content was calculated. Cu and Zn were determined with ICP AES (Perkin Elmer Optima 7300 DV) in the freshly ground sample after treatment with a mixture of perchloric acid and sulphuric acid at 300°C. For bile analysis, Cu was determined with ICP AES (Perkin Elmer Optima 7300 DV) in 1 g of homogenised sample after treatment with a mixture of perchloric acid and sulphuric acid at 300°C.

For gene expression analysis, frozen mucosa scrapings were ground in liquid nitrogen with pestle and mortar. Total RNA was extracted using Trizol Reagent (Life Technologies, Bleiswijk, The Netherlands) and subsequently subjected to an on-column DNase digestion to eliminate DNA contamination (NucleoSpin RNA II kit; Macherey-Nagel GmbH & Co. KG, Düren, Germany). Quality of RNA was

assessed using a 2100 Bioanalyzer and RNA 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, CA). To quantify mRNA expression, cDNA was synthesized from total RNA with Superscript® III (Life technologies) in the presence of random random hexamers and dNTPs according to the manufacturer's protocol and subjected to SYBR® Green real-time PCR using a ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Amplification conditions consisted of 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 5 s, and 72°C for 5 s. Specific primers were designed using Primer Express® Software (Life Technologies). Melting curve analysis and fractionation of the qPCR products on an ethidum bromide-stained agarose gel confirmed primer specificity. Quantitative mRNA measurement was performed by establishing a linear calibration curve using 10-fold serial dilutions of cDNA template for corresponding genes. Absolute expression levels were normalized to the expression of housekeeping gene importin 8.

2.7 Statistics

The data were analysed with analysis of variance as a randomised block design using Genstat statistical software. The pen was the experimental unit for the response parameters. The general model including the effects of period, block (replicate) within period and dietary treatment was:

$$Y_{ij} = \mu + Period_i + Block_j / Period_i + Diet_k + e_{ijk}$$

in which:

Y_{ijk}	= dependent variable,
μ	= overall mean
Period	= period effect (i=1,2,3)
$Block_i$	= block effect (j=1 … 4) within period
$Diet_k$	= effect of dietary treatment, (j= 1 … 8)
e_{ijk}	= residual error.

This model was used to analyse results of the performance parameters (e.g. body weight, ADG, ADFI and FCR). Analyses were performed for treatments 1-8 to determine the effects of dietary copper content, and for treatments 1-4 to determine linear and quadratic effects of copper content. A Fisher protected t-test has been used for comparison of treatment means at an overall treatment effect of $P<0.1$. Pairwise differences are marked with superscripted indices when significant ($P<0.05$). The same model with block and pen effects to account for replicate animals within a pen was used to analyse data of dissected animals: serum (copper content), liver samples (dry matter, zinc and copper content), and bile (copper content). The animal effect was taken into account for analysis of mRNA-expression of copper and zinc transporters to account for repeated measurement of transporters in different segments of the digestive tract within animals.

Number of lost animals and required medical treatments were analysed using the chi-square test. Faecal consistency scorings were analysed using the IRClass procedure of Genstat.

3 Results

In paragraph 3.1 the analysed diet composition is presented, in paragraph 3.2 the effect of copper content on animal performance, in paragraph 3.3 health and medical treatments and in paragraph 3.4 the physiological characteristics.

3.1 Analysed diet composition

In Table 2 the analysed nutrient composition of the experimental diets is summarised. The results indicate that the required amounts of Cu and Zn were included in the diet according to plan. The results of the proximate analyses were included in the diet composition in Annex 1.

Table 2
Analysed Cu and Zn contents in the basal mixture of ingredients and the experimental diets, in mg/kg as fed basis.

	Calculated and analysed Cu-content, mg/kg					Mean Zn content, mg/kg	
Added cu, mg/kg	Basal	15	80	120	160	basal	total
Pre-starter diets, Day 0-14	5	23	81	121	151	29	77
Starter diets, Day 14-40	5	17	89	112	183	37	82
Grower diets, Day 40-56	5	20	93	135	164	34	85

3.2 Effects on growth performance

The piglets were weaned at a mean age of 26.5 days (SD 1.8) with a mean weaning weight of 7.94 kg (SD 0.73). In Table 3 we present the development in body weight and the performance of the piglets in the nursery period from Day 0 to 40 and in the whole experimental period from Day 0 to 56. Figure 1 shows the ADG of the weaned pigs in 2-week periods relative to the performance of pigs in Treatment 4 (T4) that continuously received the 160 mg/kg Cu supplement. In Annex 2, the performance parameters for each 2-week period are included. The dietary treatments significantly affected ADFI, ADG and FCR in each of the 2-week periods. Effects of level and duration of the Cu supplementation are addressed below.

3.2.1 Effect of dose of copper supplementation

In T1 to T4, the Cu supplementation was increased from 15 to 160 mg/kg during the total 8-week period. In the total period, ADG was linearly increased ($P<0.001$) from 467 to 527 g/d and ADFI from 760 to 845 g/d ($P<0.001$) (Table 3, Annex 2). The strongest effect was realised in the nursery period until Day 40. The effect on ADG was relatively small from Day 40-56, without significant effect on ADFI and FCR in this period (Annex 2). From Day 0 to 28, the increase in Cu supplementation also improved FCR, without any effect on FCR from Day 28 onwards. The final BW on Day 56 increased from 34.1 to 37.5 kg in pigs fed diets supplemented with 15 and 160 mg Cu/kg, without any effect on the variation between pigs in a pen.

3.2.2 Effect of duration of copper supplementation

The duration of the 160 mg/kg Cu supplementation also affected the performance of the piglets. The withdrawal after Day 14 (T7 and T8), after Day 28 (T6) and after Day 40 (T5) transiently decreased ADG compared to pigs that continuously received 160 mg Cu/kg (T4)(Annex 2). The withdrawal after

Day 14 also decreased ADFI and deteriorated FCR, whereas the effects on ADFI and FCR were somewhat less consistent for withdrawal after Day 28 or Day 40. In the nursery period (Day 0-40) and the total experimental period (Day 0-56) the performance of pigs that received diets with 160 mg Cu/kg until Day 28 was not significantly different from that of pigs receiving the high Cu supplement until Day 56, whereas the withdrawal after Day 14 significantly reduced the overall performance of the pigs. The withdrawal of the high Cu supplement after Day 40 (T5), i.e. after the pigs were moved to the grow-finish unit, reduced the ADFI and ADG. On the other hand, pigs that received the high Cu supplement after earlier withdrawal (T8) realised an increased ADG, similar to pigs that continuously received this supplement.

Tabel 3

Influence of Cu content in diets of weaned piglets supplied from Day 0 to 56 post-weaning on body weight and growth performance of the animals in two-week periods.

Added Cu, mg/kg	T1	T2	T3	T4	T5	T6	T7	T8	SEM[1]	P T1-8[2]	PL T1-4	PQ T1-4
Day 0-14	15	80	120	160	160	160	160	160				
Day 14-28	15	80	120	160	160	160	15	15				
Day28-40	15	80	120	160	160	15	15	15				
Day 40-56	15	80	120	160	15	15	15	160				
BW Day 0	7.93	7.96	7.93	7.96	7.92	7.94	7.94	7.93	0.013	0.288	0.253	0.513
BW Day 14	10.28^{a}	10.65^{b}	10.85^{bc}	10.93^{bcd}	10.96^{bcd}	11.24^{d}	10.92^{bcd}	11.01^{cd}	0.12	<0.001	<0.001	0.417
BW Day 28	15.1^{a}	16.0^{b}	16.8^{c}	17.0^{cd}	16.9^{c}	17.6^{d}	15.9^{b}	15.7^{ab}	0.23	<0.001	<0.001	0.646
BW Day 40	22.4^{a}	23.7^{bcd}	24.7^{de}	25.2^{e}	24.6^{cde}	24.9^{e}	23.7^{bc}	22.9^{ab}	0.36	<0.001	<0.001	0.687
BW Day 56	34.1^{a}	35.5^{ab}	37.3^{cd}	37.5^{d}	35.8^{bcd}	37.2^{cd}	35.2^{ab}	35.7^{abc}	0.59	<0.001	<0.001	0.714
SD BW D56, kg	3.8	4.2	4.7	3.9	4.2	4.3	4.3	4.6	0.33	0.649	0.458	0.127
Day 0-40												
ADG, g/d	358^{a}	390^{bc}	416^{d}	428^{d}	414^{cd}	421^{d}	390^{bc}	372^{ab}	8.9	<0.001	<0.001	0.699
ADFI, kg/d	556^{a}	592^{bc}	631^{d}	635^{d}	618^{cd}	643^{d}	586^{ab}	572^{ab}	11.1	<0.001	<0.001	0.474
FCR	1.56^{c}	1.52^{abc}	1.52^{abc}	1.48^{a}	1.50^{ab}	1.53^{bc}	1.50^{ab}	1.54^{bc}	0.015	0.026	0.003	0.808
Day 0-56												
ADG, g/d	467^{a}	491^{ab}	524^{cd}	527^{d}	498^{bcd}	522^{cd}	486^{ab}	496^{abc}	10.4	<0.001	<0.001	0.722
ADFI, kg/d	760^{a}	805^{abc}	840^{bcd}	845^{cd}	793^{a}	855^{d}	786^{a}	800^{ab}	16.0	<0.001	<0.001	0.480
FCR	1.63	1.64	1.60	1.61	1.59	1.64	1.62	1.62	0.019	0.631	0.240	0.567
Feed usage nursery												
Pre-starter, kg	3.14^{a}	3.47^{b}	3.74^{bc}	3.68^{bc}	3.72^{bc}	4.05^{d}	3.68^{bc}	3.81^{cd}	0.10	<0.001	<0.001	0.098
Starter, kg	19.3^{ab}	20.4^{bc}	21.7^{d}	21.9^{d}	21.2^{cd}	21.9^{d}	19.9^{ab}	19.2^{a}	0.45	<0.001	<0.001	0.661
Total, kg	22.4^{a}	23.9^{bc}	25.4^{d}	25.6^{d}	24.9^{cd}	25.9^{d}	23.6^{ab}	23.1^{ab}	0.4	<0.001	<0.001	0.468
Gain birth D40[3], g/d	314^{a}	333^{bc}	348^{cd}	356^{d}	347^{cd}	352^{d}	332^{bc}	322^{ab}	5.6	<0.001	<0.001	0.808

[1] SEM, pooled standard error of least square means

[2] P T1-8, P-value of Cu effect in the complete model. PL T1-4 and PQ T1-4, linear and quadratic effects of Cu addition in T1 to T4

[3] Calculated with mean birth weight of 1.4 kg

3.2.3 Consequences for performance of growing finishing pigs

Table 4 and Annex 3 include the performance of pigs in the growing-finishing period. The experimental treatments from Day 0 to 56 did not significantly affect the subsequent performance of the growing pigs until slaughter. Pigs of T4 only realised a numerically lower ADG from Day 56-70 after withdrawal of the high Cu supplement. In the grower period from Day 40-70, pigs that received 160 mg Cu/kg during Day 40-56 (T4) had a numerically higher ADG of 30 g/d compared to pigs that received the high Cu supplement in the nursery phase but no longer in the grower phase (T5) (Annex 3). The overall performance in the growing-finishing period (Day 40-slaughter) was not affected by the dietary treatments (Annex 3). However, ADG from weaning to slaughter tended (P=0.078) to increase with increasing Cu content in the diets until Day 56 (T1 to T4). The overall difference in growth rate was almost 20 g/d, 779 g/d (T4) versus 761 g/d (T1). The carcass quality showed somewhat variation between the dietary treatments without consistent differences.

