Universitat Autònoma de Barcelona

EXPLORING THE Zn DEFICIENCY HYPOTHESIS TO EXPLAIN THE BENEFICIAL EFFECT OF THERAPEUTIC ZnO IN WEANING PIGS

TESI DOCTORAL PRESENTADA PER:

Roger Davin Cardona

SOTA LA DIRECCIÓ DELS DOCTORS:

Edgar Garcia Manzanilla i José Francisco Pérez Hernández

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José Francisco Pérez Hernández, professor titular del Departament de Ciència Animal i dels Aliments de la Facultat de Veterinària de la Universitat Autònoma de Barcelona i **Edgar Garcia Manzanilla,** research officer at the pig development department animal and grassland research and innovation centre de Teagasc (Irlanda).

Certifiquen:

Que la memòria titulada "Exploring the Zn defficiency hypothesis to explain the beneficial effect of therapeutic ZnO in weaning pigs", presentada per Roger Davin Cardona per optar al grau de Doctor, ha estat realizada sota la seva direcció i, considerant-la acabada, autoritzen la seva presentación per a que sigui jutjada per la comissió corresponent.

I perquè consti als efecte oportuns, signen la present a Bellaterra, 12 de maig de 2014.

Dr. Edgar Garcia Manzanilla

Dr. José Francisco Pérez Hernández

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Summary

The general aim of this PhD thesis is to study Zn status of weaned piglets and whether it is affected by weaning or dietary Zn supplementation, like pharmacological levels of ZnO that are usually administered in post-weaning piglet's feed to prevent and/or treat diarrhea. To achieve this goal the following experiments (Chapter 3 to 6) were designed.

In **Chapter 3** we wanted to explore to what extent sow's age or productive weariness could affect piglet's Zn status at weaning by an impaired mineral status of sows and consequently an altered mineral colostrum and milk composition. At the same experiment we wanted to assess how low Ca, P and Zn sow's diet with or without phytase supplementation could affect sow reproductive and litter performance as well as their digestibilities and mineral plasma and milk concentrations. Results showed that colostrum and milk mineral concentrations remain nearly constant meaning that sows make a great effort to provide a constant mineral supply to their offspring regardless of its age and variations in mineral composition and phytase supplementation of its diet.

In **Chapter 4 (Davin et al., 2013)**, we wanted to assess whether weaning and dietary pharmacological levels of Zn as ZnO affect piglets Zn status. We intended to evaluate the effect of weaning and dietary ZnO supplementation on plasma, organs (liver, pancreas and spleen) and GIT contents mineral concentrations. Results showed that weaned animals presented lower plasma Zn levels compared to unweaned animals, but ZnO supplementation counteracts this drop. Supplementation with high doses of ZnO during one week increased levels of Zn in liver, pancreas and in the gastrointestinal tract (GIT) contents. Weaned animals showed a similar Zn concentration in the organs but a higher total concentration of Zn in GIT contents compared to unweaned animals.

Chapter 5 (Davin et al., 2012), intended to add detail on how Zn, Fe and Cu were distributed into the soluble or insoluble fraction of the different GIT contents obtained from the animals of the previous chapter. Concentration of Zn clearly increased along the GIT for animals receiving high levels of ZnO compared to weaned animals receiving the control diet and to unweaned animals. The proportion of Zn in the soluble fraction in jejunum, ileum and cecum and Fe along the GIT of unweaned pigs was higher than in those weaned animals. In contrast, Cu concentrations were lower in unweaned pigs than in weaned pigs along the GIT and were increased in cecum and colon when dietary high levels of Zn were fed.

Chapter 6 includes two experiments in piglets to assess how Zn serum concentration changes around weaning and as affected by different levels, sources and posologies of supplemented Zn. Few days after weaning Zn serum concentrations decreased and pharmacological levels of Zn as ZnO was the only treatment able to rapidly increase Zn serum concentration back to physiological levels.

Results exposed in this thesis support the idea that Zn is highly regulated in the sow during lactation to satisfy Zn requirements of piglets. However, Zn status is compromised in piglets after weaning, which could have a role on growth and predisposition to diarrhea during this period. Among the different explored strategies, therapeutic doses of ZnO that are routinely used in commercial farms were the only efficient Zn treatment to counteract this transient situation.

Resum

L'objectiu general de la present tesi doctoral és estudiar l'estatus de Zn dels garrins deslletats i com aquest es veu afectat pel deslletament i la suplementació dietària de Zn, com són els nivells farmacològics de ZnO administrats de forma rutinària en les dietes dels garrins després del deslletament per a prevenir i/o tractar la diarrea. Per aconseguir aquest objectiu els següents experiments (Capítols 3 al 6) van ser dissenyats.

En el **Capítol 3** vam voler esbrinar fins a quin punt l'edat de la truja i el seu desgast productiu poden afectar l'estatus de Zn dels garrins al deslletament a causa d'un estatus mineral de les truges malmès que a més pugui alterar la composició mineral del calostre i de la llet. Al mateix temps vam voler avaluar si una dieta per a truges baixa en Ca, P i Zn amb o sense suplementació de fitassa poden afectar el rendiment reproductiu de les truges i el creixement dels garrins, a més d'afectar la digestibilitat del Ca, P i Zn de les truges i les seves concentracions en el plasma i llet. Els resultats van mostrar que les concentracions dels minerals en el calostre i la llet es van mantenir constants evidenciant que les truges fan un gran esforç per a proveir un subministrament constant de minerals a la seva descendència independentment de la seva edat i de la diferent composició mineral i suplementació en fitassa del seu pinso.

En el **Capítol 4 (Davin et al., 2013)**, volíem avaluar com el deslletament i nivells farmacològics de Zn en forma de ZnO poden afectar l'estatus de Zn dels garrins. Vam avaluar aquests dos efectes sobre la concentració mineral del plasma, òrgans (fetge, pàncrees i melsa) i dels continguts del tracte gastrointestinal (GIT). Els resultats van mostrar que els animals deslletats presentaven una concentració plasmàtica de Zn inferior a la dels animals no-deslletats, però la suplementació amb ZnO va contrarestar aquesta caiguda. La suplementació amb ZnO durant una setmana va produir un increment en els nivells de Zn al fetge, al pàncrees i als continguts del GIT. Els animals deslletats van presentar concentracions de Zn en els òrgans semblants als animals no-deslletats en canvi, la concentració de Zn en els continguts del GIT va ser superior.

El **Capítol 5 (Davin et al., 2012)** pretenia mostrar de forma més detallada com el Zn, Fe i Cu es distribuïen en les fraccions solubles i insolubles dels diferents continguts del GIT, que es van obtenir dels animals del capítol anterior. La concentració de Zn va augmentar de forma

clara al llarg del GIT dels animals que reberen nivells elevats de ZnO, en comparació als animals deslletats que reberen una dieta control i els animals no-deslletats. La proporció de Zn en la fracció soluble en el jejú, ili i cec i del Fe al llarg del GIT dels animals no-deslletats va ser superior a la dels animals deslletats. Per contra, les concentracions de Cu dels animals no-deslletats van ser inferiors a les dels animals deslletats al llarg del GIT i es van veure incrementades al cec i al colon quan els animals van rebre alts nivells de Zn a la dieta.

El **Capítol 6** inclou dos experiments en garrins per avaluar com les concentracions sèriques de Zn canvien al voltant del deslletament i com es veuen afectades al suplementar diferents nivells, fonts i posologies de Zn. Pocs dies després del deslletament les concentracions de Zn sèriques disminuïren i el tractament amb alts nivells de Zn en forma de ZnO a la dieta fou l'únic capaç d'incrementar de forma ràpida la concentració sèrica de Zn fins a nivells fisiològics.

Els resultats exposats en aquesta tesi recolzen la idea que el Zn es troba fortament regulat en la truja durant la lactació per poder satisfer els requeriments dels garrins. Tot i això, l'estatus de Zn en els garrins es veu compromès després del deslletament, malgrat que pugui tenir un paper important en el creixement dels garrins i la seva predisposició a manifestar diarrea durant aquest període. Entre les diferents estratègies investigades, les dosis terapèutiques de ZnO que són usades de forma rutinària en les dietes dels garrins criats en granges comercials va ser l'únic tractament capaç de contrarestar aquesta situació transitòria.

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Abbreviations used

AA: amino acid
ADFI: average daily feed intake
ADG: average daily gain
AP: alkaline phosphatase
BW: body weight
$CuSO_4$: copper sulfate
DM: dry matter
DNA: deoxyribonucleic acid
DRI: dietary reference intake
EDTA: ethylenediaminetetraacetic acid
EE: environmental enteropathy
EFSA: European food safety authority
ETEC: Enterotoxigenic Escherichia coli
EU: European Union
EZP: exchangeable zinc pool
FI: feed intake
FEDNA: federación española para el desarrollo de la nutrición animal
FEEDAP: panel on additives and products or substances used in animal feed
FFEBW: fat-free empty body weight
FTU: phytase unit
G/F: gain/feed ratio
GIT: gastrointestinal tract
HDL: high-density lipoprotein
IAEA: international atomic energy agency
Ig: immunoglobulin
IGF: insulin-like growth factor
IL: interleukin
IOM: institute of medicine
IZiNCG: international zinc nutrition consultative group
LD_{50}: lethal dose 50%
mEq: milliequivalents
MT: metallothionein
MTL: maximum tolerable levels
NRC: national research council
ORS: oral rehydration solutions
PMNC: peripheral blood mononuclear cells
PU: phytase units
PWD: post-weaning diarrhea
RBV: relative bioavailability
RDA: recommended dietary allowances
RNA: ribonucleic acid
SCAN: Scientific committee on animal nutrition
SCFA: short chain fatty acids
SEM: standard error of the mean
SOD: superoxide dismutases
TLR: toll-like receptor
UNICEF: united nations children's fund
VFA: volatile fatty acids
WG: weight gain
WHO: world health organization
ZIP: Zrt/Irt-like protein
ZnAA: zinc amino acid complex
ZnO: zinc oxide
$ZnSO_4$: zinc sulfate

Chapter 1

Literature review

1.1. General introduction.

Zinc (Zn) is an essential micronutrient for living organisms from the unicellular level to the vertebrate species (Solomons, 2013). In mammals, Zn acts as a cofactor and as structural element for more than 300 enzymes and is required for multiple biological functions. A deficiency of this element in mammals results in stunted growth, poor immune function and impaired homeostasis among other effects (Brown et al., 2002; Walker et al., 2009). In addition, Zn has many uses in daily live as antifungal, antimicrobial, antidandruff or sunburn protection. In the current review we discuss different aspects related to one of these applications, in particular, how Zn is empirically used as an antidiarrheal compound affecting the survival of young individuals in both humans and pigs.

Post-weaning diarrhea (PWD) in the pig industry is one of the main problems in pig production affecting the survival and performance of piglets in the post-weaning period. Diarrhea episodes are frequent few days after weaning due to the presence of stressful factors (environmental, social and dietary) and the immaturity of piglet's intestinal and immune systems (Lallès et al., 2007). The most common and successful strategy to overcome these problems is the use of antibiotics (in-feed, water or injected). However, the use of antibiotics is controversial as they promote antibiotic-resistant bacteria. Alternatively, ZnO and $CuSO_4$ are been used in the diets for weaned pigs at high levels to control post-weaning diarrhea and optimize growth performance (Hill et al., 2000). However, concerns arise due to environmental accumulation of these minerals (Berenguer et al., 2008), and recently antimicrobial resistance concerns have also raised (Bednorz et al., 2013).

Reported effects of high in-feed ZnO include increased gene expression of antimicrobial peptides in the small intestine (Wang et al., 2004), increased IGF-1 and IGF-1R expression in the small intestinal mucosa (Li et al., 2006), positive effects on the stability and diversity of the microbiota (Katouli et al., 1999), bactericidal effects (Jensen-Waern et al., 1998), and reductions in electrolyte secretion from enterocytes (Carlson et al., 2006). Hojberg et al., (2005) speculated that the influence of ZnO on the GIT microbiota resembled the working mechanism suggested for growth-promoting antibiotics.

In children, diarrhea causes almost 1.3 million deaths annually in children less than 5 years of age. Zn deficiency is one of the ten most important factors contributing to the burden of disease in developing countries with high children mortality (Shrimpton et al., 2005) and it is so far one of the main hypotheses considered in humans to cause childhood diarrhea in developing countries (WHO, 2005). Zn deficiency is counteracted by Zn supplementation that is one of the major preventive public health strategies and the only treatment

recommended by the WHO. Zn is provided as $ZnSO_4$ in combination with oral rehydration solution (WHO/UNICEF, 2004).

Thus, Zn is used as a solution for diarrhea in both piglets and children although its mode of action as antidiarrheal treatment (ZnO for piglets; $ZnSO_4$ for children) is not yet fully understood. In the present thesis I expect to bring more knowledge to further understand the effect of dietary Zn on mineral behavior in the GIT and in the organism of young individuals.

1.2. Zinc as a nutrient

"*Zinc is indispensable to the growth and development of microorganisms, plants and animals"*(Chasapis et al., 2012) Zinc essentiality was first established for *Aspergilus niger* in 1869, and for plants in 1926 (Raulin, 1869; Sommer and Lipman, 1926). Zn was shown to be an essential nutrient for rats and mice in the 1930s, for pigs in 1955 and for humans in 1963 (Todd et al., 1933; Tucker and Salmon, 1955; Prasad et al., 1963). Tucker and Salmon (1955) reported that Zn supplementation in pigs prevented and cured growth and feed intake retardation, abnormal skin and other symptoms of parakeratosis. However, since the discovery of Zn deficiency as a human health problem in 1961, interest in the biochemical and clinical aspects of Zn nutrition has increased markedly (Roohani et al., 2013).

1.2.1. Biological functions

Most nutrients are required for specific metabolic functions rather than for in general metabolism as is the case for Zn (Golden, 1989). When a deficiency of nutrient with specific functions ("type 1" nutrient, e.g. Fe, vitamin C, folic acid) occurs, the diagnosis of a deficiency is relatively straightforward as tissue nutrient concentrations decline and a defect in one or more specific metabolic pathways develop, causing the onset of specific clinical or biochemical signs (King, 2011). In contrast "type 2" nutrients, as Zn, P or Mg, are required for multiple general metabolic functions and therefore respond to insufficient intakes quite differently (Golden, 1989), more details about Zn deficiency will be explained in the following sections.

Zn is required by general metabolism because among other reasons is the only metal that is a cofactor to more than 300 metallo-enzymes and is required for the structural and functional integrity of over 2,000 transcription factors. Almost every signaling and metabolic pathway is dependent on one or more zinc-requiring proteins (Rink and Gabriel, 2000; Beattie and Kwun, 2004; Cousins et al., 2006) including carbohydrate, lipid, protein and nucleic acid

metabolism (O'Dell and Sunde, 1997; Keen et al., 2003) and is also involved in stabilizing the structures of DNA, RNA, proteins or other molecules (Keen et al., 2003) through Zn-finger proteins. Zn has a big impact on growth because it stimulates connective tissue development and maintenance (Fukada et al., 2008), bone mineralization (Yamaguchi, 1998) and regulates the function of insulin-like growth factor 1 (IGF1). It is also essential also for both innate and adaptive immunity (Prasad, 2009) and is a good antioxidant agent (it keeps biomembrane stability, it is a structural component of Cu/Zn-SOD and MT) (Powell, 2000; Klotz et al., 2003; Tapiero and Tew, 2003; Eide, 2011). Zn is also involved in other functions like wound healing and blood clotting, taste acuity and appetite control that can be further consulted at Stefanidou et al., 2006; Murakami and Hirano, 2008; Prasad, 2009; Bhowmik et al., 2010; Plum et al., 2010.