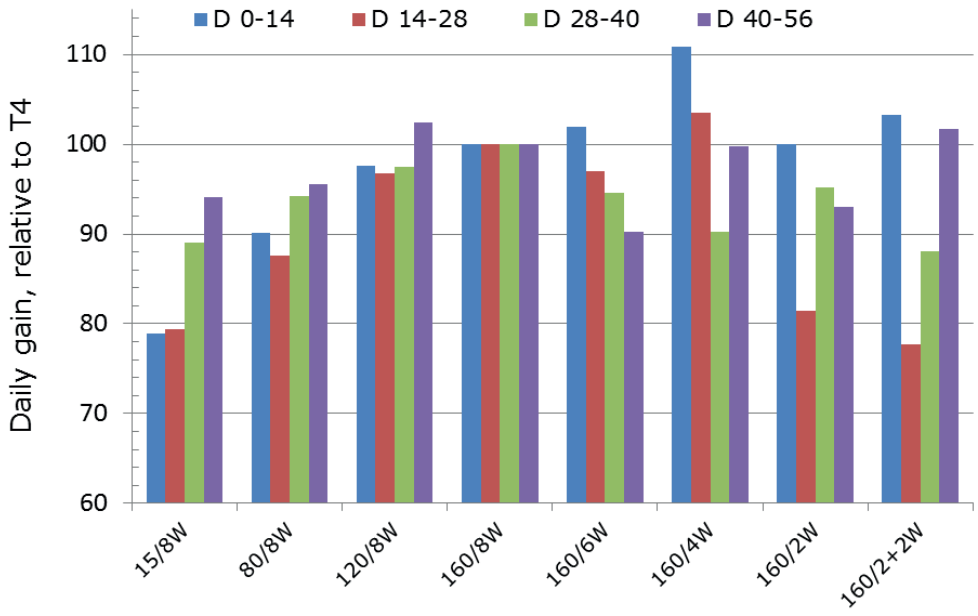

Figure 1. *Influence of level (15, 80, 120 or 160 mg/kg) and duration (8, 6, 4, or 2 weeks post-weaning or 2 weeks plus weeks 7-8 post weaning) of Cu supplementation of pig diets during 56 days post-weaning on daily gain in 2-week periods, relative to pigs receiving 160 mg Cu/kg in the entire period (T4, 160/8W).*

Table 4

Influence of Cu content in diets of weaned piglets supplied from Day 0 to 56 post-weaning on subsequent body weight and growth performance in growing finishing pigs.

Added Cu, mg/kg	T1	T2	T3	T4	T5	T6	T7	T8	SEM[1]	P T1-8[2]	PL T1-4	PQ T1-4
Day 0-14	15	80	120	160	160	160	160	160				
Day 14-28	15	80	120	160	160	160	15	15				
Day 28-40	15	80	120	160	160	15	15	15				
Day 40-56	15	80	120	160	15	15	15	160				
BW Day 40	22.4a	23.7bcd	24.7de	25.2e	24.6cde	24.9e	23.7bc	22.9ab	0.36	<0.001	<0.001	0.687
BW Day 56	34.1a	35.5ab	37.3cd	37.5d	35.8bcd	37.2cd	35.2ab	35.7abc	0.59	<0.001	<0.001	0.714
BW Day 70	46.8a	48.2ab	50.1b	49.9b	48.5ab	50.1a	48.3ab	48.9b	0.72	0.020	<0.001	0.582
BW End of trial	119.3	118.8	119.3	120.8	119.6	120.4	119.3	119.5	0.81	0.687	0.168	0.126
Days in GF period	106.0	105.8	104.4	104.6	105.4	104.2	105.7	105.9	0.64	0.241	0.094	0.958
Day 56-70												
ADG, g/d	912	911	915	891	905	924	935	942	25.2	0.883	0.615	0.632
ADFI, kg/d	1745	1755	1799	1764	1753	1810	1737	1777	0.035	0.798	0.492	0.632
FCR	1.93	1.93	2.00	1.98	1.96	1.96	1.87	1.89	0.047	0.521	0.246	0.993
Day 70-slaughter												
ADG, g/d	951	927	926	947	940	944	935	927	10.8	0.587	0.651	0.062
ADFI, kg/d	2538	2471	2547	2541	2526	2548	2527	2471	0.030	0.349	0.663	0.182
FCR	2.67	2.67	2.75	2.68	2.69	2.70	2.70	2.66	0.026	0.316	0.234	0.397
Day 0-slaughter												
ADG, g/d	761	758	770	779	767	779	763	763	7.2	0.332	0.078	0.271
ADFI, kg/d	1.80	1.78	1.82	1.83	1.80	1.82	1.79	1.77	0.018	0.224	0.136	0.318
FCR	2.36	2.35	2.37	2.35	2.34	2.34	2.35	2.32	0.016	0.564	0.541	0.844
Carcass characteristics												
Carcass weight, kg	91.8	92.0	92.2	93.1	92.1	93.2	92.5	92.3	0.66	0.761	0.091	0.348
Dressing, %	77.1	77.4	77.3	77.2	77.1	77.5	77.7	77.3	0.25	0.725	0.696	0.365
Lean meat, %	59.0	58.6	58.4	58.9	58.9	58.6	58.7	58.6	0.19	0.371	0.457	0.048
Muscle, mm	60.2ab	60.7ab	59.6a	61.0ab	59.5a	61.6b	60.4ab	59.5a	0.54	0.067	0.490	0.414
Backfat, mm	13.9	14.5	14.8	14.1	14.0	14.5	14.4	14.5	0.30	0.380	0.444	0.050

[1] SEM, pooled standard error of least square means

[2] P T1-8, P-value of Cu effect in the complete model. PL T1-4 and PQ 1-4, linear and quadratic effects of Cu in T1 to T4

3.3 Health and medical treatments

3.3.1 Faecal consistency

We registered the faecal consistency twice a week as the percentage of piglets per pen with normal, soft or watery faeces (Figure 2 and Annex 4). Figure 2 demonstrates that the Cu supplementation from Day 0-56 significantly affected the faecal consistency of the growing pigs. The increase in Cu supplementation from 15 to 160 mg/kg (T1 to T4) reduced the incidence of soft faeces and increased the percentage of piglets with normal faeces. The pigs of T1 had 11-18% of pigs with soft faeces and diarrhoea, versus only 3-6% in T4. The results of T5 to T8 show a transient increase in pigs with soft faeces during about 4 weeks after withdrawal of the high Cu supplement. Especially the reduction in Cu supplementation after Day 14 (T7 and T8) caused a drastic increase in the percentage of pigs with soft faeces during Day 14-28 post-weaning compared to T4 to T6. The increase in Cu supplementation in pigs of T8 during Day 40-56 increased the percentage of pigs with normal faeces in that period. Pigs of

T2, T3, T4 and T8 showed a transient increase in percentage of pigs with soft faeces after reduction of Cu supplementation after Day 56 (Annex 4).

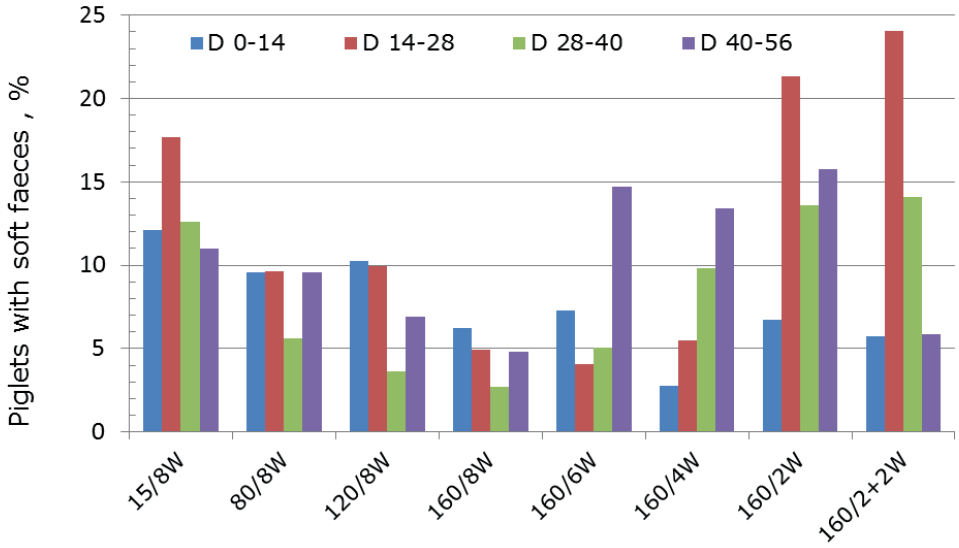

***Figure 2**. Influence of level (15, 80, 120 or 160 mg/kg) and duration (8, 6, 4, or 2 weeks post weaning or 2 weeks plus weeks 7-8 post weaning) of Cu supplementation in pig diets during 8 weeks post-weaning on percentage of piglets with soft faeces in 2-week periods.*

3.3.2 Medical treatments

In Table 6 we summarised the required medical treatments of individual piglets during the trial period. In total 110 piglets (13.3%) were medicated and 27 piglets (3.3%) died during the experimental period until Day 56. The majority of medical treatments in Table 6 were required because of arthritis (n=64), respiratory diseases (n=25), and Streptoccocus suis (n=16). In the growing-finishing period, 28 pigs (3.5%) required medical treatment and 14 pigs (1.8%) died before slaughter. The dietary Cu content did not significantly affect the number of required medical treatments. The mortality of piglets was significantly affected by the dietary treatments (P=0.04).

Table 6

Influence of Cu content in diets of weaned piglets supplied from Day 0 to 56 post weaning on required medical treatments and mortality of individual animals during the experimental period and in the growing-finishing period (GF pigs).