1.2.2. Digestion, absorption, body flows and excretion

Zn bioavailability and absorption are complex to understand and to assess as there are several factors that have an impact on them:

- Dietary factors: presence of nutrients that interact with Zn (e.g. Ca, Fe) or anti-nutritional factors (e.g. phytate) in the diet.
- Zn source: the specific chemical properties of each Zn source (Zn from foodstuffs vs. Zn from supplements, different chemical properties between Zn compounds) and their level in the diet
- Physiological factors: Zn status of the individuals, expression of several proteins (MT, Zn transporters) that regulate its absorption, gastric pH and fasting situation of the individual.

In the following sections all these points will be discussed.

1.2.2.1. Zn in the diet

Zn nutriture is based on the quantity and bioavailability of Zn in the diet (Maret and Sandstead, 2006). Worldwide, pulses and cereals are the major sources of Zn for most people (Gibson, 1994) and are the only sources of Zn for most farm animals due to the ban of animal-origin ingredients usage in feeds. In developed countries pulses and cereals provide about 20-40%, meat about 40-60% and dairy products about 10-30% of dietary Zn (Nriagu, 2007). Red meats, organ meats (e.g. liver) and shellfish are the best dietary sources as they provide highly bioavailable Zn whereas whole grain cereals are rich sources of Zn although the presence of phytate is known to inhibit Zn bioavailability. Refined grain products are

poor sources because Zn is found primarily in the bran and germ and vegetable and fruits contain low Zn concentrations (Table 1.1.)

Zn bioavailability is influenced by other food constituents like phytate, Ca, some dietary fibers, lignin and products from Maillard reactions that bind Zn and inhibit its absorption (Sandstead and Smith Jr, 1996). High concentrations of Fe and folic acid also affect Zn bioavailability (Milne et al., 1984; Solomons, 1986; Simmer et al., 1987). The bioavailability of Zn in most common foods typically is in the range of 10-30% (Nriagu, 2007), lower values would be expected for vegetable-based diets.

Table 1.1. Zn content of foods; mg/100gr. Adapted from the International MiniList.

Food groups	**Zn content (mg/kg)**	**Phytate content (g/kg)**
Liver, kidney (beef, poultry)	42-61	0
Meat (beef, pork)	29-47	0
Poultry (chicken, duck)	18-30	0
Seafood (fish, etc.)	5-52	0
Eggs	11-14	0
Dairy (milk, cheese)	4-31	0
Seeds, nuts (sesame, almond)	29-78	17.6-47.1
Beans, lentils (soy, chickpea)	10-20	1.1-6.2
Whole-grain cereals (wheat, maize, brown rice)	5-32	2.1-6.2
Refined cereal grains (white flour, white rice)	4-8	0.3-4.4
Bread	9	0.3
Tubers	3-5	0.9-1.3
Vegetables	1-8	0-1.2
Fruits	0-2	0-0.6

Swine and poultry plant-based diets are supplemented with exogenous sources of vitamins and minerals as a vitamin and mineral premix in order to meet animal requirements. This is the case for pig diets as most of feed ingredients have an average low content of Zn and relatively high content of phytate (Table 1.2).

Table 1.2. Zn, P and phytic P content of common feed ingredients. Source: FEDNA 2010.

	Zn (mg/kg)	P (g/kg)	Phytic P (g/kg)
Rice	21	1.0	0.6
Dehulled Oats	23	3.8	1.8
Barley	30	3.6	2.1
Corn	24	2.7	1.9
Wheat	50	3.	2.0
Wheat bran	83	9.5	7.5
Rice bran	45	13.5	12.0
Peas	45	4.0	2.1
Soymeal 44	48	6.1	4.0
Soybean hulls	42	1.5	1.1

Phytate P concentrations of 0.5-1.3 % are noted in wheat by-products, rice bran and maize/wheat gluten feed and of 0.3-0.5 % in triticale and oilseed meals, while in cereals and legumes the content is < 0.3 %, representing between 50 and 80 % of total phosphorus (EFSA, 2014).

Several Zn compounds are available to be used in animal premixes or for human intervention programs (either mixed in the food or dispersed in water). They are usually divided in two categories: inorganic (oxide, sulfate, chloride, carbonate) and organic (lactate, acetate, chelate of amino acids) Zn compounds. Yet, ZnO and $ZnSO_4$ are least expensive and most commonly used by the food and feed industries (Brown et al., 2004); Zn chelates usage is increasing as feed additive for pigs but still ZnO represents around 90%, $ZnSO_4$ 5-10% and Zn chelates just around 2-3% of the sales (own data from a feed industry, Spain).

For human intervention programs the choice of a particular chemical form is based on the Zn intervention strategy (supplementation vs. fortification), solubility in water, intragastric solubility, taste, cost, side effects and safety (Brown et al., 2004).

Water-soluble compounds are considered (acetate, gluconate, and sulfate) to be more readily absorbable than compounds with limited solubility at neutral pH. $ZnSO_4$ theoretically provides more absorbable Zn because of its greater solubility (Wolfe et al., 1994) but results have been variable and sometimes conflicting in terms of relatively absorption of the different Zn compounds administered as a supplement (Barrie et al., 1987; Schölmerich et al., 1987; Prasad et al., 1993; Henderson et al., 1995). Recently Zn citrate has been considered as a useful alternative; it is as well absorbed as gluconate, an higher than ZnO (Wegmüller et al., 2014). In Zn fortification studies in humans no differences are shown when comparing $ZnSO_4$ with ZnO (Herman et al., 2002; López de Romaña et al., 2003; Hotz et al., 2005). Some studies suggest that ZnO is poorly absorbed because its low solubility at the basic pH

of the small intestine may prevent it from dissociating in the GIT (Sturniolo et al., 1991; Henderson et al., 1995). However, this may only present a problem when gastric acidity is reduced, as may occur in malnourished children (Brown et al., 2004) or in weaned piglets (Heo et al., 2013).

Table 1.3. Zn compounds characteristics. Adapted from (Brown and Wuehler, 2000; NRC, 2012)

Zinc compound	Chemical formula	Zn content (%)	Color	Taste	Solubility in water (20°C)	RBV (%)	Price	
							$/kg compound	$/kg Zn
Zinc acetate	$(CH_3CO_2)_2Zn$	30	White/slightly efflorescent	Astringent	Soluble		10.2	28.6
Zinc carbonate	$ZnCO_3$	56	White	Astringent	Insoluble	100	16.0	30.7
Zinc chloride	$ZnCl_2$	48	White	Astringent	Soluble	100	32.5	67.8
Zinc citrate, dihydrate	$(C_6H_5O_7)_2Zn_3 \cdot 2 H_2O$	32	White	-	Slightly soluble		8.0	23.4
Zinc gluconate	$C_{12}H_{22}O_{14}Zn$	14	White	-	Soluble		20.9	145.6
Zinc methionine	$C_{10}H_{20}ON_2O_4SZn$	10-18.5	White	Slightly sour and bitter	Soluble		25.4	83.4
Zinc oxide	ZnO	72	White, gray, yellowish white	Bitter, astringent	Insoluble	50 to 80	4.5	5.6
Zinc sulfate (monohydrate)	$ZnSO_4 \cdot H_2O$	35.5	Colorless	-	Soluble	100	-	-
Zinc sulfate (heptahydrate)	$ZnSO_4 \cdot 7H_2O$	22.3	Colorless	Astringent	Soluble	100	10.4	25.7

RBV: Relative bioavailability.

The assessment of Zn availability of different Zn compounds is not easy as there is no single criterion response in both human or pigs. The special features of Zn metabolism (high endogenous intestinal excretion, a rapid turnover of Zn in plasma and a constant urinary excretion over a wide range of dietary intakes) limit the possible range of methods that can be used to measure Zn absorption. The apparent absorption is calculated as the difference between dietary Zn intake and fecal Zn content. To measure the true absorption of Zn, endogenous Zn should be separated from unabsorbed dietary Zn and isotope techniques are necessary (Brown et al., 2004).

In pigs, the Nutrient requirements of swine tables of NRC, 2012 estimates the bioavailability of Zn from inorganic Zn salts expressed as a percentage of a recognized standard and do not refer to percentage absorbed or retained; the absorbed and retained Zn as a percentage of intake is usually less than 50% of the intake (NRC, 2012). Variability of ZnO availability is huge, ranging from 39 to 88 relative to sulfate (Hahn and Baker, 1993; Wedekind et al., 1994; Schell and Kornegay, 1996). Zn from organic complexes seems to have approximately equal bioavailability to the Zn sulfate (Hahn and Baker, 1993; Wedekind et al., 1994; Schell and Kornegay, 1996; Swinkels et al., 1996; Cheng et al., 1998). However the different chelate

compounds and results obtained are probably an important factor to take into account (Revy et al., 2004; de Souza et al., 2007; Nitrayova et al., 2012).

Table 1.4. Overview of animal feeding trials evaluating the relative bioavailability of different Zn sources in pigs. Adapted from Lena Martin PhD Thesis, 2013

Zn sources	**Response criteria**	**Zinc (mg/kg diet)**	**Results**	**Specie (initial BW, kg)**	**Reference**
ZnO Metallic Zn dust	Performance Serum Zn	25/50	Growth and serum Zn: independent of source. RBV of Zn dust ≈ 30% greater than that from ZnO.	Swine (8)	Miller et al., 1981
$ZnSO_4$ Zn methionine	Performance Bone Zn Serum Zn	9/12/15	Zn sources are of similar biological value.	Swine (13)	Hill et al., 1986
$ZnSO_4$ Zn methionine Zn lysine ZnO	Performance Serum Zn Bone Zn	5/10/20/40/80	RBV: $ZnSO_4$ > Zn-Met > ZnO > Zn-Lys	Swine (23)	Wedekind et al., 1994
$ZnSO_4$ Zn methionine Zn lysine ZnO	Performance Serum Zn Liver, bone, kidney Zn	1,000/2,000/3,000	Performance: no diff. btw. sources. Serum, liver and kidney Zn: $ZnSO_4$ > ZnO (not at wk 4 liver & kidney) RBV: $ZnSO_4$ > Zn-Lys = Zn-Met > ZnO	Swine (8)	Schell and Kornegay, 1996
$ZnSO_4$ Zn AA chelate	Serum Zn Liver, pancreas, kidney, brain Zn	17(depleted)/45	Clear effect of Zn supplementation. No effect of Zn source	Swine (5)	Swinkels et al., 1996
$ZnSO_4$ Zn lysine	Absorption Serum Zn Bone Zn	100	No differences of Zn source	Swine (7)	Cheng et al., 1998
Zn polysaccharide ZnO	Performance Plasma Zn	Zn-PS: 150/300/450 ZnO: 2,000	Performance: no differences 300/450 Zn-PS vs. 2,000 ZnO Plasma Zn: ZnO > other treatments	Swine (6)	Buff et al., 2005
ZnO Zn methionine Zn chelates AA	Performance	Zn-Met: 125/250/500 ZnO:500/2,000/2,500	Performance: 2,000/2,500 ZnO > 500 any organic Zn source or ZnO	Swine (6)	Hollis et al., 2005
ZnO Zn methioneine Zn glycine Zn yeast Zn proteinate	Performance Serum Zn Enzyme activity	ZnO: 10/100 Other Zn sources: 10	WG:100ZnO&10Zn-Met&10Zn-yeast >10ZnO Serum Zn: 100 ZnO > 10 Zn-prot RBV: Zn-yeast&Zn-Met > ZnO	Swine (24)	Nitrayova et al., 2012
ZnO Zn montmorillonite	Performance Intestinal permeability Enzyme activity	Zn-MMT: 250/500/750 ZnO: 2,000	Performance: 500/750 Zn-MMT = 2,000 ZnO	Swine (7)	Hu et al., 2012

Recently, a new experimental model has been developed to determine Zn requirement in piglets and hence to also assess bioavailability of dietary Zn sources (Brugger et al., 2014) based on Zn digestibility, Zn concentration in different tissues (liver, bone, blood) and gene expression (MT).

1.2.2.2. Chemical behavior in the digestive tract

Zn is released from food or supplements as free ions during digestion. These liberated ions may then bind endogenously secreted ligands before their transport into the enterocytes in the duodenum and jejunum (Roohani et al., 2013).

From a chemical point of view when a Zn compound is ingested, in normal physiological conditions it is expected to finish as ionic zinc ($Zn^{2+}_{(aq)}$) once it reaches the stomach. For example, for ZnO there are at least two main reactions that take pace in the stomach:

$$ZnO_{(s)} + 2\ HCl_{(aq)} \rightarrow Zn^{2+}_{(aq)} + 2\ Cl^{-}_{(aq)} + H_2O_{(l)}$$

$$ZnO_{(s)} + 2\ H_3O^{-}_{(aq)} \rightarrow Zn^{2+}_{(aq)} + 3\ H_2O_{(l)}$$

To what extent this reaction will take place depends on the pH in the stomach; the pH should be as low as 2-3. This form of Zn ($Zn^{2+}_{(aq)}$) will allow its absorption in stomach and in proximal parts of small intestine producing increases on Zn levels in serum, liver and other organs.

Individuals not able to achieve this low gastric pH will have less available Zn to be absorbed. In that case, more ZnO will pass by the stomach and will reach distal parts of the GIT with higher pH (around 8). This reaction is produced to a greater extent when high levels of ZnO are fed. In these situations Zn could react with water producing a new solid form of Zn following the equation here described. Zn hydroxide is formed but it is not well known and we still cannot tell to what extent it will appear and what would be its effect.

$$ZnO_{(s)} + H_2O_{(l)} \leftrightarrows Zn(OH)_{2(s)}$$

Some other ZnO will pass by the stomach and the GIT without reacting. This fact can be important as the antimicrobial effect of Zn is produced by the solid form ZnO. Zn compounds like $ZnSO_4$ and $ZnCl_2$ are easily and completely solubilized in both stomach and GIT, and pH does not have a great effect on them, in the case of zinc sulfate, Zn^{2+} and SO_4^{2-} are formed. However when dissociated, the sulfate and chloride may have an osmotic effect on the intestine inducing watery feces. Zn chelates absorption may be better as they are enveloped with an organic structure that is more compatible with hydrophobic organic barrier, like GIT ones.

Another important chemical fact that can be critical for Zn bioavailability and effect is the ionic structure size. Theoretically the size of the ions forming ZnO and the ZnO itself is: Zn^{2+} = 0.88 Å + O^{2-} = 1.24 Å = ZnO = 2.12 Å or 0.2 nm), so the basic structure of ZnO is 1-5 nm.

However, ZnO is normally found in much bigger particles, 90% of bulk ZnO particles are < 250 μm. Small commercial particles as nanoparticles are in sizes around < 50 and < 100 nm. These different sizes have huge implications for the effects of ZnO. Lower size particles have bigger surfaces of interaction with GIT and bacteria and are much more available for interactions in aqueous media. This is probably the reason why ZnO nanoparticles showed increased in-vitro antimicrobial activity against *E. coli*, *Bacillus subtilis* and, *Enterobacter aerogens* gram-negative and, gram-positive bacterial strain compared to commercial ZnO powder (Newati et al., 2013). Moreover, ZnO nanoparticles have selective toxicity and are generally regarded as a safe reagent to humans and animals (Reddy et al., 2007; Liu et al., 2009) which could be an ideal potential antibacterial reagent to replace some antibiotics. More information regarding antibacterial activity, antibacterial mechanisms and food applications of ZnO nanoparticles can be found at Shi et al., (2014), so further research on ZnO nanoparticles applications in piglets feed can be of interest.