Item	T1	T2	T3	T4	T5	T6	T7	T8	P-value[1]
Day 0-14	15	80	120	160	160	160	160	160	
Day 14-28	15	80	120	160	160	160	15	15	
Day 28-40	15	80	120	160	160	15	15	15	
Day 40-56	15	80	120	160	15	15	15	160	
Number of piglets[3]	108	108	100	108	100	100	100	100	
Medicated piglets (<D56), n	17	20	13	7	14	12	12	15	0.33
Reasons for medication									
- arthritis	11	12	9	5	5	8	6	8	0.57
- respiratory diseases	4	4	2	0	7	3	1	4	0.13
- Streptococcus suis	2	3	1	0	2	1	4	3	0.51
- miscellaneous	0	1	1	2	0	0	1	0	ND[2]
Medicated GF pigs (>D56), n	2	5	3	1	5	3	3	6	0.52
Mortality of piglets, n	2^{ab}	0^{a}	3^{ab}	2^{ab}	7^{b}	2^{ab}	7^{b}	4^{b}	0.04
Causes of death									
- respiratory diseases	1	0	2	0	1	0	0	0	ND[2]
- Streptococcus suis	0	0	0	2	4	0	2	2	ND[2]
- miscellaneous	1	0	1	0	2	2	5	2	ND[2]
Mortality of GF pigs, n	1	2	1	2	3	1	2	2	0.95

[1] P-value of the effect of copper level determined for Treatments 1-8 by chi-square test

[2] ND, not determined, number of piglets too low for statistical analysis

[3] Treatments groups T1, T2 and T4 comprised 8 extra pigs to be sacrificed on Day 56

3.4 Physiological characteristics

3.4.1 Cu in blood and liver

At weaning (Day 0), 8 representative female and castrated male piglets of Period 3 were sacrificed, blood samples collected and tissues harvested. Similarly, 8 pigs of T1, T2 and T4 were sacrificed at the end of the intervention period on Day 56. From the same treatments, livers of 8 slaughter pigs at approximately 120 kg BW, male and female, were collected at the slaughterhouse. Cu content in plasma and bile, and Cu and Zn in liver were determined. The results are presented in Table 7. The increase in Cu supplementation from 15 to 160 mg/kg linearly increased the plasma Cu level, and the Cu content in liver and bile, in piglets killed on Day 56. No differences in Cu and Zn content in the liver were observed in growing pigs killed at 120 kg BW (Table 8).

Table 7

Influence of Cu content in diets of weaned piglets from Day 0 to 56 post-weaning on relative liver mass, Cu and Zn content in the liver (in mg/kg) and copper content in bile on Day 56.

	Day 0	T1	T2	T4	SEM[1]	P T1-4[2]	PL T1-4	PQ T1-4
Added cu, mg/kg		15	80	160				
Piglets, n	8	8	8	8				
Body weight, kg	7.9 ± 0.72	35.4	34.7	37.8	1.72	0.450	0.332	0.425
Plasma Cu, µmol/L	31.6 ± 3.3	23.8[a]	24.4[a]	27.5[b]	0.80	0.022	0.009	0.336
Liver								
Weight, g	194 ± 26	1064	1030	1046	60.4	0.923	0.852	0.731
Relative weight, g/kg BW	24.6 ± 2.7	30.1	29.6	27.6	1.06	0.261	0.121	0.664
DM, g/kg	257.1 ± 6.1	259.4	258.5	260.8	2.9	0.852	0.726	0.669
Cu, mg/kg DM	244 ± 96	23.0[a]	20.4[a]	32.1[b]	2.6	0.023	0.025	0.062
Cu, total mg	12.0 ± 4.7	6.34[ab]	5.44[a]	8.84[b]	0.88	0.057	0.063	0.095
Zn, mg/kg DM	285 ± 125	253	240	264	22.0	0.747	0.698	0.522
Zn, total mg	14.1 ± 6.2	69.9	66.1	72.9	9.7	0.883	0.811	0.671
Bile, mg/L	5.76 ± 2.7	1.36[a]	1.60[a]	2.40[b]	0.23	0.026	0.010	0.438

[1] SEM, pooled standard error of least square mean

[2] P T1-4, P-value of Cu effect in the complete model. PL T1-4 and PQ 1-4, linear and quadratic effects of Cu addition

[a,b] Treatment means in a row without common superscript letter are significantly different (P<0.05)

Tabel 8

Influence of Cu content in diets of weaned piglets from Day 0 to 56 post-weaning on relative liver mass, Cu and Zn content in the liver (in mg/kg) at slaughter at approximately 120 kg BW.

	T1	T2	T4	SEM[1]	P T1-4[2]	PL T1-4	PQ T1-4
Added cu, mg/kg	15	80	160				
Pigs, n	8	8	8				
Body weight, kg	117.9	119.8	123.2	2.63	0.398	0.188	0.896
Liver							
Weight, g	1703	1748	1762	58.9	0.768	0.506	0.803
Relative weight, g/kg BW	14.5	14.6	14.3	0.48	0.994	0.839	0.793
DM, g/kg	273.6	272.9	270.4	3.4	0.801	0.531	0.866
Cu, mg/kg DM	39.7	37.0	49.6	5.3	0.267	0.202	0.307
Cu total, mg	18.5	17.4	23.9	2.8	0.261	0.188	0.322
Zn, mg/kg DM	229	276	256	32.3	0.601	0.595	0.402
Zn total, mg	106	129	122	12.8	0.454	0.418	0.343

[1] SEM, pooled standard error of least square mean

[2] P T1-4, P-value of Cu effect in the complete model. PL T1-4 and PQ 1-4, linear and quadratic effects of Cu addition

[a,b] Treatment means in a row without common superscript letter are significantly different (P<0.05)

3.4.2 Expression levels of genes related to intestinal Cu- and Zn absorption

Table 9 shows the effects of a constant low (15 mg/kg, T1) or high (160 mg/kg, T4) dose of dietary Cu for a period of 8 weeks (56 days) on the mRNA abundance of key genes related to Cu and Zn transport in the mucosa of four different segments of the small intestine, including duodenum, proximal and distal jejunum and ileum. We selected the following genes: metallothionein 1A (MT1A), the intracellular Cu transport protein ATOX1, cytochrome c oxidase copper chaperone COX17,the cupric reductase STEAP2, the integral membrane Cu transporters SLC31A1/Ctr1,SLC31A2/Ctr2, ATP7A and ATP7B, the integral membrane Zn transports SLC30A1/ZnT1 and SLC30A5/ZnT5, and the proton-coupled divalent metal ion transporter SLC11A2/DMT1. In addition, as a measure of amino acid uptake to support body growth we measured the transcript level of proton-coupled amino acid transporter SLC36A1/PAT1.

Table 9

Influence of Cu content in diets of piglets from Day 0 to 56 post-weaning on mRNA levels of genes involved in Cu and Zn absorption in various intestinal segments of male and female piglets on Day 56 post-weaning.

Added Cu, mg/kg	All	Male	Female	Duod.	Prox.J.	Dist.J.	Ileum	SEM[1]	PS[2]	PG	PT	PSxG	PSxT	PGxT
MT1A	0.46	0.36	0.57	0.89	0.41	0.22	0.33	0.15	0.02	0.31	0.05	0.61	0.04	0.56
15 mg/kg	0.24	0.20	0.29	0.30^{y}	0.23^{y}	0.25^{y}	0.19^{y}	0.22						
160 mg/kg	0.69	0.53	0.84	1.48^{z}	0.60^{y}	0.19^{y}	0.48^{y}							
SLC31A1	1.45	1.52	1.38	1.23^{b}	2.17^{d}	1.55^{c}	0.85^{a}	0.085	<0.001	0.03	0.61	0.02	0.82	0.15
15 mg/kg	1.41	1.52	1.30	1.19	2.10	1.47	0.88	0.146						
160 mg/kg	1.49	1.52	1.46	1.28	2.24	1.62	0.82							
SLC31A2	0.0116	0.0115	0.0118	0.0106^{a}	0.0110^{a}	0.0113^{a}	0.0135^{b}	0.00051	0.001	0.66	0.09	0.07	0.88	0.34
15 mg/kg	0.0113	0.0107	0.0119	0.0105	0.0104	0.0110	0.0134	0.00067						
160 mg/kg	0.0120	0.0122	0.0117	0.0108	0.0117	0.0117	0.0137							
SLC30A1	0.176	0.188	0.164	0.194^{c}	0.247^{d}	0.157^{b}	0.105^{a}	0.030	<0.001	0.18	0.85	0.28	0.71	0.87
15 mg/kg	0.177	0.190	0.164	0.188	0.248	0.168	0.107	0.050						
160 mg/kg	0.174	0.185	0.164	0.201	0.246	0.146	0.104							
SLC36A1	0.197	0.217	0.178	0.254^{b}	0.349^{c}	0.101^{a}	0.086^{a}	0.019	<0.001	0.11	0.52	0.02	0.61	0.29
15 mg/kg	0.207	0.239	0.175	0.262	0.380	0.101	0.085	0.030						
160 mg/kg	0.188	0.195	0.181	0.246	0.318	0.101	0.087							
SLC30A5	0.54	0.54	0.54	0.48	0.68	0.63	0.36	0.021	<0.001	0.95	0.50	0.71	0.03	0.75
15 mg/kg	0.55	0.55	0.55	0.50^{x}	0.75^{z}	0.62^{y}	0.34^{w}	0.034						
160 mg/kg	0.53	0.52	0.53	0.47^{x}	0.62^{y}	0.64^{y}	0.38^{w}							
ATP7A	0.079	0.078	0.079	0.081^{b}	0.084^{b}	0.079^{b}	0.070^{a}	0.0023	<0.001	0.76	0.15	0.94	0.37	0.10
15 mg/kg	0.081	0.078	0.084	0.081	0.090	0.081	0.071	0.0032						
160 mg/kg	0.077	0.079	0.075	0.081	0.079	0.078	0.070							
ATP7B	0.184	0.175	0.193	0.181^{b}	0.210^{c}	0.204^{bc}	0.141^{a}	0.0095	<0.001	0.17	0.41	0.43	0.74	0.74
15 mg/kg	0.192	0.181	0.203	0.187	0.226	0.205	0.149	0.017						
160 mg/kg	0.176	0.169	0.183	0.176	0.194	0.202	0.132							
ATOX1	0.034	0.034	0.033	0.041^{c}	0.024^{a}	0.033^{b}	0.038^{c}	0.0013	<0.001	0.39	0.49	0.45	0.64	0.28
15 mg/kg	0.035	0.035	0.035	0.043	0.025	0.034	0.038	0.0026						
160 mg/kg	0.033	0.034	0.032	0.039	0.023	0.031	0.038							
STEAP2	0.0038	0.0036	0.0040	0.0057^{c}	0.0041^{b}	0.0033^{b}	0.0021^{a}	0.00039	<0.001	0.68	0.27	0.20	0.60	0.08
15 mg/kg	0.0042	0.0032	0.0052	0.0065	0.0046	0.0035	0.0024	0.00069						
160 mg/kg	0.0034	0.0040	0.0027	0.0048	0.0037	0.0032	0.0018							
COX17	0.25	0.25	0.24	0.24^{b}	0.31^{c}	0.23^{ab}	0.21^{a}	0.011	<0.001	0.33	0.48	0.51	0.88	0.02
15 mg/kg	0.25	0.24	0.27	0.25	0.32	0.24	0.21	0.018						
160 mg/kg	0.24	0.27	0.22	0.23	0.31	0.22	0.21							
SLC11A2	0.44	0.49	0.38	1.39^{b}	0.061^{a}	0.12^{a}	0.19^{a}	0.057	<0.001	0.05	0.05	0.05	<0.001	0.12
15 mg/kg	0.52	0.54	0.51	1.69	0.064	0.12	0.22	0.085						
160 mg/kg	0.36	0.45	0.26	1.08	0.058	0.12	0.16							

[1] SEM, pooled standard error of least square mean for segment and for segment × treatment classes

[2] P-value, significance of effects within small intestinal segments (PS), gender (PG), dietary Cu treatment (PT), and interactions

[a,b] Treatment means in a row without common superscript letter indicate significant differences (P<0.05) between segments

[w,x] Treatment means in a row and column without common superscript letter indicate significant differences (P<0.05) between segment × treatment classes

Quantitative PCR analysis demonstrated that the high dietary dose of Cu increased the expression of MT1A in duodenum and proximal jejunum. However, Cu content did not significantly affect mRNA levels of the Cu and Zn transporters analysed in this study and this was irrespective of the small intestinal segment. Nonetheless, the transcript level of all Cu and Zn absorption-related genes measured differed significantly along the small intestine. The expression of SLC31A1/Ctr1 and SLC30A1/ZnT1 were,

similarly to SLC36A1/PAT1 and COX17, highest in the proximal part of the jejunum. Compared to the upper parts of the small intestinal, the expression of ATP7A and ATP7B was lower in the ileum, while SLC31A2/Ctr2 mRNA was more abundant in the ileum. Highest mRNA levels of MT1A, STEAP2 and DMT1/SLC11A2 were detected in the duodenum. The expression of ATOX1 was, compared to the duodenum and ileum, lower in the jejunal mucosa. Moreover, there was an gender effect on SLC31A2/Ctr2 transcript, with an higher expression in the proximal jejunum in males than in females.