Further, ZnO possess the greatest acid binding capacity among feedstuffs (Lawlor et al., 2005), at pH 4 is 16,321 mEq (limestone is the second highest with 12,900 mEq, and sodium bicarbonate 12,600 mEq). Thus, at higher feed concentrations, ZnO can difficult the action of the acid produced by the organism or external acids.

1.2.2.3. Absorption process

Zn, together with Cu and Mn, is absorbed throughout the small intestine, mainly in jejunum, and an efficiency of 33% is accepted as the average Zn absorption in humans (Turnlund et al., 1984; Cousins, 1985) if administered in aqueous solutions to fasting subjects. Colon and cecum can contribute to Zn absorption when it is impaired in the small intestine (Hara et al., 2000). There are two mechanisms for the intestinal transport of minerals from the lumen of the intestine to the portal circulation: a/ Transcellular: regulated at the intestine level, saturable, carrier-mediated, minerals pass through apical and basolateral membrane of the cell; and b/ Paracellular: non-regulated, unsaturable, diffusional-mediated, minerals pass between cells (Menard and Cousins, 1983; Steel and Cousins, 1985).

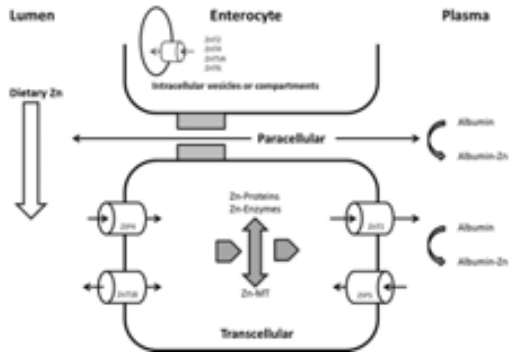


Figure 1.1. A proposed model for intestinal Zn absorption. Adapted from Grider, (2013)

a/ Transcellular: two Zn transporters that facilitate Zn uptake into the intestinal cells have been identified, ZIP4 (Zrt/Irt-like protein 4; SLC39A4) and ZnT5 (Zn transporter 5; SLC30A5). The presence of ZIP4 into the apical surface of intestinal cells is responsive to dietary Zn, increased with Zn deficiency and decreased during Zn sufficiency (Kim et al., 2004) and can also be influenced by the genotype (Siebert et al., 2013), thus genotype should be taken into consideration in animal breeding and experimental animals for the influence on Zn absorption. In the other hand, ZnT5 presence seems to respond to lumen Zn concentration although it may function as both an influx and efflux Zn transporter in the intestinal cell (Cragg et al., 2002; Valentine et al., 2007). However, the expression of ZnT5 and also ZnT1 increase in the jejunum when high levels of Zn are fed, to increase the export of Zn from epithelial cells (Chai et al., 2014). The mechanism of the intracellular transport of Zn from the apical to the basolateral intracellular surface for transport to the portal circulation is not fully known at this time.

b/ Paracellular: due to the existence of both carrier-mediated and non-regulated diffusion absorption, the efficiency of absorption falls, although the total amount of Zn entering the body increases as the dietary level of the Zn increases. This fact could have special relevance in piglets receiving high levels of ZnO.

What can affect Zn absorption?

Intestinal minerals absorption can be inhibited by (a/) metallothionein and by their (b/) chemical interactions with dietary components (bioavailability).

a/ Metallothionein is known to be induced by exposure to heavy metal cations (Ishii et al., 2001), specifically the expression level of MT1 in jejunum higher with high dietary levels of Zn in piglets (Chai et al., 2014). Zn absorption is inhibited due to the high production of MT induced by high dietary Zn. MT binds Zn in the mucosal surface of enterocytes forming a

block that prevents its movement through the cell, thereby limiting absorption. The bound metal is then lost from the body as the enterocyte is sloughed off into the intestine, Cu absorption is also reduced as MT also blocks transcellular transport. However, high levels of dietary Cu do not reduce Zn absorption as Cu is not a much stronger inducer of intestinal MT than Zn (Leone et al., 1985).

b/ The interactions between Zn and dietary components in the lumen of the GIT can influence how well Zn is absorbed from the diet. A paradigmatic example is the inhibitory effect of phytate on the absorption of Zn and many other minerals (Torre et al., 1991). Phytate is the principal storage of P in many plants and has the ability to chelate metal ions, especially Zn, Fe and Ca, but not Cu (Egli et al., 2004) forming insoluble complexes in the GIT that cannot be digested or absorbed in humans or pigs because of the absence of intestinal phytase enzymes (Iqbal et al., 1994). Phytate also complexes endogenously secreted minerals such as Zn (Sandström, 1997) and Ca (Morris and Ellis, 1985) making them unavailable for reabsorption into the body. The adverse effect of phytate on Zn absorption follows a dose-dependent response (Nävert et al., 1985) and there is no threshold for the inhibitory effects of dietary phytate on Zn bioavailability (Lonnerdal, 2000; Hambidge et al., 2004). It appears unlikely that Ca per se has a negative effect on Zn absorption (Lonnerdal, 2000). However, Ca content may affect Zn absorption from phytate containing diets because Ca has the propensity to form complexes with phytate and Zn that are insoluble and resistant to enzymatic hydrolysis by phytases and consequently have an inhibitory effect on Zn absorption (Taylor, 1965; Lonnerdal, 2000); further information of the effect of phytate, phytase and Ca on piglets diet are found in Chapter 1.5.1.3. The amount and type of protein also affect Zn absorption and the presence of even modest amounts of animal protein can substantially enhance the efficiency of absorption in addition to increasing the absolute amount of Zn (Krebs, 2000). Other authors have suggested that some ligands such as amino acids (histidine) may enhance Zn or Cu intestinal absorption, but is not clear how this enhancement occurs.

1.2.2.4. The body metabolic compartments and flows of Zn

Zn is the second most abundant transition metal ion in living organisms, after Fe (Vasák and Hasler, 2000). Due to its chemical features (is the most common intracellular metal ion with the exception of potassium (K^+) and magnesium (Mg^{2+}) it is found in the cytosol, in vesicles, organells and in the nucleus, and this is why this metal is so prevalent in protein and enzyme systems.

Transport in plasma and tissue uptake.

Once absorbed Zn is bound primarily to albumin in the plasma (Scott and Bradwell, 1983) and transported to the liver and after reaching the liver it is repackaged and released into the circulation bound to α_2-macroglubilin. So in a given time plasma Zn is primary bound to albumin (70%), with the remainder bound tightly to α_2-macroglubilin (18%) and other proteins or amino acids, specially histidine and cysteine (Gibson et al., 2008).

The uptake of Zn into cells and Zn intracellular homeostasis are extremely regulated thus a total of 24 mammalian Zn transporter and binding proteins have been identified (Lichten and Cousins, 2009). Cellular Zn is distributed between nucleus (30-40%), membrane (10%) and cytoplasm (50%) (Vallee and Falchuk, 1993). The latter contains membrane-enclosed structures rich in Zn^{2+}, so-called zincosomes, in which the ions are stored and released upon stimulation of the cell (Haase and Rink, 2013). From the 24 Zn transporters, fourteen belong to the SLC39A (Zrt, Irt-like protein: ZIP 1 to ZIP14) family and ten to the SLC30A (ZnT1 to ZnT10). a/ ZIP proteins generally transport Zn into the cytosol, either from the extracellular milieu or from intracellular organelles and b/ ZnT: exhibit the opposite function, facilitating Zn efflux from the cytosol to the outside of the cell or into intracellular organelles.

The function of transporters in Zn homeostasis is complemented by intracellular Zn binding proteins, including MT, that binds 20% of the intracellular Zn (Stefanidou et al., 2006). For example, as intracellular Zn concentration increases, the expression of MT and ZnT1 (SLC301) also increase resulting in increased cytosolic Zn binding and increased efflux of Zn across the plasma membrane (Heuchel et al., 1994; McMahon and Cousins, 1998 a; Langmade et al., 2000; Lichten and Cousins, 2009). MTs also have a redox-dependent function in Zn metabolism (Maret, 2011) and are regulated by external influences like inflammatory cytokines (Leibbrandt and Koropatnick, 1994).

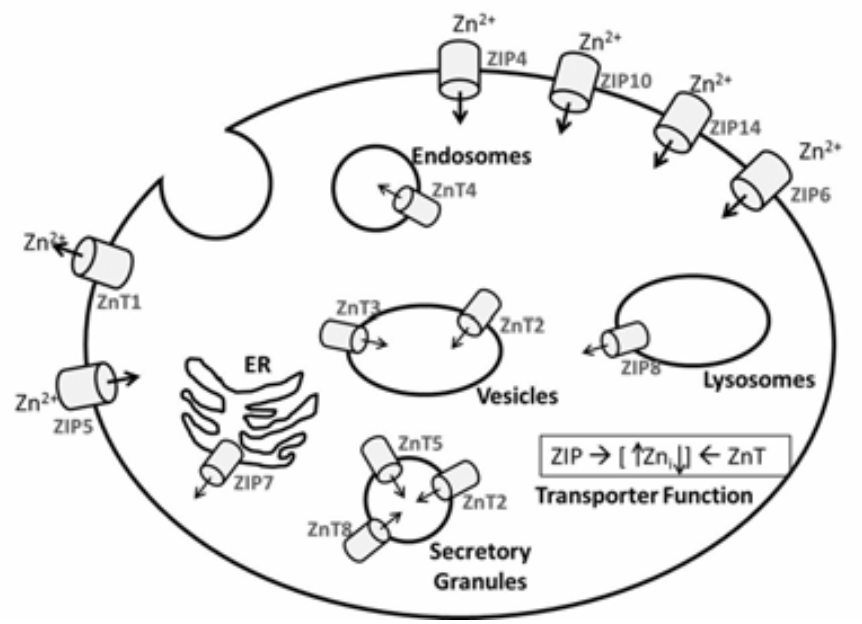


Figure 1.2. Generalized cell showing locations of some key Zn transporter proteins. Adapted from Lichten and Cousins, (2009).

Storage/Status.

In contrast to Fe or other "type 1" nutrients when animals are fed a diet lacking Cu, Mn or Zn, their status rapidly declines, suggesting that there is not a storage pool for these minerals to be used during times of low intake or increased need (Grider, 2013) also Zn stores do not last for more than 2 days in growing animals (King, 2011) and there is no storage form of Zn in the body that can be readily mobilized (King et al., 2001).

Thus, this implies that a 0.1% of the 2-3 g of total Zn human body content are exchanged daily (Maret and Sandstead, 2006) which emphasizes the need for a regular dietary supply (King et al., 2001). Other authors estimate the total amount of Zn present in a human body between 1.5 and 2.5 g (Jackson, 1989), the same estimation for a 100-kg pig (Mahan and Shields, 1998). Zn is present in all organs, tissues, fluids and secretions in the body. Most Zn is located in the fat-free mass and the Zn content of the bodies of adult animals (milligrams per kg of crude defatted tissue) ranges from 25 for swine to 50 for rabbits and in newborn animals, the Zn contents are somewhat lower: 10 for swine and 30 for cattle (Georgievskii et al., 1981). Tissue distribution of Zn varies considerably, see Table 1.5.

Table 1.5. Zn content of major organs and tissues in an adult (70 kg) man. Adapted from (Iyengar, 1998).

Tissue	Zn concentration (mg/kg wet weight)	Total Zn content (mg)	Proportion of total body Zn (%)
Skeletal muscle	50	1400	63
Skeleton			
Bone	90	450	20
Marrow	20	60	3
Cartilage	34	30	1
Periarticular tissue	11	11	< 1
Liver	40	72	3
Lung	40	40	2
Skin	15	39	2
Whole blood	6	33	1
Kidney	50	15	1
Brain	10	14	1
Teeth	250	11.5	1
Hair	200	4	< 1
Spleen	20	3.6	< 1
Lymph nodes	14	3.5	< 1
GIT	15	1.8	< 1
Prostate	100	1.6	< 1
Other	Variable	50	2
TOTAL		2240	100

Almost all Zn is found intracellularly (> 95 %) (Iyengar, 1998). The Zn located in the muscle and bone tissues (≈ 90% of total body Zn) has a slow turnover and is not readily responsive to changes in dietary Zn or especial needs; thus, the remaining ≈ 10% represents the "functional pool" (Foster et al., 1979; King, 1990; Miller et al., 1994). This small and exchanging Zn pool (EZP) is found primarily in the liver, pancreas, kidney, intestinal tissue and extracellular fluid that exchanges rapidly with plasma (Miller et al., 1994; Hess et al., 2007). EZPs are rich in MT, thus MTs constitute the only candidate for Zn storage, except for zincosomes used to regulate intracellular Zn homeostasis again together with MT (Grider, 2013). EZP mass vary directly with dietary Zn but it changes in a lesser extent than plasma Zn concentration in an experimental severe Zn depletion study in humans (King et al., 2001) suggesting that plasma Zn is more sensitive to Zn depletion than EZP. Plasma Zn only represents < 0.1-0.2% of total body Zn content but is the most frequent biomarker of Zn status (see Chapter 1.2.3.4). Although it represents a very small fraction, the circulating Zn turns over rapidly (≈150 times/day) to meet tissue needs: during the course of 24 hours, the equivalent of one-fourth to one-third (≈450 mg) of total body Zn exchanges between the bloodstream and other tissues (King et al., 2000).

There are several isoforms of MT and each MT can bind up to 7 ions (Krężel and Maret, 2007). Several metals induce MTs, Zn, Cu, Cd, Hg, Au and Bi but Zn is the primary physiological inducer since the other metals can be considered as environmental toxicants (Coyle et al., 2002). MT are mainly localized in liver, pancreas, kidney and intestine and as previously explained, they are rapidly induced by exposure to high levels of heavy metals (particularly Zn and Cd) playing an important role in detoxification of heavy metals and also detoxifies reactive oxygen species (Andrews, 2000). MTs ability to capture oxidant particles is 300 times greater than that of glutathione (Sato, 1992). Moreover, hepatic MT play a key role during the acute-phase response that follows an inflammatory or infection condition being directly induced by inflammatory signals and helping on the redistribution of Zn from the plasma to the liver. Hepatic MT binding hepatic Zn reserves accrued in utero are mobilized to maintain Zn homeostasis during late lactation period (Zlotkin and Cherian, 1988).

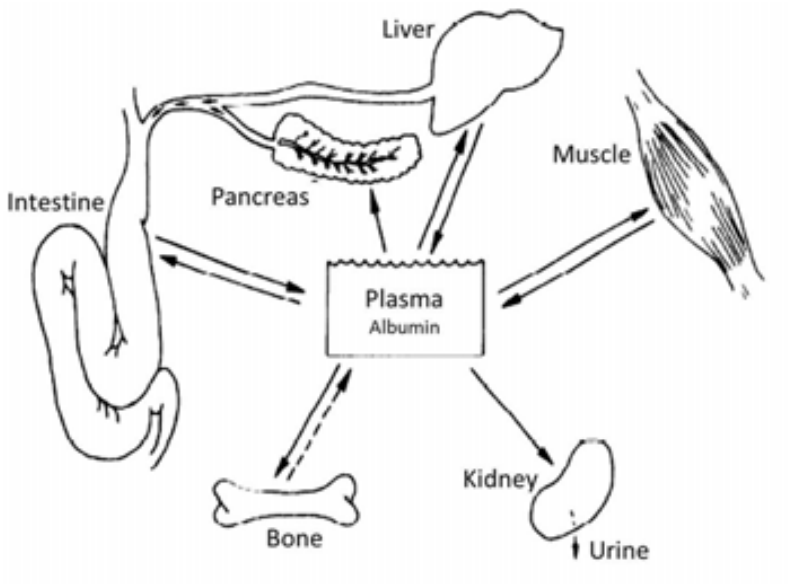


Figure 1.3. Basic aspects of mammalian Zn metabolism. From Cousins, (1985).

In Figure 1.3 the basic aspects of mammalian Zn metabolism is represented. Absorption of dietary Zn occurs from the small intestine. Endogenous Zn is secreted into the intestine from pancreatic and biliary secretions as well as from serosal-to-lumen zinc flux by intestinal cells. Zinc is not excreted in urine in appreciable amounts except during concomitant excessive nitrogen excretion. Regulation of zinc absorption occurs at cellular levels and through endogenous secretion. Absorbed zinc is transported in portal plasma bound to albumin. Hepatic uptake occurs via a saturable energy-dependent process and accounts for major initial

accumulation of newly absorbed zinc. On the whole, muscle and bone represent the largest pools. The latter probably is only returned to plasma when bone is mobilized to maintain calcium homeostasis. Marked increases in muscle catabolism may favor urinary zinc losses. Acute responses to physical stress and infection involve depression in plasma zinc, uptake of zinc by the liver and concomitant redistribution within hepatocytes.

1.2.2.5. Excretion

Under physiological conditions, most of Zn is lost through feces (≈ 90%) and a small portion through the urine (< 10 %) or through cutaneous losses. Loss of Zn through the GIT accounts for approximately half of all Zn eliminated from the body and comes from the pancreas, biliary and intestinal secretions (King and Keen, 1999). The total endogenous gastrointestinal Zn secretion may exceed the amount consumed in the diet however, much of the Zn can be reabsorbed; this process serves as an important point of regulation of Zn balance (Brown et al., 2004).

Diarrheal diseases are common in many low-income countries and also in weaned pigs. These conditions that affect intestinal integrity is doubly negative for Zn status as Zn absorption is reduced and also result in increased endogenous losses of Zn. So fecal excretion of Zn is increased during diarrhea (McMahon and Cousins, 1998 b) but is not clear to what extent this represents unabsorbed Zn or Zn of endogenous origin (Brown et al., 2004).

As previously mentioned (Chapter 1.2.2.3.), high dietary Zn induces MT binding in the intestine thus increasing Zn excretion.

1.2.3. Requirements, excess, deficiency and assessment

1.2.3.1. Requirements

Zn requirement is the individual demand for Zn under defined conditions and recommendation is the estimate of the Zn supply necessary to meet the average gross demand of the population under common conditions plus a safety factor considering the individual variability, varying bioavailabilities and interactions between nutrients.

Zn requirements of young pigs consuming a casein-glucose diet without any phytate content can be as low as 15 mg/kg (Shanklin et al., 1968), but in conventional diets without supplemented phytase maximal growth was reach between 47- 60 mg/kg (Revy et al., 2006; Paulicks et al., 2011).

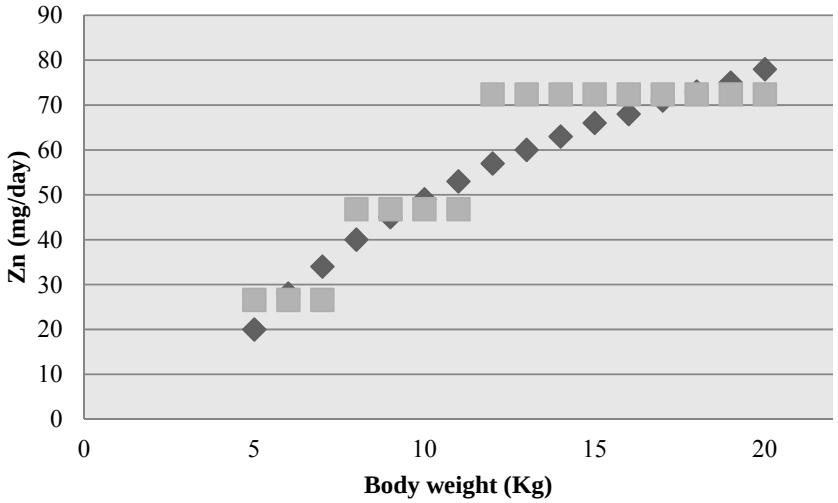
According to the NRC, recommendations for Zn requirements consider the basal requirement of the individual but also diet-related factors that interact with Zn like phytic acid or plant phytates, Ca, Cu, Cd, Co, EDTA, histidine and protein level and source, and additional safety allowances (NRC, 2012).

Zn recommendations of NRC, (2012) in pigs are presented depending on the body weight of the animals as per kg of diet or as amount per day on Table 1.6. Zn requirements increase significantly and linearly by age, in parallel with the body weight gain. For example: pigs between 5-7 kg: 100 mg/kg of diet or 26.6 mg/day; and pigs between 7-11 kg: 100 mg/kg of diet or 46.8 mg/day. In-feed Zn recommended level is maintained at different BW because of different estimated feed intake (e.g. 280 and 493 g/day including 5% feed wastage, for 5-7 and 7-11 kg pigs, respectively).

Table 1.6. Recommended dietary and daily allowances for Zn depending on body weight and productive stage of swine

BW range (kg)	**NRC, 2012**		**BW range**	**NRC, 1998**		**FEDNA, 2013**	**GfE, 2006**
	(mg/kg)	(mg/day)	(kg)	(mg/kg)	(mg/day)	(mg/kg)	(mg/kg DM)
5-7	100	26.6	**3-5**	100	25	120 (100-130)	100
7-11	100	46.8	**5-10**		50		
11-25	80	72.4	**10-20**	80	80		80
25-50	60	90.2	**20-50**	60	111.3	110 (110-120)	50-60
50-75	50	105.9	**50-80**	50	129.75	80	50-60
75-100	50	125.3	**80-120**	50	153.75	(90-110)	50-60
100-135	50	139.4					50-60
Gestation sows	100	210.0		50	93	100 (95-120)	50
Lactating sows	100	596.6		50	263	100 (95-120)	50
Sexually active Boars	50	118.8		50	100	120 (95-140)	-

In the following figure, a representation of daily Zn requirements by NRC, (2012) for post-weaning piglets is shown. The NRC and other recommendation entities make recommendations based on body weight ranges and that means that some there are some gaps for the needs of each single kg of weight (Figure 1.4).



Black, represents daily Zn recommendation for each kg of BW; grey, represents average daily Zn recommendations for each BW category.
Figure 1.4. Daily Zn requirements for swine. (NRC, 2012)

The previous (1998) and current (2012) NRC publications describe essential micromineral requirements on a total basis and state that requirements include contributions of all dietary ingredients. The current NRC (2012) indicates that micromineral contents and bioavailabilities are variable and largely unknown. Consequently, the micronutrient contributions of ingredients, other than the specific trace minerals themselves, are ignored in diet formulation and it is common to supplement micronutrients in excess of NRC (2012) requirements and therefore, "any amounts of supplies by feed ingredients then contribute to a margin of a safety". This is the case of ground limestone and monocalcium or dicalcium phosphate that are largely used and contain appreciable amounts of Fe and Mn (NRC, 2012). Several authors reported that eliminating dietary microminerals did not affect grower-finisher pig performance or carcass characteristics (Patience and Gillis, 1995; Mavromichalis et al., 1999; Shelton et al., 2004). Gowanlock et al., (2013) also showed no detrimental effect by eliminating microminerals from grower-finisher diets but a dietary level of 50% of the NRC (2012) requirement for Zn (and Cu, Fe and Mn) would be warranted.

Zn requirements in humans are presented as Dietary Reference Intakes (DRIs) by IOM, (2006), see Table 1.7. The different expert committees involved on the development of estimates of human Zn requirements use a factorial method in the determination of Zn requirements. This method seeks to estimate the Zn intake required to meet the amount of Zn lost through both intestinal and non-intestinal pathways, the amount of Zn retained for growth

(based on average Zn content of 20 µg/g wet weight of tissue) in children and pregnant women and for lactating women the Zn secreted in breast milk.

Table 1.7. Recommended daily Zn depending on body weight or reproductive stage for humans.

	Reference BW (kg)	**Physiologic req., IOM (mg/day)**	**Physiologic req. IZiNCG (mg/day)**	**DRI , IOM (mg/day)**	**DRI, IZiNCG (mg/day)**
< 6 months				2.0	
7 – 12 months	9	0.84	0.84	3.0	5
1 – 3 years	12- 13	0.74	0.53	3.0	3
4 – 8 yr.	21 - 22	1.20	0.83	5.0	5
9 – 13 yr.	38 - 40	2.12	1.53	8.0	9
14- adult Males	64	3.37	2.52	11.0	14-19
14 -18 Females	56-57	3.02	1.98	9.0	11
> 19 Females				8.0	9
Pregnant		4.1 – 5.0	2.68	11.0	13
Lactating		3.8-4.5	2.98	12.0	10

EAR (Estimated Average Requirements): Zn requirements assuming an estimated fractional absorption of 0.3 in children, 0.41 for men and 0.48 for women. DRI (Dietary Reference Intake): EAR + 20%.

Thus, daily Zn requirements are much higher in piglets than in children, probably due to much higher growing rate.

The Adequate intake of Zn for infants from birth through 6 months of age was set as 2 mg/d however it varies along this period due to the rapid physiological decline in the Zn concentration of human milk. The Zn content of human milk is growth limiting for some infants after 4 months of age and after 6 months human milk alone is an inadequate source of Zn.

Dietary Zn recommendations in humans vary widely across Europe due to the heterogeneity of approaches used by expert panels (Doets et al., 2008). Apart from the factorial method explained above, an alternative approach is to examine the dose-response relationship between intake and biomarkers of status and between intake and health status (Lowe et al., 2012).

1.2.3.2. Excess/ Toxicity

Given that Zn has multiple essential functions, it also has the potential to interact with at least as many biological functions to induce adverse effects (Maret and Sandstead, 2006). The physiological responses to Zn deficiency are well-characterized in mammals (Giugliano and Millward, 1984; Park et al., 1986; Fairweather-Tait and Hurrell, 1996), whereas less information is available regarding the effects of excess Zn intake. As a general rule, Zn is

relatively non-toxic when consumed in the diet (Grider, 2013). In rats, the oral LD_{50} for Zn salts is 237-623 mg/kg, the intraperitoneal injection LD_{50} is 28-73 mg/kg (Domingo et al., 1988; WHO, 2001) and the inhalation LD50 for $ZnCl_2$ is 2,000 mg/m^3 (Karlsson et al., 1991; WHO, 2001). In humans, high concentrations of Zn in drinks, up to 2,500 mg/L with an estimated dose of 325-650 mg have been linked to poisoning of individuals, causing nausea, abdominal cramping, vomiting, tenesmus and diarrhea with or without bleeding (Brown et al., 1964; WHO, 2001). Excess Zn during embryogenesis can be teratogenic or lethal (WHO, 2001).

One of the established effects of chronic Zn toxicity is that oral Zn intakes disproportionately high relative to Cu are a conditioning factor to induce Cu deficiency (Maret and Sandstead, 2006). Cu deficiency is characterized by anemia and neutropenia and other multiple adverse effects include decrease in Cu- dependent enzymes (Cu/Zn-SOD, ceruloplasmin and cytochrome C oxidase), impaired immune function and reduction of high-density lipoprotein (HDL) cholesterol levels (Maret and Sandstead, 2006; Grider, 2013). The negative interaction has been attributed to MT because Cu is relatively poor at the induction of MT compared to Zn, although the affinity of MT for Cu is higher than for Zn (Cousins, 1985). The reduction of tissue Cu reserves may induce the reduction of ceruloplasmin, which is required for Fe metabolism (Osaki and Johnson, 1969). Consequently an increase on plasma and liver Fe concentrations may occur when high dietary levels of Zn are administered into piglets (Rincker et al., 2005).

Severe P deficiencies and impaired performance have been registered in piglets receiving high levels of Zn. For example Vilà et al., (2010) found that feeding 3,000 mg/kg of ZnO into piglets diet decreased serum P concentrations, and no effect was registered on Ca levels.

Concentration of Zn in blood plasma/serum, urine and hair may increase when exposures are high but their measurement is not a standardized procedure to confirm exposure (Maret and Sandstead, 2006). The pancreas has been suggested as the tissue most sensitive to excess Zn (Sutomo et al., 1992) and it plays an important role in Zn metabolism (McClain, 1990) and excretes Zn via pancreatic juices into the gut in a Zn dependent manner. Recently, Siebert et al., (2013) described that a mutation in porcine ZIP4-like Zn transporter was associated with pancreatic Zn concentration and apparent Zn absorption.

Pigs are possibly the livestock species with the highest tolerance of Zn. The NRC (National Research Council, 2005) set the MTL (maximum tolerable levels) of Zn for pigs at 1,000 mg/kg diet and the NRC proposes that Zn accumulates in tissues such as liver, kidney and bone in order to protect other organs from failure induced by Zn accumulation.

According to an old study in piglets the addition of 0.1% of Zn to the diet is the maximum level tolerated (Brink et al., 1959), showing poor performance, inflammation and hemorrhages in multiple organs and locations. Studies in other species showed that Zn concentrations increased in the liver (6-fold) and in the kidney (11-fold) in preruminant calves and in sheep fed a diet supplemented with 500-700 and 700-2,100 mg Zn/kg, respectively, whereas increases were smaller in the heart and muscle (Jenkins and Hidiroglou, 1991; Henry et al., 1997). In rats, Zn concentrations in the liver, kidney and bone were higher when animals were fed a diet supplemented with 2438 mg Zn/kg compared to rats fed 38 mg Zn/kg (Ansari et al., 1976). Accumulation on the same organs was also registered in a recent short-term study in rats (Fujimura et al., 2012). This study showed that pancreas atrophy is induced in smaller Zn intake required to reach a plateau in Zn accumulation in other tissues and that Zn homeostatic mechanisms (up- or down-regulation of Zn transporters and MT) are altered when high dietary Zn concentrations are fed to rats; the similar homeostatic response was observed in piglets (Martin et al., 2013 a).

However, in contrast to most piglet studies beneficial effects of high dietary Zn on rats growth was not observed; for example (Li et al., 2006) suggested that the excess Zn ingestion stimulates IGF-1, which results in growth promotion in piglets is in contrast to (Fujimura et al., 2012) in which IGF-1 expression was not significantly in rats.

The Zn-induced growth promotion effects in piglets remains unclear (Jensen-Waern et al., 1998; Katouli et al., 1999; Heo et al., 2010; Shelton et al., 2011; Sales, 2013) but the optimum dose is about 2,500 mg Zn/kg feed, while concentrations of 4,000 and 5,000 result in adverse effects (Hill and Miller, 1983; Poulsen, 1995). Some authors found out that high Zn concentrations can stimulate the occurrence of resistance to Zn in the pig gut microbiota (Mazaheri Nezhad Fard et al., 2011; Vahjen et al., 2011) this negative findings requires further investigations. The growth-promoting effect of ZnO will be further discussed in chapter 1.5.1.1.

1.2.3.3. Deficiency

Zn deficiency causes growth retardation and a depletion of overall enzyme activity in tissues, although the deficiency is sometimes difficult to diagnose (Prasad et al., 1969; Prasad and Oberleas, 1971). There are various causes of Zn deficiency. Primary genetic causes include acrodermatitis enterophatica, a rare autosomal-recessive condition in human individuals

caused by a mutation of the ZIP4 gene (Slc39a4), leading to low Zn absorption; for more information see Geiser et al., 2012. Secondary causes of Zn deficiency include poor intake due to several situations: high phytate and low in meat diets, malabsorption states (e.g. inflammatory bowel disease), long-term parenteral nutrition; or from increased utilization such as sepsis, trauma, or associated with vitamin A and D deficiency and also Fe deficiency (Hambidge, 2000; Crook, 2006; Maret and Sandstead, 2006).