We observed an intestinal segment x treatment interaction for SLC30A5/ZnT5 expression (Table 9). While the amount of SLC30A5/ZnT5 mRNA was more abundant in the jejunal mucosa, compared to the duodal and ileal part, a higher level of SLC30A5/ZnT5 mRNA was seen in the proximal compared to the distal part of the jejunum in growing pigs receiving the lower dose of 15 mg Cu/kg. Strikingly, an intestinal segment x treatment interaction was also seen for the expression of divalent ion transporter SLC11A2/DMT1, with an higher mRNA level in the duodenum in response to low Cu intake (T1) compared to high Cu intake (T4). Furthermore, an intestinal segment x gender was obtained for SLC36A1/PAT1 with an significant higher mRNA expression in the proximal jejunum in males compared to that in the proximal jejunum in females.

4 Discussion

In practical pig production, inclusion of a dietary Cu supplement of 160 mg/kg from weaning until the end of the nursery phase at approximately 10 weeks of age, or until 12 weeks of age is a common practice. Earlier studies of Bikker et al. (2011), Jongbloed et al. (2011) and others have shown a significant enhancement of feed intake and daily gain due to this Cu supplement. However, this Cu is largely excreted in the manure and contributes to the accumulation of Cu in soil and surface water. Therefore, the aim of this study was to determine the influence of the level and the duration of supply of a Cu supplement beyond the normal physiological requirements within the legal limits of a maximum total dietary Cu content of 170 mg/kg until 12 weeks of age. The experiment was conducted according to plan, without major problems. Also, the analysis of the diets confirmed the inclusion of Cu according to plan.

4.1 Performance and health

4.1.1 Influence of level of copper supplementation

4.1.1.1 Growth performance

The effect of dietary Cu level was determined in T1-T4. The Cu supplement linearly enhanced the daily gain in each of the 2-week periods, in the 40-day nursery period and in the 56-day period until 12 weeks of age, being the legal limit to the period of inclusion in the EU (Table 3 and Annex 2). Hence, the maximum effect of Cu supplementation on growth performance may not be realised, but a higher Cu inclusion level is not allowed in the EU. A linear increase was also found in feed intake, apart from the period Day 40-56 in which the ADFI was no longer significantly enhanced by the increasing Cu content. The FCR was improved by Cu content in the period until Day 28. Overall, in the nursery period (1-40 days), approximately 75% of the effect on growth rate could be explained by an increase in feed intake.

In the 56-day period, the effect of the highest Cu level compared to the lowest level was 60 g/d in ADG. Compared to T1 with 15 mg Cu/kg, the highest Cu supplementation increased BW at the end of the nursery period (Day 40) with 2.8 kg and at the end of the treatment period (Day 56) with 3.4 kg. These results show that a Cu supplement of 160 mg/kg substantially improves growth rate and BW in nursery pigs. This is in agreement with our previous study in which the high Cu supplement enhanced ADG and increased BW on Day 56 by 3 kg compared to the mean of 6 treatments with a Cu supplementation of 0-18 mg/kg (Bikker et al., 2011).

Despite the absence of a quadratic effect, numerically the difference between the inclusion of 120 and 160 mg/kg was small and not significant. Until Day 40, the difference in ADG was 12 g/d. This effect, though somewhat larger, is in line with the data of Jongbloed et al. (2011), who calculated that a reduction in Cu supplement from 160 to 100 mg/kg would cause a reduction in ADG of 8 g/d between 5 and 25 kg BW. In our present study, the extra BW of 160 versus 120 mg Cu/kg was 0.5 kg on Day 40 and 0.2 kg on Day 56 (Table 3). This would be the cost of a reduction in Cu supplementation to reduce Cu excretion.

4.1.1.2 Subsequent performance

Earlier studies reported a transient drop in performance in the grower period after withdrawal of a Cu supplement (Van der Aar et al., 1986; Jongbloed et al., 2003; and Jongbloed et al., 2011). In the present study, we observed a numerical reduction in growth rate of 20 g/d in the period from Day 56-70 after withdrawal of 160 mg Cu/kg. No such loss was observed after withdrawal of the 80 and 120 mg/kg supplements (Table 4). No differences were observed from Day 70 to slaughter. Overall, the Cu supplement until day 56 tended to linearly ($P=0.078$) increase the growth rate from weaning to slaughter with almost 20 g/d for the highest supplementation. This illustrates the benefit of using the Cu supplement in the diets of weaned piglets. The overall effect from weaning to slaughter was somewhat bigger than in our previous study (Bikker et al., 2011) because of a somewhat higher

numerical loss in performance of approximately 60 g/d during the first two weeks (Day 56-70) after withdrawal of the Cu supplement.

4.1.1.3 Health

The increase in Cu supplementation reduced the percentage of piglets with diarrhoea and increased the percentage of piglets with normal faeces (Figure 2 and Annex 4). This effect tended to decrease somewhat over time but it was present in all 2-week periods until Day 56.
In our previous study (Bikker et al., 2011), we observed a significant effect of the high Cu supplement on faecal consistency until Day 28 and no effect thereafter since the percentage of pigs with soft faeces was very small on all low Cu treatments after Day 28 in that study. This suggests an overall difference in faecal consistency between the two studies and a bigger effect of a high Cu supplement in conditions of reduced faecal consistency.

A reduction from 160 to 120 mg Cu/kg significantly reduced faecal consistency in the period until day 28 and only had a minor effect thereafter. Importantly, the Cu level did not significantly influence the mortality or the percentage of piglets that required medication. Nonetheless, the number of medicated piglets was numerically higher at lower levels of Cu supplementation, so a possible effect cannot be fully excluded. In our previous study, we did not observe any effect of the high Cu supplement on the percentage of medicated piglets, although numerically the mortality was lowest in piglets receiving the high Cu supplemented diets (Bikker et al., 2011).

In general, data on faecal consistency are not included in published studies, but Meyer and Kröger (1973) reported a decrease in the number of piglets with soft faeces and diarrhoea when piglets were supplemented with 100 or 200 mg Cu per kg. In addition, the high Cu supplement did not significantly reduce the number of piglets requiring medical treatment or the mortality of pigs. Other authors who used high animal numbers reported a reduction in mortality of weaned piglets after inclusion of 250 mg Cu per kg of diet (Stahly et al., 1980).

4.1.2 Influence of duration of high copper supplementation

4.1.2.1 Growth performance

In T4 to T8 we studied the influence of withdrawal of the Cu supplement after 2, 4 or 6 weeks post-weaning. The results (Figure 1, Annex 2) showed a drastic drop in performance in the first 2 weeks after withdrawal. After withdrawal on Day 14 (T7 and T8), ADG was approximately 20% lower during Day 14-28 and 10% lower during Day 28-56. Nonetheless, even the short period of high Cu supplementation resulted in an increased ADG in the nursery period (Day 0-40) and a numerical increase in BW compared to pigs of T1 without any Cu supplement. After withdrawal on Day 28 (T6), ADG was approximately 10% lower during Day 28-40 and recovered thereafter. After withdrawal on Day 40 (T7), ADG was approximately 10% lower during Day 40-56 and gradually recovered thereafter. After withdrawal on Day 56, we observed a numerical drop in ADG from Day 56-70. Overall the withdrawal after Day 14 in the nursery period (Day 0-40) resulted in a substantial loss in gain and final BW, whereas withdrawal after Day 28 only had a minor effect on gain and BW. Similar results were found on Day 56: the pigs of T6 performed equally well as the pigs of T4 receiving 160 mg Cu/kg. However, the withdrawal of the high Cu supplement on Day 40 (T5) resulted in a 10% lower ADG during Day 40-56. In this period, the pigs also had a lower ADG than those of T6 that received the low Cu supplement from Day 28 onwards, suggesting that piglets were better adapted to the low Cu content upon movement from the nursery house to the grow-finish unit. The high Cu supplement to the growing pigs in T8 during Day 40-56, after they received low Cu diets from Day 14-40, enhanced ADG similarly to those in T4. Hence, also these pigs that may have been adapted to the low Cu diet, the Cu supplement had beneficial effects.

Our results demonstrate that withdrawal of the high Cu supplement after Day 14 evokes a drastic reduction in gain, and withdrawal after Day 28 a minor reduction in gain, whereas withdrawal after Day 40, simultaneous to the change in housing which may be a bigger challenge to the pigs, displayed a more substantial loss in performance

4.1.2.2 Subsequent performance

We discussed above the transient reduction in growth rate after early withdrawal of the Cu supplement in the nursery period (Day 0-40). The supply of high Cu in the grower period (Day 40-56, T4 vs. T5) resulted in a higher ADG of 75 g/d from Day 40-56 and a small drop of 15 g/d during Day 56-70 (Table 4, Annex 3). In the grower period from Day 40-70 the ADG was numerically 30 g/d higher in pigs of T4 versus T5. An enhancement of gain without subsequent loss in performance was observed in pigs of T8 that received the high Cu supplement during 2 weeks in the grower period after earlier withdrawal in the nursery period. Our results suggest a beneficial effect of the high Cu during the first 2 weeks of the grower period in pigs that received the high Cu supplement during the complete nursery period and in pigs that received the Cu supplement during Day 0-14 only. The trial design did not allow to determine the effect of high Cu in the grower period in pigs that received the Cu supplement during Day 0-28 (T6). Overall, the loss in subsequent performance after withdrawal of the high Cu supplement in the grower period was relatively small and transient. No significant differences were observed in performance of pigs in the period from Day 56-70 and day 70 to slaughter.