According to Chasapis et al., (2012) Zn is such a critical element in health that even a small deficiency is a disaster. Many symptoms of Zn deficiency have been characterized but the underlying biochemical defects responsible for most of them have not been found (Grider, 2013). One of the first response to an insufficient intake of type 2 nutrients is a metabolic adaptation to reduce the need for functions that have a high demand, such as growth and immunity (King, 2011). In growing animals showed a marked decline in growth within days (King, 2011), as loss of appetite is one of the first signs associated with dietary Zn inadequacy (Grider, 2013). Its effect on immunity means that there is an increased risk of infections as Zn is essential for phagocytic and bactericidal activity of macrophages, cellular and humoral action of lymphocytes (Hambidge, 2000; Küry et al., 2002; Crook, 2006; Maret and Sandstead, 2006) and reduce the total numbers of lymphocytes (B and T cells) of the peripheral immune system (Walsh et al., 1994), probably due to its actions on signaling pathways of T-cell receptors and on pre B-cell development (Haase and Rink, 2009) and from atrophy of the thymus and the loss of thymulin secretion (a Zn-dependent hormone). Other clinical signs of Zn deficiency include: reduced taste and smell, alopecia, impaired wound healing and infertility.

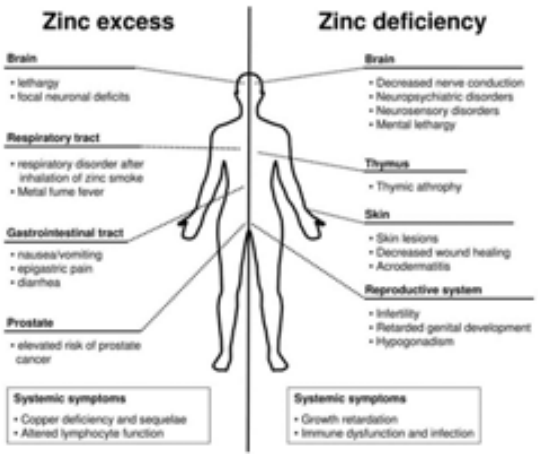


Figure 1.5. Comparison of the effects of Zn intoxication vs. deficiency. Adapted from Plum et al., 2010

1.2.3.4. Assessment of Zn status.

When thinking about Zn status marker it is important to consider that a nutritional assessment may focus on individuals or populations. Assessment of Zn status in individuals has a more clinical application to those seeking medical attention for a health condition. The diagnosis of isolated Zn deficiency is usually found in association with a variety of health conditions for which primary treatment is being sought (Brown et al., 2004). Population assessment is applied to a sample of interest and the primary goal of population-level assessment of Zn status is to characterize the degree of risk of deficiency in the population and the need of intervention (Brown et al., 2004).

Given that Zn is required for multiple general metabolic functions, the assessment of Zn status is notoriously problematic, as a sensitive, specific biomarker for Zn has not yet been identified and is unlikely that a specific biomarker of Zn exists (King, 2011).

In a systematic review and meta-analysis of biomarkers of Zn status it was concluded that erythrocytes, PMNCs, mononuclear cells, platelet Zn and plasma alkaline phosphatase are not useful biomarkers for Zn status. Further, plasma, urinary and hair Zn are reliable biomarkers for Zn status in healthy individuals. Both 24-h urinary Zn excretion and hair Zn appear to respond to Zn supplementation but the effect of Zn depletion is inconclusive due to insufficient data (Lowe et al., 2009). Other researchers have examined several Zn-dependent enzymes for their ability to serve as sensitive indicators of Zn (alkaline phosphatase, Cu/Zn-SOD, 5' nucleotidase) status (Delves, 1985) and Zn transporter proteins located within their erythrocyte membranes (Ryu et al., 2008). Although some of these assays show promise, none has to date been proved useful (Grider, 2013).

Plasma (or serum) Zn concentration responds to dietary manipulation in both depletion and supplementation studies (Hotz, 2007; Lowe et al., 2009) and is the only biochemical indicator recommended by the WHO, UNICEF, IAEA, IZiNCG for the assessment of population Zn status (Hess et al., 2007). Plasma/serum Zn is a useful test for deficiency probably because during a severe dietary Zn restriction (< 1 mg/day for 4-5 weeks) there is a marked drop (about 35%) of Zn in the rapidly exchangeable Zn pool, but little or no measurable effect is registered on the size of the slow Zn pool, which is maintained as the expense of the rapid pool (King et al., 2001) So, a plasma/serum Zn concentration of less than about 0.65 mg/L suggests deficiency (Suttle, 2010; Crook, 2011), 0.4-0.8 mg/L are associated with marginal status (Puls, 1990). Johanning et al., (1990), found plasma Zn concentrations of 0.33 mg/L in Zn-deficient piglets and 0.85-1.2 mg/L in Zn sufficient piglets.

Plasma/serum Zn concentrations may have limitations in validity and reliability for identification of mild or moderate Zn deficiency in individuals as low concentrations can occur in the presence of several conditions not necessarily indicative of low Zn status (Brown et al., 2004), but in the absence of suitable alternatives it remains by far the most commonly used method (Moran et al., 2012). Serum Zn concentrations are reduced during acute infections and inflammation due to the redistribution of Zn from the plasma to the liver (Moshage, 1997) carried out by MT. Elevated concentrations of serum C-reactive protein (CRP) or other markers of acute phase response can be used to indicate the presence of infection and should be considered in the interpretation of results (Brown et al., 2004). A possible explanation for this reduction is that low Zn levels support the production of acute phase proteins, host defense proteins (calprotectin) (Kehl-Fie and Skaar, 2010), restriction of Zn acquisition systems of pathogens (Citiulo et al., 2012; Gielda and Dirita, 2012), immune cell function (Haase and Rink, 2009). Also, it should be considered that plasma/serum Zn concentrations fluctuate as much as 20% during a 24-h period, largely due to the effects of food ingestion (Hambidge et al., 1989), metabolic changes after meal consumption results in a decrease in serum Zn concentrations and is cumulative following repeated meals in humans (Goode et al., 1991; Wallock et al., 1993). Conversely, overnight and daytime fasting in humans result in increased circulating Zn concentrations (Wallock et al., 1993). Serum Zn concentration increase by 0.01 mg/L with each additional hour of fasting during daytime (Aaron et al., 2011). Some other variation may also occur as a result of normal circadian variations in metabolism (Guillard et al., 1979). Thus, in human Zn assessment studies blood collection from all individuals should be at a specified time of day (ideally in the morning) and consistent interval after meal consumption (Arsenault et al., 2011) (ideally in fasting conditions: > 8 hours since the last meal; Brown et al., 2004) to minimize variability in serum Zn concentrations.

Hair Zn concentrations reflects the quantity of Zn that was available to the hair follicle during an earlier time interval, so it has been proposed as a useful index of longer-term Zn status (Brown et al., 2004) and has been used in several animal (Combs et al., 1983; Combs, 1987; Bobilya et al., 1994) and human studies (Hambidge et al., 1972; Gibson and DeWolfe, 1979; DeAntonio et al., 1982; Paschal et al., 1989). Its application as an indicator of Zn status has several advantages and limitations. It is more stable than serum Zn and is not affected by diurnal variations, prolonged fasting, meal consumption or acute infection and has practical advantages (less invasive, no storage requirements) (Brown et al., 2004). The main limitations of hair Zn concentration is the limited availability of reference data and some

interpretation of the results: varies according to sex (DeAntonio et al., 1982; Taylor, 1986; Vanderkooy and Gibson, 1987) and age (Hambidge et al., 1972; Gibson and DeWolfe, 1979).

1.3. Piglet's situation around weaning

In current commercial conditions piglets suffer a forced separation from the sow at an early age (21 – 28 days old) compared to wild animals (10 weeks old). Weaning imposes tremendous stress on piglets and is accompanied by marked changes in gastrointestinal tract (GIT) physiology, microbiology and immunology (Hampson, 1986; Pluske et al., 1997). The period after weaning is characterized by sub-optimal growth performance (e.g.: low feed intake, deteriorated feed efficiency, body weight loss) and a high incidence of intestinal disturbances with diarrhea often occurring that can cause morbidity and/or mortality (Bark et al., 1986; Pluske et al., 1997; Halas et al., 2007; Heo et al., 2013). Burrin and Stoll (2003) distinguish between *acute phase* (within the first 5-7 days after weaning) and *adaptive phase* (7^{th} after weaning and onwards) based on the changes in feed intake and the subsequent impacts on the GIT, because it takes 7–14 days for weaned pigs to reach a similar level of dry matter intake to the pre-weaning period (Pluske et al., 1997). Temporary low intake during the acute phase can create a deficiency in macronutrients, micronutrients and energy that can impair health, development and recovery during adaptive phase (Pluske, 2013).

1.3.1. Pre-weaning situation

The piglet's micronutrient status at weaning depends on micronutrient input received during gestation (through uterus) and lactation (through colostrum and milk, and in some cases through creep-feed) that should cover piglet's demand. Mahan and Shields, (1998) showed that during the lactation period there is a considerable increase of body Zn concentration, from 10.9 to 15.3 mg/kg FFEBW (fat-free empty body weight).

During gestation passive transfer of some nutrients occur between the maternal-fetal blood barrier but most micronutrients are more dependent on an active transport mechanism (Mahan and Vallet, 1997). Most of the macro- and micromineral are mainly transferred during the late pregnancy (Figure 1.6) and there may be an increased sow mineral requirement particularly with high-producing sows having larger litter sizes as mineral contents of fetal pigs increase during this phase (Mahan et al., 2009).

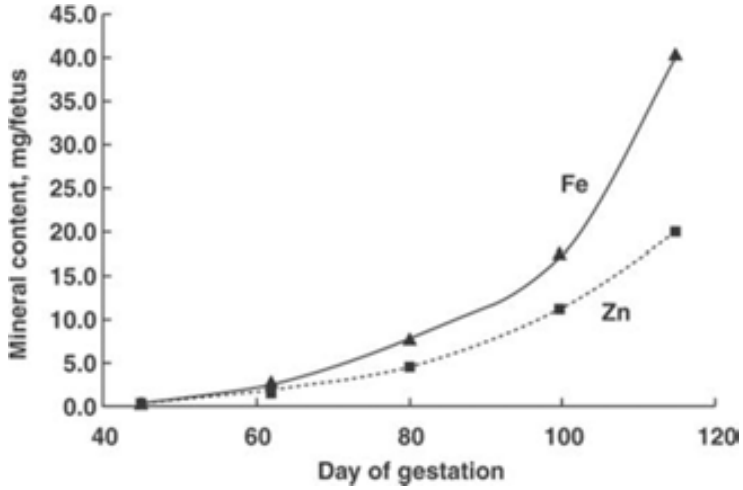


Figure 1.6. Deposition of Fe and Zn in pig fetuses from 45 d postcoitum to birth. From Mahan et al., 2009.

However, the transfer of macro- and microminerals to the fetus in utero is not greatly affected by the dietary sources or dietary level of minerals fed to sows during gestation (Peters et al., 2010) except when sow gestation diets contain extremely elevated mineral content, for example 5000 mg Zn/kg of diet (Hill et al., 1983a). In recent article it was found that supplementing sow's gestation diet with 250 mg/kg of Zn as ZnAA enhanced serum concentrations of Zn in pigs on days 7 and 14 after birth (Caine et al., 2009).

In contrast, colostrum mineral composition can be influenced by factors affecting the minerals needs of pregnant sows and dietary mineral supplementation and sow mineral status as colostrum synthesis is largely initiated before parturition (Mahan et al., 2009; Peters et al., 2010). Macro- and micromineral concentrations (except for Ca) are higher in colostrum than in the later milk of lactating sows (Hill et al., 1983 b; Csapo et al., 1996; Peters et al., 2010). The Zn content in colostrum is highest at parturition (15.70 mg/kg) and decrease continuously in milk to the 3rd day of lactation (5.75 mg/kg) and remain constant at 5.69 to 6.49 mg/kg of milk until the end of lactation (Csapo et al., 1996), a more sharply decrease compared to humans (Brown et al., 2009).

Most microminerals increase at a rapid rate in piglet tissues during nursing period (Mahan and Shields, 1998). Recently, Matte et al., (2014) showed this rapid increase measuring micromineral concentrations in serum of both sow and piglets (Table 1.8). Thus, the mammary gland makes a great effort to export minerals, especially Zn, from maternal mineral tissue stores to milk to meet piglet's requirement. Ullrey et al., (1967) showed that serum Zn concentrations of piglets decline after one week of lactation, likely sow milk was not providing enough Zn.

Table 1.8. Sow and piglets serum mineral concentrations. From (Matte et al., 2014).

Micronutrient	Sow serum concentration (110d of gestation)	Piglet serum concentration (0 d of life)	Piglet serum concentration (3 d of life)	Prepartum transfer, 0d / 110d	Postpartum transfer, 3d/0d	Peripartum transfer, 3d / 110d
Fe, µM	38.1 ± 2.0	23.6 ± 0.9	30.8 ± 1.9	0.62	1.3	0.8
Zn, µM	9.1 ± 0.2	11.9 ± 0.7	18.6 ± 0.8	1.3	1.6	2.0
Cu, µM	31.7 ± 0.8	9.4 ± 0.4	15.1 ± 0.6	0.30	1.6	0.48
Se, µM	2.02 ± 0.06	0.71 ± 0.02	0.98 ± 0.04	0.36	1.4	0.49

Tight regulation of Zn transporting mechanisms (Figure 1.7) is critical to provide Zn for secretion into milk and for maintaining optimal cellular function in the mammary gland (McMahon and Cousins, 1998 a; Kambe et al., 2004; Eide, 2006; Kelleher et al., 2009; McCormick et al., 2014).

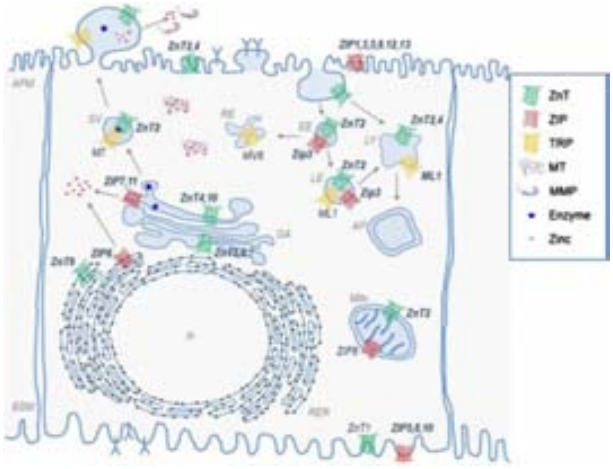


Figure 1.7. A model for the transport of Zn for specialized functions in mammary epithelial cells. From (McCormick et al., 2014).

According to Peters et al., (2010) milk mineral compositions are considered to be influenced largely by the postpartum feed and mineral intakes, sow body mineral status and milk production differences. However, milk Zn concentration didn't change when dietary Zn concentrations were increased from NRC (50 mg/kg) to industry levels (120 mg/kg) at the same work or increases up to 500 ppm Zn (Hill et al., 1983 b). Only after excessive dietary Zn (5,000 ppm), an increase in the level of Zn in milk and piglet mineral reserves was observed (Hill et al., 1983 b). In recent article (Metzler-Zebeli et al., 2010) piglets from sows

receiving a lactation diet supplemented with 250 mg/kg of Zn as ZnAA showed no change on Zn serum concentration 7 days after weaning.

Sow mineral status can be progressively depleted with greater parity number likely affecting colostrum, milk and weanling pig body mineral concentrations. The total body mineral content of sows (particularly for Ca, P and Zn) was less after 3 parities when compared with similarly aged group of non-gravid females (Mahan and Newton, 1995). However in Peters et al., (2010) milk minerals concentrations were not decreased by parity number and it had no major effect on the mineral composition of piglets at birth. In Chapter 4 we further investigate the effect of sow's age on colostrum and milk mineral content and its effect on piglet's status. In humans, no relation between plasma Zn concentration and breast milk Zn concentration was observed which confirms that breast milk Zn concentration is not affected by maternal Zn intake or status (Islam and Brown, 2014)

1.3.2. Post-weaning situation

As previously introduced, the period following weaning is generally characterized by sub-optimal growth due to low feed intake (anorexia) and body weight loss (Bark et al., 1986; Pluske et al., 1997). Under practical commercial conditions and both before and after weaning, young pigs achieve less than 50% of their growth performance potential (Pluske et al., 1997). The GIT of young pigs around the time of weaning undergoes rapid changes in size, protein turnover rate, microbiota mass and composition and quick and marked alterations in digestive, absorptive, barrier and immune functions (Pluske et al., 1997; Vente-Spreeuwenberg and Beynen, 2003; Lallès et al., 2004; Domeneghini et al., 2006) that could affect Zn bioavailability and absorption.

The effect of weaning on the activity of gastric enzymes is equivocal: Hedemann and Jensen, (2004) reported decreased pepsin activity and no change on lipase activity while Cranwell, (1985) and Jensen et al., (1997) reported increased pepsin and lipase activities in the stomach mucosa after weaning. Moreover, weaned pigs have higher gastric pH value than unweaned pigs, probably due to a lower acid secretion capacity (Manners, 1976; Efird et al., 1982). Piglets also show reduced gastric motility and emptying rate (Snoeck et al., 2004) that may contribute to development of PWD in piglets.

In the small intestine significant changes occur in the structure and in the digestive function during the immediate post-weaning period (Hopwood and Hampson, 2003). Post-weaning anorexia and stress during weaning causes transient villus atrophy and crypt hyperplasia

(Pluske et al., 1997). Alteration of brush-border enzymes (e.g. decreased lactase, increased sucrose) is another characteristic consequence of weaning, however discrepancies between studies result of multiple variations such as experimental diets, age of the animals and days post-weaning at which measurements were taken (Heo et al., 2013). Enzyme activities usually reach minimum levels 3-5 d postweaning and increase thereafter probably due to an increase of feed intake (Hampson, 1986; Pluske et al., 1997). Weaning also results in a reduction in the net absorption of fluid and electrolytes, and a malabsorption of nutrients in the small intestine of piglets (Nabuurs et al., 1994; Miller and Skadhauge, 1997). For example, it is possible that newly-weaned pigs do not consume enough feed during the first week after weaning to stimulate MT production (Carlson et al., 1999) and this could affect Zn absorption capacity.

However, all these effects of weaning on the GIT do not seem to have a clear impact on Zn status. Zn concentration in plasma remained constant until 15 d after weaning in several articles (Ullrey et al., 1967; Poulsen, 1995; Carlson et al., 2007 a) indicating that weaning did not influence the Zn status of the piglets. However, in (Carlson et al., 2007 a) some animals were marginally Zn-deficient even before weaning. In contrast, plasma Cu concentration were reduced from 1-2 days after weaning until 14-15 d after weaning, but this is not in agreement with Poulsen, (1995) who found that Cu concentration increased during the first 7 d after weaning, and thereafter decreased.

Martin et al., (2011) suggested that during the initial period postweaning there may be adequate body reserves derived from the lactation period that can prevent from an imminent deficiency, and that NRC recommendations are adequate for Cu and Mn but there is a supplemental need for Fe and Zn.

Piglet's age at weaning could be an important factor for Zn status/stores and thus to successfully face the challenge of weaning, for example Schell and Kornegay, (1994) showed that the earlier the pigs were weaned the larger the magnitude of the decline in serum Zn 1 week after weaning. But further investigation is needed.

1.4. The example of the Zn status in children's in underdeveloped countries

Human Zn deficiency worldwide affects nearly 2 billion subjects (Prasad, 2012), most prevalent in children under 5 years of age in developing countries (Gibson and Ferguson, 1998) who are most affected by diarrhea, malaria and pneumonia; Zn deficiency may result in approximately 453,000 deaths each year (Walker et al., 2009). In growing children Zn

deficiency results in growth retardation gonadal failure, intercurrent infections, premature death and impaired cognitive functions (Prasad, 2012). Over the past two decades, strong evidence has come forward from multiple randomized controlled trials, in both developed and developing countries, showing an effect of Zn in decreasing morbidity and mortality in children due to gastrointestinal and respiratory infections (Sazawal et al., 1998; Hambidge and Krebs, 1999). This effect of Zn against infectious diseases is therapeutic as well as preventive (Yakoob et al., 2011).

Diarrheal diseases remain a major public health problem especially in children in developing countries with approximately 1.5 billion episodes per year and 1.5-2.5 million deaths each year among children younger than age 5 and contribute substantially to malnutrition in surviving children (Black et al., 2003; Kosek et al., 2003; Hoque and Binder, 2006).

Other nutrients may also be involved in Zn deficiency, vitamin A deficiency, also widespread in humans, can aggravate both Fe and Zn deficiencies, so correcting any one of these deficiencies can make more of the other two nutrients available (Thurlow et al., 2005). For example, in humans some, if not most, of the Fe deficiency may be a consequence of the underlying Zn deficiency (Graham et al., 2012).

Zn deficiency in populations is assessed by a shift of the population distribution of serum Zn concentrations to lower values as recommended by the IZiNCG (Brown et al., 2004). According to this method, an estimated 17 % of the world's population has an inadequate Zn intake; substantial regional variation exists, with Asia and Africa having the highest prevalences (Black et al., 2013), see Figure 1.8 for more detail.

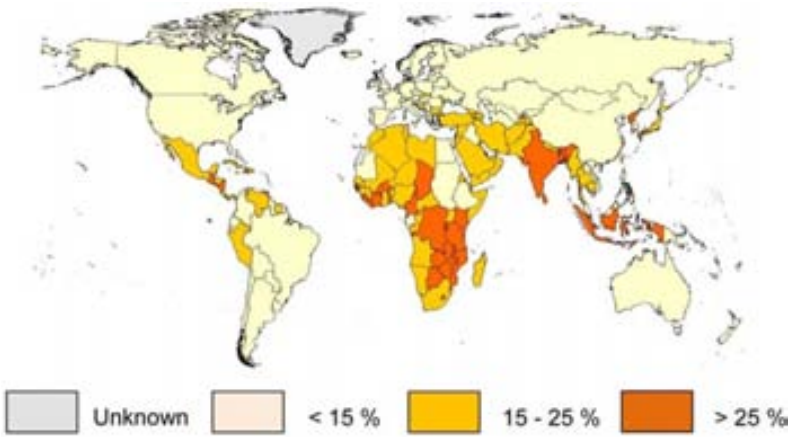


Figure 1.8. Estimated country-specific prevalence of inadequate Zn intake. From (Wessells and Brown, 2012).

The general causes of Zn deficiency include inadequate intake, increased requirements, malabsorption, increased losses (e.g. diarrhea) and impaired utilization (King and Cousins, 2006). Inadequate dietary intake of absorbable Zn is the primary cause of Zn deficiency in most situations (Lonnerdal, 2000). However, is important to have in mind that zinc deficiency increases the susceptibility to childhood diarrhea while increased losses of endogenous zinc associated with diarrhea further deplete body zinc and results in a vicious cycle that merits further study (Maret, 2001). This may result from low dietary intake or heavy reliance on foods with little or poorly absorbable Zn. The paradigmatic examples of this situation are children under 5 years old from underdeveloped countries consuming a cereal-rich diet with high content in phytates. Other factors can also be contributing to low Zn intake. In a recent publication Graham et al., (2012) reported that cereal and pulse micronutrients density have decreased due to the green-revolution and also highlighted that Zn-deficient soils are widespread on Earth, about half of the major agriculturally productive soil types (Sillanpää, 1982, 1990).

The major intervention strategies to improve Zn status in high-risk populations are dietary diversification/modification, Zn supplementation, Zn fortification and Zn bio-fortification.

In chapter 1.5.2. Zn supplementation and Zn fortification are further explained.

1.5. Zinc as an antidiarrheal treatment.

In both piglets and in children Zn is used as a preventive and as a therapeutic to overcome digestive disturbances. The main differences between the two species are the posology, the source of Zn used and the dose.

1.5.1. The use of ZnO in piglet diets

During the late 1980s, it was discovered that high dietary concentrations of ZnO (2,000 – 3,000 mg/kg) resulted in reduced diarrhea and increased growth rates in weanling pigs (Poulsen, 1989; Hahn and Baker, 1993; Hill et al., 2001; Fairbrother et al., 2005). In-feed pharmacological ZnO concentrations (i.e. concentrations in excess of normal dietary requirements) are commonly used for enteric disease prevention and growth promotion in weaned pig but there are concerns about its accumulation in the environment (Pluske, 2013). Its use is common in industrialized countries all around the world, despite the exact mechanisms whereby it improves growth and has an antidiarrheal are yet to be elucidated (Heo et al., 2010; Shelton et al., 2011). However, this practice is prohibited in the EU and a veterinary prescription is needed.

1.5.1.1. Positive effects and working assumption

Pharmacological levels (up to 3,000 mg Zn/kg) have been used as an effective dietary tool to ameliorate and (or) prevent PWD (Katouli et al., 1999; Hojberg et al., 2005), thereby acting as a growth promoter after weaning (Poulsen, 1995; Hill et al., 2000, 2001; Case and Carlson, 2002). The exact mode(s) of action for these effects have not been fully elucidated (Heo et al., 2013).

A recent meta-analysis (Sales, 2013) presented conclusive results that pharmacological levels of ZnO (1,000 to 3,000 mg/kg of Zn as ZnO) increased ADG, ADFI and G/F in post-weaning pigs. The ADG and ADFI increased linearly when dietary Zn concentrations increased, however that growth responses to added ZnO seem to reach a plateau at 1,500-2,000 mg Zn/kg (Hill et al., 2001), or up to 3,000 according to (Windisch et al., 1998; Mavromichalis et al., 2000; Shelton et al., 2011), the differences in the optimum level might be due to environmental variations and weanling age of the piglets (Zhang and Guo, 2007). The same meta-analysis did not reveal any influence of the duration of supplementation on growth (Sales, 2013), however, ADG and ADFI tend to increase when the supplementation period increase from 7 to 35 days. Carlson et al., (1999), showed that feeding high levels of ZnO the first week after weaning or solely during weeks 2 and 3, did not result in any better growth compared to no supplementation.

Enhanced feed intake account for a part of the improvement in growth of high ZnO concentrations, for example Yin et al., (2009) showed that it stimulates ghrelin secretion, a peptide that plays an important role in food-intake regulation. However, increased growth was also registered without an association to increased feed intake (Smith et al., 1997; Case and Carlson, 2002; Li et al., 2006; Yin et al., 2009), indicating that the effects of ZnO on growth involve several mechanisms (Sales, 2013), and the exact mechanisms is yet to be elucidated (Heo et al., 2010; Shelton et al., 2011).

It was previously assumed that high levels of dietary ZnO enhanced the growth of weaned pigs by controlling pathogenic bacterial scours, because ZnO possess antimicrobial properties (Hill et al., 2001). However, ZnO promotes growth in early- and conventionally weaned pigs, regardless of diarrhea prevalence or intestinal microbial numbers (Poulsen, 1995; Jensen-Waern et al., 1998; Katouli et al., 1999). These studies and others suggest several hypotheses that may explain the growth promoting effect of Zn in weaned pigs are summarized in Table 1.9.

Table 1.9. Overview of in-feed high levels of ZnO in weaned pigs.

Effect location	Zn regime	Initial/final age (days)	Results	Reference
Performance	0; 3,000 Zn as ZnO	22/50	-Improved ADG, FI, F/G - Elevated plasma Zn -Zn and Cu: no additive effects	(Hill et al., 2000)
Performance	0, 500, 1,000; 2,000; 3,000	<15/<43 >20/>48	- Increased ADG, FI, F/G as ZnO increased. Plateau at 2,000. - Plasma Zn concentration quadratically increased, when ZnO >1,000 -Antimicrobial agent: additive effect.	(Hill et al., 2001)
Performance	150Zn as ZnO 500 Zn as ZnO, ZnAA, Zn-PS 3,000Zn as ZnO	24, 17, 18 / 52,46,45	- Greater ADG of 500Zn-PS (polysaccharide) & 3,000ZnO -Plasma, fecal, urinary, liver Zn: greatest in 3,000ZnO Under certain nursery conditions the use of 500 ppm added Zn as Zn polysaccharide may also enhance performance	(Case and Carlson, 2002)
Performance	0, 550, 1,500; 2,250; 3,000 Zn as ZnO and TBZC (Tetrabasic $ZnCl_2$)	15/38	- ADG increased in both ZnO and TBZC - F/G inconsistently TBZC>ZnO TBZC is effective for enhancing growth performance	(Mavromichalis et al., 2001)
Performance	0, 150, 300, 450 Zn as Zn-PS vs.2,000 Zn as ZnO	21/56 (phase 1: 21-35d; phase 2: 35-56d)	-Phase 2 growth performance 300 & 450 Zn-PS (polysaccharide) was no different to 2,000ZnO. - Plasma Zn: greater 2,000ZnO than any other Zn-PS &Ct	(Buff et al., 2005)
Performance	500, 2,500 Zn as ZnO, 500 Zn as ZnMet, ZnAA, Zn-PS, Znprot.	20/48	- ADG, FI 2,500ZnO> 500 Zn as ZnO, ZnAA, ZnMethionine, Zn proteinate, Zn polysaccharide	(Hollis et al., 2005)
Performance	0, 50, 100 Zn as ZnGly 3,000 Zn as ZnO	35/70	- ADG: 100Gly(glycine chelate)&3,000ZnO>Control - Zn muscle: 100Gly&3,000ZnO>Control - Zn, Fe liver, spleen Cu, kidney Cu of 3,000ZnO>100 ZnGly - Zn&Mn fecal 3,000ZnO>100 ZnGly - AP, SOD 100Gly&3,000ZnO>Control	(Wang et al., 2010)
Performance	Exp1: 0, 250, 500, 1,000; 5,000 Zn as ZnO; 1,500; 2,500 Zn as ZnLys, ZnSO4	28/49	- Plasma Zn: did not increase until 1,000 mg Zn/kg. - Above 1,000 mg/kg, it increased linearly for all 3 sources	(Hahn and Baker, 1993)
	Exp2: 3,000; 5,000 Zn as ZnO, ZnSO4		- ADG, FI: increased for 3,000 ZnO and ZnSO4 and 5,000 ZnO - Plasma Zn: 2x ZnSO4>ZnO	
	Exp 3: 3,000 Zn as ZnO, ZnSO4, Zn Met		- Plasma Zn: ZnSO4, ZnMet > ZnO	
Performance	0-500 Zn as Zn-PS, 0-800 Zn as Zn-prot, 2,000 Zn as ZnO	17/42	- ADG, G/F: no differences, except for wk1 2,000ZnO was best. -Plasma Zn: 2,000 ZnO > Ct -Fecal Zn: 2,000ZnO has the higher. Organic Zn either as a polysaccharide or a proteinate had no effect on growth performance at lower inclusion rates; greatly decreased the amount of Zn excreted.	(Carlson et al., 2004)
Microbiological	2,500 ppm ZnO	35/63 d old	-Increased growth (2wks). -Higher serum, liver (x4-5) & kidney Zn values. -No effect on E.coli nor enterococci number	(Jensen-Waern et al., 1998)
Microbiological	2,500 ppm ZnO	35/63	-No effect on WG -No effect on coliform numbers and fermentative capacity. -ZnO has a + impact on microflora stability and maintains high diversity of coliforms, the first 2wks postweaning.	(Katouli et al., 1999)
Microbiological	2,500 ppm ZnO	28/42	-Reduced bacterial activity -Reduced lactic acid-producing bacteria and lactobacilli -Increased coliforms and enterococci -No reduced pH in stomach and distal small intestine	(Hojberg et al., 2005)
Microbiological	3,000 ppm Zn as ZnO	28/42	-Increased bacterial diversity -Increased enterobacteria… could lead to increased competition among enterobacteria and consequently, reduced pathogenic E.coli strains.	(Vahjen et al., 2011)
Microbiological	50, 150 250; 1,000; 2,500	25/40	-Performance: no difference -Serum Zn: quadratic curve for dose effects. -Ileal microbial composition and DGGE: dose-dependent	(Pieper et al., 2012)