4.1.2.3 Health

The high Cu supplementation reduced the percentage of piglets with diarrhoea and increased the percentage of piglets with normal faeces (Figure 1 and Annex 4). After withdrawal of the high supplement on Day 14, the percentage of piglets with soft faeces increased from approximately 5% to 20-25% during Day 14-28 and to 10-15% during day 28-56. Hence the effect of this early withdrawal on faecal consistency was quite dramatic and is not to be recommended. The withdrawal on Day 28 increased the percentage of piglets with soft faeces to 10% during Day 28-40. In the grower period (Day 40-56), 10-15% of piglets on the low Cu treatments (T1, T5, T6, and T7) had soft faeces irrespective of the time of withdrawal, whereas in pigs receiving the high Cu supplement (T4 and T8) 5% had soft faeces. Annex 4 shows that after Day 70, when all pigs received the same finisher diets with low Cu content, the percentage of piglets with soft faeces was less than 5%. From Day 56-70, when all pigs received a grower diet with low Cu content, the percentage of piglets with soft was 10-15%, and numerically the lowest in pigs of T4 and T8 that received the high Cu supplement from Day 40-56. Hence, no drawback of the Cu withdrawal was observed in these pigs, indicating that any early withdrawal of a high Cu supplement increases the percentage of pigs with soft faeces, with the most dramatic effects during Day 14-28 post-weaning.

Cu dosage did not significantly influence the percentage of piglets that required medication, whereas the mortality was relatively high in T5 and T7, although not significantly different from T4 (Table 6). Consequently, it is difficult to draw any firm conclusion. Moreover, the mortality and medication were spread over the experimental period and difficult to relate to either the period of high or low Cu supplementation. For example, relative high mortality of piglets was seen in T5 (which were given high Cu supplementation during the nursery phase), but also in T7 (i.e. after withdrawal of the high Cu supplement on Day 14). Furthermore, a relatively high number of piglets in T7 and T8 was medicated after Day 14 (period of mediaction not included in Table 6). Furthermore, in T5 and T6 a relatively high number of pigs was medicated after Day 40 in the early grower phase, when these pigs received a low Cu diet. Taken together, although our data does not provide conclusive evidence, it is possible that Cu withdrawal in the post-weaning period increases the risk that medication of the piglets is required.

4.2 Physiological characteristics

4.2.1 Cu levels in blood and liver

A selection of 8 pigs from each of the treatments T1, T2 and T4 that continuously received 15, 80 and 160 mg Cu/kg of diet, respectively, were used to determine plasma Cu level at the end of the intervention period (Day 56) and liver characteristics on Day 56 and at slaughter. In addition, 8 pigs were sacrificed at weaning (Day 0) as a reference group. The results for this reference group, including the relatively high plasma Cu level and Cu content in liver dry matter were fully in agreement with our previous study. Also the lower levels of plasma and liver Cu were earlier reported (Bikker et al., 2011).

As expected, the increase in Cu supplement linearly increased the plasma Cu level and the liver Cu content (Tables 7 and 8). However, the extent was much less than in our previous study in which plasma Cu increased to 30.9 µmol/L, liver Cu content to 105 mg/kg dry matter and Cu in bile to 7.4 mg/L (Bikker et al., 2011). The hepatic zinc content was higher in the present study (264 mg/kg) compared to that in the previous study (140 mg/kg). We postulate that the interaction between Zn, Cu and phytase may have caused differences between studies. In the previous study, no phytase was used in the Cu supplemented diets. In one treatment without added Cu, the effect of phytase was studied. Phytase inclusion only enhanced the liver Zn content from 128 to 194 mg/kg without having an effect on liver Cu content. In another study investigating the effects of dietary Zn content using phytase-free diets with 160 mg Cu/kg, we observed that the increase in dietary Zn supplementation from 0 to 100 mg/kg enhanced the liver Zn content from 97 to 165 mg/kg dry matter, and reduced the Cu content from 183 to 48 mg/kg (Bikker et al., 2010). Addition of phytase to the diet with 15 mg supplemented Zn increased the liver Zn content from 105 to 297 mg/kg dry matter and reduced the Cu content from 109 to 74 mg/kg. Hence, an increase in dietary Zn content drastically reduced the liver Cu content and the inclusion of phytase reduced the liver Cu content as well, presumably mediated by an increased availability of dietary Zn. This effect may be explained by interactions in absorption and excretion of Zn in the digestive tract, as also suggested by Zacharias et al. (2003). A higher Zn supply, either by an increased dietary Zn content or the use of phytase stimulates the expression of methallothioneins (MT) in the enterocytes. These MT have a higher affinity for binding Cu than Zn. Consequently, a relatively high portion of Cu may be bound by MT and excreted, e.g. after sloughing off of enterocytes (Fischer et al., 1983). Others (Reeves et al., 1998) suggested that Zn influences Cu influx and efflux from the intestinal cells at the transporter level. In the present study, the combination of a moderate Zn supplement of 45 mg/kg and the inclusion of 500 FTU phytase presumably reduced the absorption and storage of Cu compared to our previous studies with low dietary Zn content and/or phytase-free diets (Bikker et al., 2010; 2011).

Apgar et al. (1995) reported a liver copper content of 28, 36, 80 and 138 mg/kg dry matter in weaned pigs fed a basal diet with 15 mg total copper per kg and 0, 100, 150 and 200 mg/kg copper from copper sulphate, respectively, during a 5-week period. Cromwell et al. (1989) reported an increase in dietary copper content at the end of five 4-5-week experiments from 22 mg/kg dry matter on a basal diet with 30 mg copper per kg to 30 and 240 mg/kg with a 125 and 250 mg copper supplement, respectively. Coffey et al. (1994) reported liver copper contents of 23, 34 and 272 mg/kg dry matter in pigs that received a basal diet with 17 mg copper per kg or supplemented with 100 and 200 mg/kg, respectively, during a 5-week experiment. Bradley et al. (1983) reported liver copper contents of 23, 20, 34, 34, 122 and 439 in pigs fed diets with 7.5, 15, 30, 60, 120, and 240 mg copper per kg from weaning to 90 kg body weight, respectively. Zacharias et al. (2003) reported a liver copper content of approximately 40 mg/kg dry matter in pigs receiving diets with 35 or 75 mg copper/kg from 23 to 55 kg and 90 mg/kg dry matter when receiving 170 mg/kg of diet. Our results are well in the range of these published studies (Tables 7 and 8). Especially at Cu supplementation above 150 mg/kg published liver Cu contents showed a large variation and sometimes a drastic increase, suggesting that above this concentration growing pigs were no longer able to maintain Cu homeostasis adequately (Jondreville et al. (2002).

In pigs at slaughter, the hepatic Cu and Zn content were not significantly affected by the level of Cu supplementation until Day 56 (Tables 7 and 8). Hepatic Cu content was only numerically higher in pigs that received the highest Cu supplement in the nursery and early grower phase. Despite the low Cu inclusion in the commercial diets in the finisher phase, the mean liver Cu content was higher than in pigs killed on Day 56 of the experiment, 42 vs 25 mg/kg dry matter. This may be related to the relative weight of the liver. The relative weight of the liver was 14 g/kg at slaughter versus 26 g/kg BW on Day 56. Thus, the absorbed Cu during the finisher phase was stored in a relatively smaller liver mass, leading to higher calculated hepatic Cu contents.

4.2.2 Intestinal gene expression

Absorption of minerals, such as Cu, Zn and iron (Fe), predominantly takes place in the small intestine and is mediated by specialized epithelial cells called enterocytes. According to the model presented in Figure 3, Cu can be absorbed from the diet in the cuprous (i.e. Cu^{+}) form only. Cu in the cupric (Cu^{2+})

state must first be reduced at the apical membrane probably by Steap2, a novel identified Fe^{3+} reductase that can also reduce Cu^{2+} (Ohgami et al., 2006). The cuprous ion comes then in contact with SLC31A1/Ctr1 which is believed to be the primary protein responsible for the uptake of dietary Cu across the brush border microvilli (Nose et al., 2010). Alternatively, Cu may enter the enterocyte via DMT1, the nonspecific divalent cation transporter (Arredondo et al., 2003). In the current model, Cu exit and delivery of cytosolic Cu into the secretory pathway is accomplished by two ATP-dependent intrinsic membrane Cu transporters, ATP7A and ATP7B, that both utilize the energy of ATP hydrolysis to fuel the transport of Cu across membranes (Lutsenko and Petris, 2003). ATP7A is designated as the main exit transporter of Cu in a process that involves trafficking of the transporter from the trans-Golgi network (TGN) towards the basolateral membrane of enterocytes (Monty et al., 2005). ATP7B is also located to the TGN and may be mainly involved in the sequestration of copper in intracellular vesicles either for extracellular release or internal storage of Cu (Weiss et al., 2008). Since Cu is a powerful oxidant and able to generate free radicals, it is toxic and must be for this reason bound to cytosolic proteins. On apical entry, Cu is generally handed to pathway-specific chaperones including antioxidant protein 1 (ATOX1) and COX17, that transfer Cu to various organelles for the delivery of Cu to Cu-requiring enzymes (Palumaa, 2013). ATOX1 delivers Cu to ATP7A and ATP7B, whereas COX17 transfers Cu to the mitochondrion for assembly of cytochrome c oxidase. To buffer intracellular Cu and reduce Cu export from enterocytes, free Cu can be scavenged by small cytosolic, cysteine-rich heavy metal-binding proteins of the family of metallothionein (MT) (Lalioti et al., 2009). The expression of MT can be induced by metals such as Cu and Zn via the interaction of metal-responsive transcription factor 1 (MTF1) with metal response elements (MREs) in the promoter regions of the MT genes (Zhang et al., 2003).

We observed differences in the level of expression of Cu and Zn transports along the various segments of the small intestine (Table 9). The lowest mRNA levels for the transporters SLC31A1/Ctr1, SLC30A1/ZnT1, SLC30A5/ZnT5, ATP7A and ATP7B were found most distally of the small intestine, that is the ileum, which is in line with the concept that the bulk of dietary Zn, and probably also that of Cu, is taken up in the jejunum (Lee et al., 1989; Condomina et al., 2002). Our data are in line with the observation in mice that SLC30A1/ZnT1 and SLC30A5/ZnT5 are mainly expressed in the duodenum and jejunum (Yu et al., 2007). Our data in pigs are also in concert with the finding that both ATP7A and ATP7B are highly expressed in the upper part of the small intestine in mice (Nyasae et al., 2007; Weiss et al., 2008) and that SLC31A1/Ctr1is abundantly expressed in mouse duodenum and pig jejunum (Nose et al., 2010).

SLC31A2/Ctr2 contains sequence homology with SLC31A1/Ctr1 (Zhou and Gitschier, 1997)and facilitated Cu uptake when overexpressed in COS-7 cells (Bertinato et al., 2008). Compared to SLC31A1/Ctr1, it has a relative low affinity for Cu (Bertinato et al., 2008). Contrary to the other Cu transporters (SLC31A1/Ctr1, ATP7A, ATP7B), SLC31A2/Ctr2 mRNA abundance was higher in the ileum compared to the proximal segments of the porcine gut (Table 9). It is conceivable that SLC31A2/Ctr2 has a function different from SLC31A1/Ctr1 with another affinity for Cu from different sources and where the local concentrations of Cu are different.