			clustering; richest as higher Zn level, high enterobacteria, no effect on lactic acid bacteria, decreased clostridial XIVa SCFA, lactate NH_3: no effects lactate, propionate, VFA, acetate and butyrate increased from 50 to 150ppm, then decreased until 2,500ppm. NH_3 decreased with increasing ZnO.	
Microbiological	200; 3,000 ZnO		high dietary zinc oxide has a major impact on ileal bacterial communities in piglets	(Vahjen et al., 2010)
Microbiological	57; 2,425 Zn as ZnO	32/39/46/53	Stomach &Jejunum: -Increased diversity of enterobacteria -Increased nº bifidobactera, enterobacteria, enterococci. -Reduced ex-vivo bacterial growth from stomach and jejunum Rapid bacterial adaptation to Zn (2-3 wks), the beneficial effect might only be beneficial in a short period after weaning.	(Starke et al., 2013)
Microbiological	0, 100; 3,000 ZnO	1, 14, 28, 42, 56	Increased gene expression of antimicrobial peptides in the small intestine.	(Wang et al., 2004)
Microbiological	In-vitro		- The resistance of intestinal bacteria against ZnO is species specific and the antibacterial effect of Zn cannot be assigned to a specific bacterial group: instead of a direct inhibition of E.coli, modifications of enterobacterial diversity and changes in other bacterial groups can be more relevant.	(Liedtke and Vahjen, 2012)
Intestinal architecture	100; 3,000 Zn as ZnO	28/42	- Intestinal growth through increasing IGF-I and IGF-IR expression in the small-intestinal mucosa	(Li et al., 2006)
Intestinal architecture	57, 164, 2,425 Zn as ZnO	28, 33, 40, 47, 54	- Maturation of barrier function of colonic mucosa, increase mucin production and down-regulation IL-8 and TLR-4. Suggesting, stimulation of protective mechanisms in colonic function.	(Liu et al., 2014)
Intestine	In vitro	28, 35	Reductions in electrolyte secretion from enterocytes.	(Carlson et al., 2006)
Intestine architecture. Performance.	100; 2,500 Zn as ZnO	28, 42	- Increased activity of enzymes in the pancreatic tissue and increased the mucin staining area in the large intestine. - No definite answers for growth promoting and diarrhea reductions.	(Hedemann et al., 2006)
Microbiological Performance	0; 3,100 ZnO	23, 43	-No effect on growth performance. -Reduction of bacterial translocation to the MLN… modulation immune system and enhanced IgA	(Broom et al., 2006)
Antioxidant	100; 3,000 Zn as ZnO	28/43	- Improvement of the redox state (glutathione)and prevention of apoptosis in the jejunum, alleviating intestinal dysfunction and malabsorption of nutrients	(Wang et al., 2009)
Antioxidant	0; 2,500 Zn as ZnO	20, 32	- Reduction of systemic oxidation and improvement of antioxidant status in jejunal and ileal mucosae.	(Bergeron et al., 2014)
Intestinal transporters	57, 164; 2,425 Zn as ZnO + invitro	26/54	Increased intracell. Zn, Zn export to extracell., decreased Zn uptake from gut: ZnT1, ZnT2, ZIP4, MT. The adaptive process appears to be established within 24 h; however, it does not prevent tissue zinc accumulation.	(Martin et al., 2013 a)
Intestinal transporters. Performance	50, 150; 2,500 Zn as ZnO	26/33/40/47/54	- Increased ADG, ADFI until 2wks pstweaning. -Zn acmulation in tissues - Upregulation ZnT1, down-regulation ZIP4. - Increased intestinal AP: resistance to pathogen induced inflammatory response?	(Martin et al., 2013 b)
Immunity Antiviral Intestinal transporters	50, 150; 2,500	28, 29, 46	- Enhanced protection in the intestinal tract (modulation of cytokines, prevention of intestinal barrier integrity) and earlier and grater stimulation of the systemic humoral immune response against TGEV infection. - Increased expression ZnT1, ZnT5, MT1, decreased ZIP4 with increasing Zn.	(Chai et al., 2014)
Liver	50, 150; 2,500	25/46	- Increased hepatic Zn and MT expression and altered the protein expression profiles. - There is a complex regulatory network of Zn-induced protein expression pattern alterations in the liver, involved in transport, stress response, metabolism, apoptosis and cellular signaling.	(Bondzio et al., 2013 a)
Intestine-histology	100; 3,000 Zn, as ZnO	28/38	- ZnO inhibits stem cell factor , leading to reductions in the nº of mast cels and histamine release	(Ou et al., 2007)
Intestine	100; 2,500 Zn as ZnO	28/33	- Zn reduces diarrhea directly through a regulatory role of serosal Zn and Xl- secretion and indirectly by improving the nutritional status..	(Carlson et al., 2007 b)
Intestine-inflammatory	0; 3,100 of ZnO	28/38	- Reduction in inteswtinal expression of immune response genes. Therefore, it may improve piglet performance when there is an ETEC challenge.	(Sargeant et al., 2010)
Inflammatory	In vitro (IPEC-J2)	-	- Reduction of innate immune response genes. - It inhibits the induction of NF-κB in response to pathogens. - A host effect is involved in the mechanism of action of ZnO.	(Sargeant et al., 2011)

Hojberg et al., (2005) suggested that reduced fermentation of digestible nutrients in the proximal part of the GIT might render more energy available for the host animal and contribute to the growth-promoting effect of high levels of dietary ZnO.

Interestingly, some studies found that dietary ZnO concentrations need to be higher than 1,000 mg/kg to elicit increased serum Zn concentrations in weaning pigs, and at the same time plasma Zn concentrations seem to be related to ADG (Hahn and Baker, 1993; Carlson et al., 2007 a). Zn treatments that resulted in plasma Zn values of approximately 1.5 mg/L (e.g. 3,000 mg/kg Zn as ZnO) were associated with weight gains that tended to be higher than those that resulted from pigs having plasma Zn values that were < 1.5 or > 3.0 mg/L (Figure 1.9).

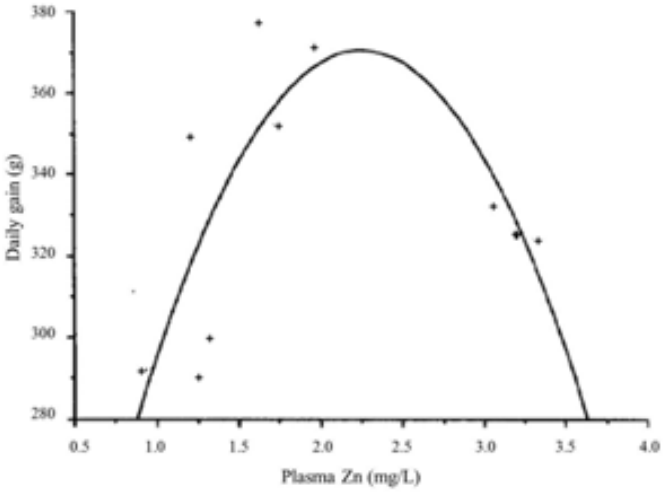


Figure 1.9. Polynomial pot of ADG as a function of plasma Zn concentration. From (Hahn and Baker, 1993)

Finally, Poulsen, (1995) and Carlson et al., (1999) reported increased growth of weaned pigs when plasma Zn concentrations reached a level of about 2.5 mg/L. Further explanation and investigation are found in **Chapter 4.**

1.5.1.2. Environmental consequences and legislation

When high levels of Zn are fed for longer than 10 days to weaned pigs, tissues become loaded and homeostatic mechanisms excrete excess Zn (Rincker et al., 2005). Most of the dietary Zn supply is excreted and manure highly concentrated in Zn may concentrate in top soil (L'Herroux et al., 1997) and can cause toxicity to plants and soil microorganisms, becoming an environmental concern in some areas of intensive pig farming (Coppenet et al., 1993; McGrath et al., 1995). Teira, (2008) reported that in Catalan farms the average Zn content of pig manure during nursing phase is 6776 ± 2645 mg/kg of DM, during growing-fattening phase concentration is 3101 ± 1044 mg/kg DM and in breeding herds 1740 ± 1705 mg/kg DM. However, the volume of manure produced by each space and year for nursing phase represents less than 3% of the manure produced throughout the production cycle.

The recent EFSA report (EFSA, 2014) showed that mean Zn concentration of piglet's feed in Europe is 139 mg/kg. It seems that therapeutic ZnO administration in farms has been ignored. Feed samples of this report were mainly from Germany. No samples from Spain were included in this report.

Zn, together with Cu, was initially authorized for all species under Directive 70/524/EEC concerning additives in feeding stuffs. In 2003, SCAN (Scientific Committee on Animal Nutrition) issued two opinions on the use of elements in feed additives and considered that there may be a risk associated with Cu and Zn. Consequently maximum limits of Cu and Zn in feed additives were reduced (Commission Regulation (EC) 1334/2003); the maximum content of Zn in mg/kg of the complete feedstuff for swine was set to 150. Very recently (EFSA, 2014), the FEEDAP (the EFSA Panel on Additives and Products or Substances used in Animal Feed) published a scientific opinion suggesting that the use of phytase in piglets, pigs and sows would allow a further reduction of 30% of total maximum Zn contents.

In Europe, therapeutic ZnO must be registered by each producing company in the drug agency. Like any other drug, each product has its recommended posology, that in the case of ZnO is 100 mg ZnO / kg of live weight/ day for 14 days post-weaning, equivalent to 3.1 kg of product/ ton of feed (2,500 g Zn/ ton of feed), assuming an average feed consumption of 0.225 kg. It is not illegal to use a higher dose than recommended and it is also not illegal to use the treatment for longer. However there should always be a veterinarian prescription. In contrast, in other parts of the world like Asia or South America there is no control on the use of Zn either as an additive or as a therapeutic. Anyway, therapeutic ZnO is extensively used worldwide, probably because it is the cheapest treatment in swine production.

So there are basically two different products with two different objectives when adding Zn in pig diets: its use as an additive or as a therapeutic. In the EU two different legislation rules apply. A summary of the main characteristics are found in Table 1.10.

Table 1.10. Main characteristics of Zn compounds depending on its use in swine feed. (own data from a feed industry, Spain)

	"Additive" Zn	**"Therapeutic" Zn**
Color	Yellowish	White
Particle size	90% < 250 µm	99.9%, < 45 µm
Density	+ (2 kg/dm^3)	- (0.55 kg/dm^3)
Impurities	≈ 5% Fe, ≈ 4% CaO, < 400 ppm Pb	No
Legislation	1831/2003	Drug agency
Control of its use	No	Yes
Price	1.3 €/kg	1.9 €/kg

General concerns about agriculture and environmental problems related to Zn excretion provoked changes in the EU legislation in order to reduce mineral surpluses. The maximum authorized amount of Zn as an additive in swine diets is 150 mg/kg of the complete feedstuff (Comission Regulation (EC) No 1334/2003, 2003). There is a total of ten Zn additives registered that can be added to feedstuffs in the EU: Zn lactate, trihydrate; Zn acetate, dehydrate; Zn carbonate; Zn chelate of AAs, hydrate; Zn chloride, monohydrate; ZnO, Zn sulfate, heptahydrate; Zn sulfate, monohydrate; Zn chelate of glycine, hydrate; and Zn chelate of hydroxyl analogue of methionine.

1.5.1.3. Alternatives

In a recent review article Pluske, (2013) pointed out that in contrast to in-feed antibiotics, ZnO is purported to have a wide variety of modes of action and that makes it difficult to interpret what the precise mechanism is (or precise mechanisms are) for its efficacy; and that makes the search/development of products to replace ZnO more problematic. Regardless, such understanding is critical for finding effective, sustainable and consumer-acceptable replacements for these products.

Alternative Zn compounds to replace ZnO when used as a nutrient are multiple. Organic Zn compounds (amino acid chelates, polysaccharides, e.g. Zn methionine, Zn lysine) are suggested to have the ability to replace higher doses of inorganic Zn compounds thus, to be more environmentally friendly however performance results are inconsistent (van Heugten et al., 2003) and the kind of chelate seem to be an important factor (Nitrayova et al., 2012).

Some Zn compounds formulated to replace pharmacological ZnO levels at a lower dose have been studied and summarized on the Table 1.9. (section 1.5.1.1.), (Hahn and Baker, 1993; Swinkels et al., 1996; Mavromichalis et al., 2001; Case and Carlson, 2002; Buff et al., 2005; Hollis et al., 2005; Wang et al., 2010). There are few studies testing alternative Zn products with the aim to replace pharmacological levels of ZnO (therapeutic). Performance results of alternative Zn compounds are inconsistent, in no case are better, than pharmacological levels of ZnO. None of these studies with alternative Zn compounds claim for an antidiarrheal effect. They showed a reduced excretion of Zn because the lower dietary level, that may claim for environmental friendly. For example, nutritional levels (150 ppm) of a "potentiated" ZnO showed comparable performance results to pharmacological dosage of regular ZnO in low-medium sanitary conditions farm attributed to a lower inflammatory protein expression (Morales et al., 2012).

In the present PhD thesis we try to shed some light into the chemical behavior (i.e. availability) of several Zn compounds. We also tested the same Zn compound in different posologies (i.e. in-feed, dispersible tablets) as used in children (**Chapter 6**) on piglets Zn status, as a previous step to assess its antidiarrheal effect.

Another nutritional interesting alternative is the use of phytase to make Zn more available, this strategy is useful in order to reduce Zn nutritional levels and provides an important means of environmental protection (Jondreville et al., 2003; Revy et al., 2004, 2006), also in accordance to very recent FEEDAP scientific opinion (EFSA, 2014). According to a recent meta-analysis (Schlegel et al., 2013), piglets are limited in the use of native Zn (Zn of the own vegetable ingredient) because of the antagonism of phytate. This fact explains the higher efficacy of phytase in improving Zn availability in piglets compared to other species like poultry. Phytase is efficient in increasing digestive soluble Zn and improving Zn status in piglets (Schlegel et al., 2010). Its use also becomes interesting in piglets to counteract the negative interaction with other nutrients (further described in Chapter 1.2.3.2.) and between wheat bran (rich in phytate) and pharmacological levels of ZnO on piglets performance and intestinal health found in Molist et al., (2011). Martinez et al., (2004) showed that feeding 1,000 mg Zn/kg as ZnO with phytase enhances MT mRNA abundance and protein in liver and Zn absorption to the same level as 2,000 mg Zn/kg as ZnO with or without phytase. An another study (Walk et al., 2013), showed that at high Zn:phytate ratio (> 4:1) the benefits of both phytase and ZnO may be severely diminished, suggesting a reduction of dietary ZnO; and that the use of high doses of phytase (2,500 FTU/kg) with moderate pharmacological dosages of Zn (1,750 mg/kg) can result in plasma Zn level similar to that observed with

greater Zn (3,500 mg/kg) in absence of phytase, suggesting that the levels of supplemental Zn needed to elicit a beneficial performance effect may be reduced in the presence of phytase.