Similar to the distribution of the expression of SLC31A1/Ctr1, ATP7A and ATP7B, the expression level of the Cu chaperone COX17 was lower in the ileum, supporting the notion that Cu chaperones are involved in the process of intestinal Cu absorption (Prohaska, 2008). There is evidence that the Cu chaperone ATOX1 binds to lipid membranes (Flores and Unger, 2013) and possibly physically interact with Ctr1 (Xiao and Wedd, 2002), which may be important step in the distribution of Cu to downstream Cu-binding proteins, including ATP7A and ATP7B (Larin et al., 1999; Strausak et al., 2003). However, we found lower mRNA levels of ATOX1 in jejunum compared to duodenum and ileum. Reason for this contradiction may be that protein levels of ATOX1 do not always correlate with that of its transcript as described under certain conditions in endothelial cells (Dong et al., 2014).

Cupric- and ferrireductases such as STEAP2 are thought to stimulate cellular uptake of Cu and Fe (Ohgami et al., 2006). STEAP2 expression was highest in duodenum and gradually decreased distally in the small intestine of growing pigs, suggesting that cupric reductase activity is the highest most proximally, in the duodenum. Under physiological conditions, SLC11A2/DMT1 is proposed to act as a major entry pathway for metals such as Cu and Fe (Garrick et al., 2003; Arredondo et al., 2014), but

not for Zn (Tandy et al., 2000). Similar to STEAP2, the highest mRNA levels of SLC11A2/DMT1 were detected in the porcine duodenum (Table 9), which is in agreement that the upper small intestine is also the main site for iron absorption (Hunt, 2005).

Treatment effects

The expression of MT1A in the duodenum was significantly increased by dietary high Cu content. This indicates that entry of Cu into the enterocyte was highest in the upper small intestine as compared to the lower part. Our data support the notion that metallothionein mRNA levels increases at the higher copper exposures (Bauerly et al., 2004). It is important to stress that Cu bound to MT is not directly available for ATP7A-dependent Cu export, resulting in decreased copper uptake from the diet. In combination with induced MT expression, Cu uptake can be prevented by shedding cells that were loaded with Cu (Lonnerdal, 2008; Prohaska, 2008).

The dietary Cu content did not alter the expression of Cu transporters (SLC31A1/Ctr1, SLC31A2, Ctr2, ATP7A, ATP7B) in the small intestine of growing pigs. However, the mRNA abundance of SCL11A2/DMT1 was higher in the duodenum when pigs received the low Cu content. These data suggest that SLC11A2/DMT1 protein expression was also increased, thereby increasing the capacity of Cu uptake. Further studies are needed to investigate the role of SLC11A2/DMT1 in dietary Cu absorption in the porcine intestine.

Neither the expression of Zn transporter SLC30A1/ZnT1 nor amino acid transporter SLC36A1/PAT1 were affected by the dietary Cu content (Table 9). ZnT1 is an important transporter controlling intestinal Zn efflux (Cousins et al., 2006; Liuzzi and Cousins, 2004) and its expression is reported to be influenced by dietary Zn supply. For example, rats fed a diet deficient in Zn showed decreased ZnT1 mRNA expression, whereas rats fed a diet rich in Zn exhibited increased ZnT1 mRNA abundance (Liuzzi et al., 2001), probably under control of the transcription factor MTF-1, which is zinc-responsive (Langmade et al., 2000). Dietary Cu effects on SLC30A1/ZnT1 are not reported in the literature. Also no evidence for Cu-induced gene expression of ATP7A or ATP7B exists, except for a slight increase in ATP7A transcript in mouse pups when exposed to high dietary copper (Bauerly et al., 2005).

For SLC30A5/ZnT5 we found an Intestinal segment x treatment interaction with higher expression in the proximal jejunum in pigs receiving low Cu content. SLC30A5/ZnT5 has been reported to be present on the apical membrane of Caco-2 cells mediating both Zn uptake and efflux(Jackson et al., 2007). There are no reports describing effects of Cu content on the expression of SLC30A5/ZnT5. Interestingly, Condomina et al. (2002) reported that luminal Cu is capable to inhibit the uptake of Zn in the small intestine of the rat. It is conceivable that high Cu content hindered Zn uptake in our piglets thereby reduced intracellular Zn acting as a stimulator of SLC30A5/ZnT5 gene expression as seen in CaCo-2 cells (Cragg et al., 2002). However, Martin et al. (2013) reported no changes in ZnT5 mRNA levels with different amounts of dietary zinc in jejunal tissue of pigs. Several interactions with other genes and/or factors may affect SLC30A5/ZnT5 expression at the level of transcriptional repression and increased mRNA stability (Jackson et al., 2007).

Gender effects

We found in the proximal jejunum significant higher levels of Ctr1 mRNA in males than in females suggesting that female growing pigs have a lesser ability to absorb Cu. Gender differences have been also reported for cattle with higher Ctr1 expression and Cu content in livers of male subjects (Fry et al., 2013). Also, SLC11A2/DMT1 mRNA was more abundant in the duodenum of males compared to females (Table 9). Furthermore, we observed an intestinal segment x gender interaction for SLC36A1/PAT1, with a 50% higher amount of SLC36A1/PAT1 mRNA in the proximal jejunum in male compared to female growing pigs. The proton-coupled amino acid transporter SLC36A1/PAT1 is responsible for the luminal uptake of amino acids in the brush border of the proximal segments of the small intestine (Thwaites and Anderson, 2007). It could be that the higher jejunal SLC36A1/PAT1 expression in males reflected the faster growth rate and increased amino acid requirements for protein deposition of entire male growing pigs as derived from recent publications and model calculation by Dunshea et al. (2013). Taken together, it is possible that the capacity to import luminal Cu^{2+} and other divalent metals including $Fe^{2+}$and Mn^{2+} and amino acids is higher in the male porcine upper small intestine and that

this may contribute to the lower feed conversion rate after the nursery fase in male compared to female growing pigs (REF).

There was a tendency of an intestinal segment x gender interaction for SLC31A2/Ctr2 showing higher levels of SLC31A2/Ctr2mRNA in the ileum in females than in males. It is tempting to speculate that the higher expression of Ctr1 in the proximal jejunum in males is compensated in females by an higher expression of ileal SLC31A2/Ctr2.

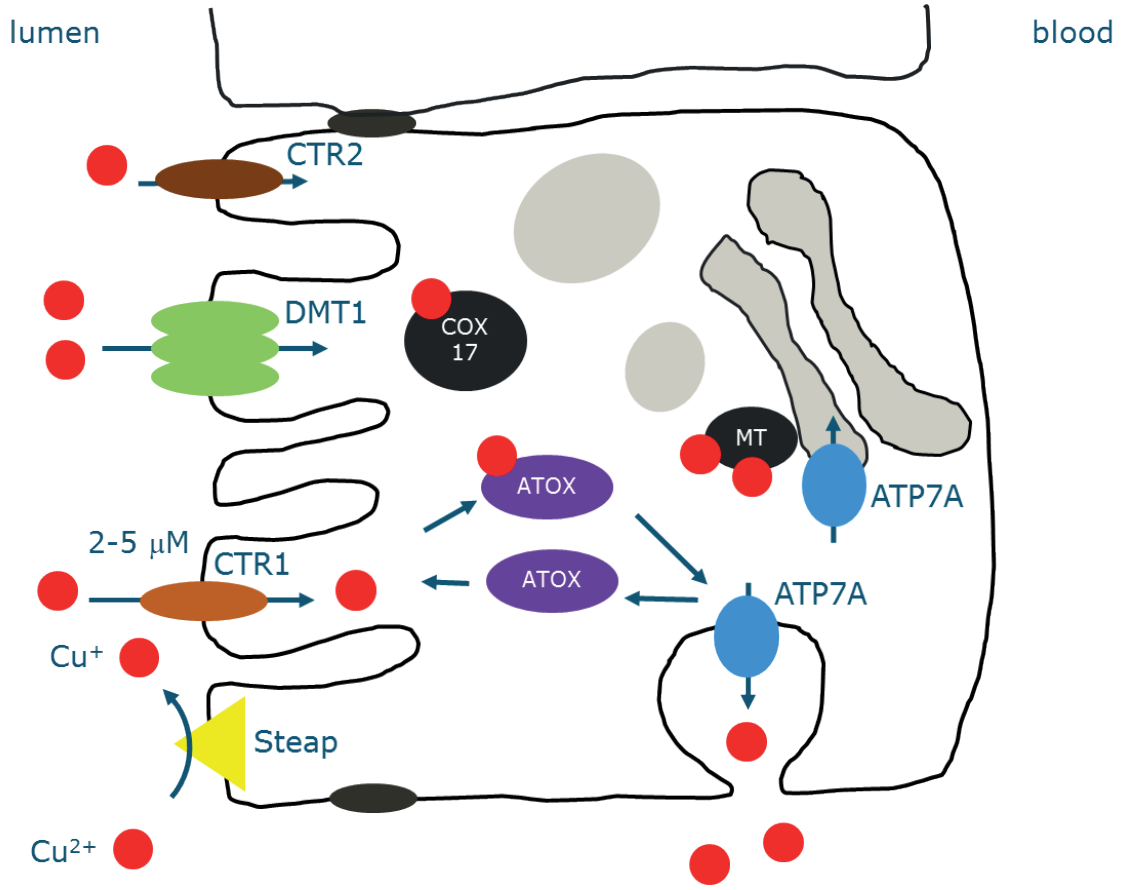

***Figure 3**. Transport of Cu in the enterocyte. Proteins are indicated with abbreviated names and their role are explained in the text.*

Conclusions

- The feed intake and daily gain responded linearly to an increase in Cu supplement from 15 to 160 mg/kg. In addition, an increase in Cu supplement reduced the percentage of piglets with soft faeces.
- The inclusion of the highest Cu supplemention of 160 mg/kg, within the legal limits of a total Cu content of 170 mg/kg until 12 weeks of age, increased the growth rate of the pigs by 60 g/d and the final body weight by 3.4 kg.
- This effect was largely mediated by an increase in feed intake. The Cu supplementation only improved the FCR in the first 28 days post weaning without overall effect after 56 days.
- The high Cu supplementation reduced the percentage of piglets with soft faeces from 10-15% to approximately 5% in the experimental period from Day 0-56 compared to Cu supplement of 15 mg/kg.
- Extrapolating these results, a reduction of the Cu inclusion level from 160 to 120 mg/kg would result in a relatively small reduction in growth performance and body weight of the pigs, approximately 0.5 kg at the end of the nursery period. In addition, it would increase the percentage of piglets with soft faeces by approximately 5% during the first 4 weeks post weaning. On Day 70, no more differences in body weight were observed between these treatments.
- Each early withdrawal of the high Cu supplement in the nursery period resulted in a (transient) reduction in growth rate. This effect was most drastic, a transient loss of 20% in ADG, after withdrawal on Day 14 post weaning. The effect was relatively small and of short duration after withdrawal on Day 28.
- Early withdrawal also enhanced the percentage of pigs with soft faeces, with most dramatic effects after withdrawal on Day 14 in the subsequent 2 week period.
- Inclusion of the high Cu supplement in the first 2 weeks of the grower period (Day 40-56) increased pig performance in this period and numerically increased the growth rate in the grower period from Day 40-70.
- Overall, the highest performance and lowest percentage of pigs with soft faeces was realised when the Cu supplement of 160 mg/kg was used in the entire experimental period. Relatively good results were realised in pigs fed the 120 mg/kg supplement and in pigs fed the 160 mg/kg with subsequent adaptation to a low Cu diet in the nursery period.
- The results suggest that withdrawal at Day 28 caused less reduction in gain and faecal consistence than withdrawal on Day 40, at the transition to the grower unit. Because of the large reduction in usage and excretion of Cu, this would reward further evaluation.
- It should be emphasised that in this experiment, no alternative health supporting products were included in the diet when the Cu content was reduced. Hence, we cannot advice whether these might alleviate the effect of a reduction in level or duration of Cu supplementation.
- Cu- and Zn-related absorption genes are differently expressed along the small intestine of growing pigs. Overall, gene expression was higher in the upper part with the exception of SLC31A2/Ctr2 which might have a different physiological role in the uptake of dietary Cu.
- A high dietary Cu content increased the level of duodenal MT1A mRNA. Pigs receiving a low Cu content displayed more SLC11A2/DMT1 and SLC30A5 mRNA in the duodenum and proximal jejunum, respectively, which may be a compensatory mechanism to maintain metal homeostasis.
- SLC31A1/Ctr1, SLC11A2/DMT1 and SLC36A1 were more abundantly expressed in the upper small intestine in male growing pigs, which may be related to increased nutrient requirements of male growing pigs compared to female pigs.

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Appendix 1 Diet composition

Composition and calculated nutrient content of experimental diets.

Ingredients	Pre starter, D1-14	Starter, D14-40	Grower, D40-56	Grower, D56-70
Barley	30.00	25.00	25.00	25.00
Wheat	24.26	26.66	28.59	28.20
Maize	20.00	20.00	20.00	20.00
Soya bean meal, extracted, CP>48%	-	12.00	13.97	13.97
Rapeseed meal, extracted	1.00	5.00	5.00	5.00
Delactosed whey powder	8.00	-	-	-
Soycomill-P, soya concentrate	6.00	-	-	-
Protastar, potato protein, ash<10	3.91	-	-	-
Protamyl, potato protein, ash>10	-	2.14	-	-
Soya bean oil	1.44	2.06	0.46	0.46
Palm oil	-	-	1.50	1.50
Cane molasses	1.00	2.00	2.00	2.00
Limestone	1.05	1.28	1.06	1.06
Lactic acid (liquid)	0.80	0.80	-	-
Vit. and min. premix (RDS 264)[1)]	1.00	1.00	1.00	-
Mono calcium phosphate	0.52	0.83	0.40	0.40
Sodium chloride	0.29	0.49	0.36	0.36
L-Lysine HCL	0.42	0.45	0.41	-
DL-Methionine	0.16	0.13	0.10	-
L-Threonine	0.1	0.13	0.12	-
L-Tryptophan	0.05	0.04	0.03	-
Vit. and min. premix (m2220, 15 Cu)	-	-	-	0.40
Phytase premix (m2345)	-	-	-	0.33
L-Lysine/tryptophan (premix m2713)	-	-	-	0.50
L-Lysine (premix m2710)	-	-	-	0.35
DL-Methionine (premix m2711)	-	-	-	0.17
L-Threonine (premix m2715)	-	-	-	0.30
Nutrients, g/kg				
Net energy, EW	1.12	1.10	1.10	1.10
Net energy, MJ	9.86	9.68	9.68	9.68
Crude ash	53 (46)	53 (46)	47 (42)	47 (45)
Crude protein	176 (172)	172 (169)	166 (162)	164 (162)
Crude fat (B)	42 (37)	47 (48)	46 (46)	44 (46)
Crude fibre	28 (23)	33 (27)	34 (30)	34 (31)
Starch (enzymatic)	410 (422)	399 (429)	410 (453)	426 (423)
Sugars	66	43	45	41
NSP	139	161	166	161
Digestible P	3.6	3.4	2.6	2.7
Calcium	7.5	8.0	6.6	6.4
Phosphorus	5.4	5.5	4.7	4.3
Sodium	2.5	2.0	1.5	1.5
Lysine	12.4	11.6	10.7	10.6
AID lysine	11.0	10.0	9.1	9.3
AID threonine	6.6	6.0	5.5	5.5
AID met + cys	6.6	6.0	5.5	5.5
AID tryptophan	2.09	1.90	1.73	1.75

[1)] Provided per kg of diet: vitamin A, 10,000 IU; vitamin D3, 2,000 IU; vitamin E, 40 IU; vitamin K3, 1.5 mg; thiamine, 1.0 mg; riboflavin, 4.0 mg; D-pantothenic acid, 15 mg; niacin, 30 mg; vitamin B_{12}, 20 µg; folic acid, 0.3 mg; pyridoxine, 1.5 mg; choline chloride 150 mg; biotin, 50 µg, Fe ($FeSO_4$-H_2O), 150 mg; Zn ($ZnSO_4$-H_2O) 45 mg; Mn (MnO), 30 mg; Co ($CoSO_4$-$7H_2O$), 0.15 mg; I (KI), 0.5 mg; Se (Na_2SeO_3-$5H_2O$), 0.3 mg. Additionally, this premix provided 500 FTU phytase (Natuphos® 5000 G, BASF) to diets. Cooked maize was used as carrier material

Appendix 2 Animal performance

Influence of Cu content in diets of weaned piglets supplied from Day 0 to 56 post weaning on body weight and growth performance of the animals in two-week periods.

Added Cu, mg/kg	T1	T2	T3	T4	T5	T6	T7	T8	SEM[1]	P T1-8[2]	PL T1-4	PQ T1-4
Day 0-14	15	80	120	160	160	160	160	160				
Day 14-28	15	80	120	160	160	160	15	15				
Day28-40	15	80	120	160	160	15	15	15				
Day 40-56	15	80	120	160	15	15	15	160				
BW Day 0	7.93	7.96	7.93	7.96	7.92	7.94	7.94	7.93	0.013	0.288	0.253	0.513
BW Day 14	10.28^{a}	10.65^{b}	10.85^{bc}	10.93^{bcd}	10.96^{bcd}	11.24^{d}	10.92^{bcd}	11.01^{cd}	0.12	<0.001	<0.001	0.417
BW Day 28	15.1^{a}	16.0^{b}	16.8^{c}	17.0^{cd}	16.9^{c}	17.6^{d}	15.9^{b}	15.7^{ab}	0.23	<0.001	<0.001	0.646
BW Day 40	22.4^{a}	23.7^{bcd}	24.7^{de}	25.2^{e}	24.6^{cde}	24.9^{e}	23.7^{bc}	22.9^{ab}	0.36	<0.001	<0.001	0.687
BW Day 56	34.1^{a}	35.5^{ab}	37.3^{cd}	37.5^{d}	35.8^{bcd}	37.2^{cd}	35.2^{ab}	35.7^{abc}	0.59	<0.001	<0.001	0.714
SD BW D56, kg	3.8	4.2	4.7	3.9	4.2	4.3	4.3	4.6	0.33	0.649	0.458	0.127
Day 0-14												
ADG, g/d	168^{a}	192^{b}	208^{bc}	213^{bcd}	217^{cd}	236^{d}	213^{bcd}	220^{cd}	8.5	<0.001	<0.001	0.441
ADFI, kg/d	225^{a}	248^{b}	267^{bc}	263^{bc}	266^{bc}	290^{d}	263^{bc}	272^{cd}	7.4	<0.001	<0.001	0.098
FCR	1.35^{b}	1.30^{ab}	1.28^{ab}	1.24^{a}	1.23^{a}	1.23^{a}	1.25^{a}	1.25^{a}	0.028	0.049	0.011	0.852
G/F	0.746	0.771	0.781	0.807	0.817	0.815	0.802	0.804	0.048	0.056	0.011	0.772
Day 14-28												
ADG, g/d	346^{a}	382^{b}	422^{c}	436^{c}	423^{c}	451^{c}	355^{ab}	339^{a}	12.1	<0.001	<0.001	0.876
ADFI, kg/d	562^{a}	607^{bc}	642^{cd}	661de	643^{cde}	682^{e}	581^{ab}	569^{ab}	13.9	<0.001	<0.001	0.800
FCR	1.64^{cd}	1.59^{bc}	1.53^{ab}	1.52^{ab}	1.52^{ab}	1.52^{a}	1.64^{cd}	1.69^{d}	0.026	<0.001	0.003	0.815
G/F	0.613^{abc}	0.630^{abcd}	0.655^{bcd}	0.658^{d}	0.658^{cd}	0.661^{d}	0.611^{ab}	0.594^{a}	0.028	<0.001	0.003	0.909
Day 28-40												
ADG, g/d	591^{a}	626^{abc}	647^{bc}	664^{c}	628^{abc}	599^{ab}	632^{abc}	585^{a}	17.2	0.020	0.002	0.869
ADFI, kg/d	927^{a}	969^{abc}	1033^{d}	1027^{cd}	993^{bcd}	1000^{bcd}	957^{ab}	915^{a}	22.5	0.002	<0.001	0.641
FCR	1.57^{ab}	1.55^{ab}	1.60^{b}	1.55^{ab}	1.59^{ab}	1.69^{c}	1.51^{a}	1.57^{ab}	0.032	0.013	0.867	0.665
G/F	0.640^{a}	0.647^{b}	0.626^{ab}	0.648^{b}	0.632^{ab}	0.601^{a}	0.662^{b}	0.641^{ab}	0.026	0.002	0.939	0.523
Day 40-56												
ADG, g/d	737^{ab}	748^{abc}	802^{c}	783^{bc}	707^{a}	781^{bc}	728^{ab}	796^{c}	19.9	0.007	0.024	0.750
ADFI, kg/d	1418^{bc}	1510^{cd}	1421^{bc}	1531^{d}	1305^{a}	1411^{abc}	1342^{ab}	1413^{bc}	38	<0.001	0.146	0.928
FCR	1.92^{bcd}	2.03^{d}	1.77^{a}	1.96^{cd}	1.85^{abc}	1.80^{ab}	1.85^{abc}	1.78^{a}	0.045	<0.001	0.522	0.818
G/F	0.527^{ab}	0.499^{a}	0.565^{b}	0.515^{ab}	0.543^{ab}	0.557^{b}	0.543^{ab}	0.564^{b}	0.034	<0.001	0.603	0.740
Day 0-40												
ADG, g/d	358^{a}	390^{bc}	416^{d}	428^{d}	414^{cd}	421^{d}	390^{bc}	372^{ab}	8.9	<0.001	<0.001	0.699
ADFI, kg/d	556^{a}	592^{bc}	631^{d}	635^{d}	618^{cd}	643^{d}	586^{ab}	572^{ab}	11.1	<0.001	<0.001	0.474
FCR	1.56^{c}	1.52^{abc}	1.52^{abc}	1.48^{a}	1.50^{ab}	1.53^{bc}	1.50^{ab}	1.54^{bc}	0.015	0.026	0.003	0.808
G/F	0.645^{a}	0.659^{ab}	0.659^{ab}	0.674^{ab}	0.669^{b}	0.655^{ab}	0.667^{ab}	0.652^{ab}	0.017	0.020	0.003	0.727
Day 0-56												
ADG, g/d	467^{a}	491^{ab}	524^{cd}	527^{d}	498^{bcd}	522^{cd}	486^{ab}	496^{abc}	10.4	<0.001	<0.001	0.722
ADFI, kg/d	760^{a}	805^{abc}	840^{bcd}	845^{cd}	793^{a}	855^{d}	786^{a}	800^{ab}	16.0	<0.001	<0.001	0.480
FCR	1.63	1.64	1.60	1.61	1.59	1.64	1.62	1.62	0.019	0.631	0.240	0.567
G/F	0.615	0.611	0.624	0.624	0.629	0.612	0.620	0.619	0.021	0.646	0.237	0.625
Feed usage												
Pre-starter, kg	3.14^{a}	3.47^{b}	3.74^{bc}	3.68^{bc}	3.72^{bc}	4.05^{d}	3.68^{bc}	3.81^{cd}	0.10	<0.001	<0.001	0.098
Starter, kg	19.3^{ab}	20.4^{bc}	21.7^{d}	21.9^{d}	21.2^{cd}	21.9^{d}	19.9^{ab}	19.2^{a}	0.45	<0.001	<0.001	0.661
Total, kg	22.4^{a}	23.9^{bc}	25.4^{d}	25.6^{d}	24.9^{cd}	25.9^{d}	23.6^{ab}	23.1^{ab}	0.4	<0.001	<0.001	0.468
Gain birth – D40[3], g/d	314^{a}	333^{bc}	348^{cd}	356^{d}	347^{cd}	352^{d}	332^{bc}	322^{ab}	5.6	<0.001	<0.001	0.808

[1] SEM, pooled standard error of least square means

[2] P T1-8, P-value of Cu effect in the complete model. PL T1-4 and PQ 1-4, linear and quadratic effects of Cu addition in T1 to T4

[3] Calculated with mean birth weight of 1.4 kg

Appendix 3 Subsequent animal performance

Influence of Cu content in diets of weaned piglets supplied from Day 0 to 56 post weaning on subsequent body weight and growth performance in growing finishing pigs.

Added Cu mg/kg	T1	T2	T3	T4	T5	T6	T7	T8	SEM[1]	P T1-8[2]	PL T1-4	PQ T1-4
Day 0-14	15	80	120	160	160	160	160	160				
Day 14-28	15	80	120	160	160	160	15	15				
Day 28-40	15	80	120	160	160	15	15	15				
Day 40-56	15	80	120	160	15	15	15	160				
BW Day 40	22.4^{a}	23.7^{bcd}	24.7^{de}	25.2^{e}	24.6^{cde}	24.9^{e}	23.7^{bc}	22.9^{ab}	0.36	<0.001	<0.001	0.687
BW Day 56	34.1^{a}	35.5^{ab}	37.3^{cd}	37.5^{d}	35.8^{bcd}	37.2^{cd}	35.2^{ab}	35.7^{abc}	0.59	<0.001	<0.001	0.714
BW Day 70	46.8^{a}	48.2^{ab}	50.1^{b}	49.9^{b}	48.5^{ab}	50.1^{a}	48.3^{ab}	48.9^{b}	0.72	0.020	<0.001	0.582
BW End of trial	119.3	118.8	119.3	120.8	119.6	120.4	119.3	119.5	0.81	0.687	0.168	0.126
Days in GF period	106.0	105.8	104.4	104.6	105.4	104.2	105.7	105.9	0.64	0.241	0.094	0.958
Day 56-70												
ADG, g/d	912	911	915	891	905	924	935	942	25.2	0.883	0.615	0.632
ADFI, kg/d	1745	1755	1799	1764	1753	1810	1737	1777	0.035	0.798	0.492	0.632
FCR	1.93	1.93	2.00	1.98	1.96	1.96	1.87	1.89	0.047	0.521	0.246	0.993
G/F	0.524	0.520	0.506	0.506	0.516	0.512	0.538	0.530	0.012	0.488	0.163	0.922
Day 70-slaughter												
ADG, g/d	951	927	926	947	940	944	935	927	10.8	0.587	0.651	0.062
ADFI, kg/d	2538	2471	2547	2541	2526	2548	2527	2471	0.030	0.349	0.663	0.182
FCR	2.67	2.67	2.75	2.68	2.69	2.70	2.70	2.66	0.026	0.316	0.234	0.397
G/F	0.374	0.376	0.364	0.373	0.373	0.371	0.370	0.376	0.0037	0.382	0.246	0.505
Day 40-70												
ADG, g/d	824	830	859	837	806	853	831	869	16.4	0.165	0.338	0.576
ADFI, kg/d	1582^{ab}	1633^{b}	1610^{ab}	1647^{b}	1529^{a}	1610^{ab}	1540^{a}	1595^{ab}	0.030	0.083	0.171	0.829
FCR	1.92^{bc}	1.97^{c}	1.88^{ab}	1.97^{c}	1.90^{abc}	1.89^{ab}	1.85^{ab}	1.84^{a}	0.026	0.003	0.554	0.713
G/F	0.523^{ab}	0.509^{a}	0.53^{bc}	0.510^{a}	0.527^{abc}	0.531^{bc}	0.540^{bc}	0.545^{c}	0.0069	0.002	0.453	0.723
Day 40-slaughter												
ADG, g/d	913	899	906	915	901	917	904	910	8.8	0.767	0.886	0.176
ADFI, kg/d	2270	2236	2280	2287	2245	2281	2250	2226	0.024	0.481	0.441	0.261
FCR	2.49	2.49	2.52	2.50	2.49	2.49	2.49	2.44	0.020	0.396	0.345	0.783
G/F	0.402	0.402	0.398	0.400	0.401	0.403	0.402	0.410	0.0033	0.383	0.339	0.818
Day 0-slaughter												
ADG, g/d	761	758	770	779	767	779	763	763	7.2	0.332	0.078	0.271
ADFI, kg/d	1.80	1.78	1.82	1.83	1.80	1.82	1.79	1.77	0.018	0.224	0.136	0.318
FCR	2.36	2.35	2.37	2.35	2.34	2.34	2.35	2.32	0.016	0.564	0.541	0.844
G/F	0.423	0.426	0.423	0.426	0.427	0.427	0.426	0.432	0.0030	0.535	0.563	0.311
Carcass weight, kg	91.8	92.0	92.2	93.1	92.1	93.2	92.5	92.3	0.66	0.761	0.091	0.348
Dressing %	77.1	77.4	77.3	77.2	77.1	77.5	77.7	77.3	0.25	0.725	0.696	0.365
Lean meat %	59.0	58.6	58.4	58.9	58.9	58.6	58.7	58.6	0.19	0.371	0.457	0.048
Muscle mm	60.2^{ab}	60.7^{ab}	59.6^{a}	61.0^{ab}	59.5^{a}	61.6^{b}	60.4^{ab}	59.5^{a}	0.54	0.067	0.490	0.414
Backfatmm	13.9	14.5	14.8	14.1	14.0	14.5	14.4	14.5	0.30	0.380	0.444	0.050

[1] SEM, pooled standard error of least square means

[2] P T1-8, P-value of copper effect in the complete model. PL T1-4 and PQ 1-4, linear and quadratic effects of Cu addition in T1 to T4

Appendix 4 Faecal consistency

Influence of Cu content in diets of weaned piglets supplied from Day 0 to 56 post weaning on faecal consistency of the pigs, in percentage of observed animals (pen basis) in each category.

Item	T1	T2	T3	T4	T5	T6	T7	T8	P-value[1]
Day 0-14	15	80	120	160	160	160	160	160	
Day 14-28	15	80	120	160	160	160	15	15	
Day 28-40	15	80	120	160	160	15	15	15	
Day 40-56	15	80	120	160	15	15	15	160	
Day 0-14	a	abc	ab	bc	abc	d	bc	cd	<0.001
Normal faeces, %	87.9	90.4	89.8	93.8	92.8	97.3	93.3	94.3	
Soft faeces, %	10.1	7.5	7.5	3.8	5.0	1.3	4.3	3.8	
Watery diarrhoea, %	2.0	2.1	2.8	2.5	2.3	1.5	2.5	2.0	
Day 14-28	a	b	b	c	c	c	a	a	<0.001
Normal faeces, %	82.4	90.4	90.1	95.1	96.0	94.5	78.7	76.0	
Soft faeces, %	14.5	7.9	6.7	2.6	2.5	3.8	18.1	18.5	
Watery diarrhoea, %	3.2	1.8	3.3	2.3	1.6	1.8	3.3	5.6	
Day 28-40	a	bc	c	c	c	ab	a	a	<0.001
Normal faeces, %	87.4	94.4	96.4	97.3	95.0	90.2	86.5	86.7	
Soft faeces, %	10.1	4.0	2.3	1.6	2.6	8.8	11.9	12.0	
Watery diarrhoea, %	2.6	1.6	1.4	1.1	2.5	1.1	1.7	2.1	
Day 40-56	ab	ab	bc	c	a	ab	a	bc	<0.001
Normal faeces, %	89.0	90.4	93.1	95.2	85.3	86.6	84.3	94.2	
Soft faeces, %	8.7	7.3	4.7	3.3	11.2	10.8	12.7	3.3	
Watery diarrhoea, %	2.3	2.3	2.2	1.6	3.6	2.6	3.1	2.6	
Day 56-70									0.484
Normal faeces, %	87.6	87.9	87.7	90.1	84.9	87.4	86.3	90.1	
Soft faeces, %	9.1	8.3	7.6	6.5	11.9	9.6	10.2	8.4	
Watery diarrhoea, %	3.4	3.8	4.8	3.5	3.3	3.1	3.6	1.6	
Day 70-84									0.181
Normal faeces, %	95.5	97.0	92.6	96.0	95.2	97.2	95.9	96.6	
Soft faeces, %	4.5	3.0	7.0	3.8	4.6	2.8	3.9	3.2	
Watery diarrhoea, %	0.0	0.0	0.5	0.3	0.3	0.0	0.3	0.3	
Day 84-98									0.846
Normal faeces, %	96.7	97.7	96.5	96.7	96.8	97.0	97.3	96.4	
Soft faeces, %	2.8	1.9	3.3	3.1	2.7	2.5	2.7	3.4	
Watery diarrhoea, %	0.6	0.5	0.3	0.3	0.6	0.5	0.0	0.3	
Day 98-112	ab	b	b	ab	a	ab	ab	ab	<0.001
Normal faeces, %	98.7	99.0	99.0	98.2	96.4	98.5	98.1	97.6	
Soft faeces, %	1.4	0.8	1.0	1.6	2.6	1.0	1.7	2.4	
Watery diarrhoea, %	0.0	0.3	0.0	0.3	1.1	0.5	0.3	0.0	

[1] P-value, significance of copper effects in the complete model for Treatments 1-6 and Treatments 1-7

[2] ND, P-value not determined because of too low numbers of pigs with soft faeces

To explore the potential of nature to improve the quality of life

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