However, pharmacological levels of Zn reduce the efficacy of phytase, probably because high levels of Zn chelate the phytate complex, thereby decreasing its availability for hydrolysis by phytase (Augspurger et al., 2004). This effect was also recorded in another study as phytase was not effective in improving Zn availability and it inconsistently affected growth performance in weanling pigs compared to its better efficacy in growing-finishing pig diets (Williams et al., 2005).

According to Hambidge et al., (2010), phytate is the only substantial dietary factor that inhibits Zn absorption. Phytate with minerals such as Ca or Zn can form ternary interactions with protein in the small intestine thus, this type of interactions exerts a great effect on the digestibility of these minerals (Selle and Cowieson, 2012).

1.5.2. The use of $ZnSO_4$ in children - WHO recommendations and positive effects and working assumptions

The efficacy of Zn in treating both persistent (by 15-30%) and acute diarrhea (by 20%) is evident (Black, 2003; Patel et al., 2010), but the mechanisms of action are becoming clearer (Lazzerini and Ronfani, 2009; Canani et al., 2011). These reviews stressed the importance of Zn on intestinal transepithelial ion transport, on mucosa integrity and on intestinal defense mechanisms. Zn also decrease diarrhea prevalence in both 24-h and 2-week recall survey (Walker and Black, 2010).

The introduction of Zn community programs resulted in increased use of ORS (oral rehydration solution), decreased use of unnecessary antibiotics and a reduced need for medical visits (Bhandari et al., 2008). Zn is included in the WHO essential medicine list for diarrhea treatment and widely used in the treatment of acute diarrhea in developing countries where it is responsible for saving more than 400,000 lives per year (Walker et al., 2009). Also, a Zn containing super ORS has been proposed by various authors (Fischer Walker et al., 2009; Santosham et al., 2010).

Supplementation programs are useful for targeting vulnerable population subgroups which are high risk of Zn deficiency. The recommended Zn dosages are 5 mg/day for children between 7 months and 3 years and 10 mg/day for older children (Müller et al., 2001; Brown et al., 2002).

In supplementation programs Zn is usually administered with ORS when used as an adjunct therapy during the treatment of diarrhea in children. The recommended daily dosage for the

WHO (WHO, 2005) is twice the age-specific RDA per day for 14 days; that is 10 mg/day for children under 3 years and 20 mg for older children.

It is recommended that salts providing readily absorbable Zn, like $ZnSO_4$, Zn gluconate or Zn acetate are used because they are absorbed more efficiently (Brown et al., 2004).

Food fortification is a more cost-effective and sustainable strategy to overcome micronutrient malnutrition than supplementation, but is not used as a strategy to overcome diarrhea. Zn is usually added together with other micronutrients into staple foods. Among several Zn compounds that are available for fortification, ZnO and $ZnSO_4$ are less expensive and most commonly used. The suggested levels for fortification are 30-70 mg Zn/kg (WHO, 2009). More information on the bioavailability of different Zn compounds is required.

Chapter 2

Objectives and experimental design

The project AGL2009-07328 entitled: "Evaluation of the dietary Zn and blocking agents of the microbial intestinal adhesion in the weaning piglets feeding", focused the study on Zn status of weaned pigs and the use of Zn sources in weaned pig diets, in one hand, and on the study of blocking substances of bacterial adhesion, in the other.

In the present PhD thesis we hypothesized that sows Zn status can be affected by age or by dietary factors that can compromise their offspring Zn status at weaning and growth performance. In addition, we hypothesized that high in-feed levels of ZnO can affect Zn status of weaned pigs and more soluble Zn sources could exert similar effects.

The following main objectives were fixed for the present PhD thesis:
1- To evaluate the effect of the sow parity number and dietary factors (Ca, P, phytase) on the Zn status and reproductive performance in sows as well as Zn transfer to piglets.

2- To study how Zn, Fe and Cu concentrations are modified in plasma, liver, pancreas and spleen in littermates when unweaned or weaned to nutritional or therapeutic doses of dietary ZnO one week after weaning.

3- To study how Zn, Fe and Cu concentrations are modified in the GIT content (stomach, jejunum, ileum, cecum and colon) in littermates when unweaned or weaned to nutritional or therapeutic doses of dietary ZnO one week after weaning.

4- To assess how Zn plasma/serum concentration changes depending on different Zn levels and sources at weaning with special interest on different posologies of $ZnSO_4$

To assess these objectives, five different experiments were designed. Results are included in Chapters 3 to 6.

In Chapter 3, two experiments were designed with the aim of studying the effect of sow's age on Zn, Fe and Cu composition of colostrum and milk and on the consequent micromineral status of piglets. In the other experiment the effect of low Ca and P dietary concentrations and phytase supplementation was studied.

The experiment of **Chapter 4 (Davin et al., 2013)** aimed to consider the hypothesis that weaning by itself can affect Zn status of piglets by comparing weaned and unweaned littermates and how high levels of ZnO can counteract the weaning effects on Zn status.
In **Chapter 5 (Davin et al., 2012),** the effects on Zn, Fe and Cu concentrations at stomach, jejunum, ileum, cecum and colon of the same animals at Chapter 4 were analyzed.

In **Chapter 6**, two experiments were designed to evaluate the effect of different dietary Zn regimes on Zn status of piglets the first days following weaning. In the first experiment different dietary concentrations of ZnO and $ZnSO_4$ were administered whereas in the second experiment a Zn-tableted form was included.

Chapter 3

The Zn status in sows and piglets as affected by the diet and the number of sow parity

Abstract

The objective of the present study was to evaluate if changes on the dietary P, Ca and phytase level and the sow parity number affects the productive and reproductive performance in sows as well as the mineral transfer to their piglets during the lactation. In experiment 1, one hundred and twelve sows receiving the same gestation and lactation diets were characterized and selected according to their parity number. Colostrum and 21-days-milk samples were collected from sows and piglet's hair was also collected at the 21st day of lactation. In experiment 2, forty one sows were characterized and distributed into 3 parity number groups and 4 dietary lactation treatments: recommended Ca, P and Zn in-feed levels (Rec) or low Ca, P and Zn in-feed levels (Low) and with 250 FTU of phytase (Low250) or 500 FTU (Low500). Titanium dioxide (TiO_2) was added as indigestible marker. Fecal samples were obtained between 21st and 25th day of lactation and analyzed for Ca, P, Zn, Fe, Cu and Ti; blood and colostrum samples were obtained on the farrowing day and blood and milk samples were collected on day 21st. Blood, colostrum and milk samples were analyzed for Zn, Fe and Cu concentration and hair samples just for Zn. In Experiment 1 no differences among colostrum, milk and hair trace mineral concentrations were found among different parity number, except for milk Cu concentrations that were higher in older sows and milk Zn concentrations that tended to be higher in older sows. In Experiment 2, sows fed Rec diet had higher Ca, P and Zn intake and excretion, and higher P digested than the other three treatments. Sows fed dietary treatments supplemented with phytase had a higher P digestibility than those fed unsupplemented diets. None of the dietary treatments changed Zn, Fe and Cu concentrations in plasma and milk. None of the variables were affected by parity number groups except for Cu that was higher in plasma directly after farrowing in youngest sows compared to oldest sows. High-producing sows secrete large amounts of Zn through milk during lactation regardless the parity number of the sow and phytase application to low P and Ca sow's diets.

3.1. Introduction

Zinc is an essential trace nutrient for pigs and has numerous structural and metabolic roles, such as regulating the activity of metalloproteases, initiating transcription, immune cell function, and cell differentiation. Loss of appetite is usually the earliest clinical sign of zinc deficiency followed by higher incidence of infectious disease (diarrhea), skin problems, and impairment of functions, such as taste, smell, and cognition.

During pig's life, major concerns have been described on Zn homeostasis directly after weaning as weaning by itself may create a Zn deficiency situation (Davin et al., 2013), which it is overcome by the use of therapeutic doses of ZnO in weaning diets

It is generally accepted that piglets are well protected against Zn deficiency during the suckling period. Adequate Zn supply from sow body reserves with the sow milk is tightly regulated (McCormick et al., 2014) as piglet's Zn requirement is high for the rapid growth rate and immune function development during the suckling period. Thus, the Zn content in liver of piglets increases from d 1 to d 21 of lactation (Hill and Miller, 1983) and a considerable increase of piglet's body Zn concentration is observed (Mahan and Shields, 1998). Average levels of trace minerals in sow colostrum and milk (Peters et al., 2010) show that Zn contents (15 and 7.1 mg/L) clearly exceed those of Fe (1.78 and 1.66 mg/L) and Cu (2.41 and 0.51 mg/L, respectively). In contrast, the serum Zn levels in sows (0.79 mg Zn/L) are lower than its Fe and Cu serum levels (1.71 mg Fe/L, 1.63 mg Cu/L, (Tremblay et al., 1989). Thus, it is suggested that a substantial amount of Zn is taken up by the mammary gland and secreted into milk in order to meet offspring Zn requirements (Matte et al., 2014). Some authors suggested that the Zn pool/reserves of the sow may be progressively depleted and high parity sows and those weaning heavy litters may show a lower body mineral content compared to non-gravid gilts (Mahan and Newton, 1995). This decline was particularly evident for Ca, P, and Zn, which may have an effect on lameness, claw lesions, and sow longevity.

Dietary composition should have an impact on the whole-body Zn status of sows and Peters et al., (2010) showed that an increase on the levels of trace minerals in the diet to exceed the NRC (1998) sow recommendations increased the sow body content of Cu, Se, and Zn. Additionally, ZnAA administration to sows during gestation and lactation increased the number of pigs born alive and weaned per litter (Payne et al., 2006; Caine et al., 2009); and supplementation with microbial phytase and formic acid significantly increased Zn, Ca, and P concentration in humeral bones of sows after the lactation period. However, when gilts from 30 kg BW onwards were offered a long-lasting regime with varying levels of supplementary

Zn, milk Zn content remained unchanged for diets between 0 to 500 ppm Zn (Hill et al., 1983 b). Only excessive dietary Zn supply (5000 ppm), increased the level of Zn in milk and piglet's liver, kidney and pancreas, accompanied by a decrease in Cu concentration in sow's milk and piglet's liver, pancreas and muscle (Hill et al., 1983 a; b). Earle and Stevenson, (1965) also found no difference in colostrum Zn content attributable to different dietary level of Zn. Milk Zn concentration in mammals seem to be tightly regulated and maintained over a wide range of dietary Zn intakes (Courtney Moore et al., 1984; Krebs, 1998; Kelleher and Lönnerdal, 2005); only when applied 3-4 fold above the sows requirement Zn level in colostrum and normal milk increased (Kirchgessner et al., 1980).

The present study aimed to evaluate the effect of dietary P, Ca and phytase level and the sow parity number on the apparent mineral digestibility, milk level of Zn and reproductive performance in sows as well as the hair Zn level of their piglets. For Experiment 1, we hypothesized that high parity sows will show lower reproductive performance associated to a lower trace mineral content in milk. For Experiment 2, we hypothesized that phytase supplementation would influence total apparent digestibility of Zn, sow reproductive performance and the trace mineral content in milk.

3.2. Materials and methods

The experiments received prior approval by the Universitat Autònoma de Barcelona ethical committee (CEEAH). The treatment, management, housing and husbandry conditions conformed to the European Union Guidelines (European Parliament, 2010).

Experiment 1

Facilities, Animals and Management

The study was carried out in a commercial farm, with a breeding stock of 6,000 sows (Large White x Landrace). A total of 112 sows were selected by their age (from the 1st to 10th parity number) one week before the expected farrowing. Sows showing lesions or signs of illness were not included in the study. All sows received the same gestation and lactation diet. Via the gestation and lactation premixes the same calculated levels of Zn, Fe and Cu were included to the diets: 100 mg/kg Zn as ZnO, 80 mg/kg Fe as $FeSO_4$ and 20 mg/kg Cu as $CuSO_4 \cdot 5H_2O$. Before farrowing, 2.7 kg of feed were offered to the sows daily, except the day of farrowing. After farrowing, feed allowance was increased to reach *ad libitum* intake at 7th day post-farrowing. Water was available *ad libitum* throughout the study.

Sows were housed in individual cages until 109th day of gestation when moved to the farrowing room and housed in individual cages equipped with a nipple water drinker and a trough. At 6-12 hours after the parturition piglets were distributed with the objective to obtain equal litters of 12 piglets for young sows (< 5th parity number) and litters of 10 piglets for older sows (> 5th parity number). At two days of life piglets were injected with 200 mg of iron dextran (Ferrovall®, Mevet laboratorios, Lleida, Spain), antibiotic (Vetrimoxin®, Ceva santé animale, Libourne, France), oral administrated of coccidiostat (Baycox®, Bayer Health Care, Leverkusen, Germany), tail-cut and ear tagged.

Controls and sample collection

Sows were characterized -individual BW and P2 backfat thickness (BF; Renco Lean-Meater® series 12, MN, USA)-, on day 107 of gestation. All samples were collected following the same distribution of sow population age of the farm. Colostrum and milk samples were obtained from 95 sows (10 samples for each parity number except for the first parity with 15 samples and for the ninth and tenth parity with only 5 samples each). Colostrum samples (30-50 mL, within first 5 hours of parturition) were manually collected from sows. Milk samples (30-50 mL) were manually collected from all sows after oxytocin injection on day 21 of lactation. Colostrum and milk samples were obtained from the teats of one mammary chain of the sow. Hair samples were obtained on day 21 of lactation from 81 pairs of piglets that were previously ear-tagged to avoid cross-fostering movement. All samples were frozen at -20ºC until analyses were performed.

Experiment 2

Facilities, Animals and Management

The study was carried out in a commercial farrowing facility, with a breeding stock of 400 sows (Landrace x Large White). A total of forty one sows from two farrowing groups (separated by 3 weeks; n = 19, 1st group and n = 22, 2nd group) were selected just before farrowing (110 d of gestation) according to BW, P2 backfat thickness (65 mm off the midline at the last rib), body condition and parity number. Sows showing lesions or signs of illness were not included in the study. Sows received the same diet during gestation (Table 1). After farrowing, sows were equally distributed into four experimental treatments on the basis of BW before farrowing, parity number and number of piglets born alive. Sow litters were

standardized to 12 piglets by cross fostering. Plastic ear tags were applied to piglets for identification.

Sows were housed in individual farrowing cages equipped with a nipple water drinker and a trough. Lactation diets (Table 3.1.) were offered following the same pattern as the previous experiment from 48h after farrowing until the end of the lactation period (28 d).

Table 3.1. Composition of the experimental lactation diets (%). Experiment 1 and 2.

	Experiment 1		Experiment 2			
	Gestation	Lactation	Lactation			
			Rec	Low	Low250	Low500
Ingredients	%	%	%		%	
Maize	-	-	25.0		25.0	
Wheat	40.4	40.4	20.0		20.0	
Barley	5.0	15.4	19.6		21.6	
Sunflower	15.0	10.0	-		-	
Soybean meal 44% CP	-	13.0	18.5		18.0	
Wheat bran	-	-	10.0		10.0	
Wheat middlings	23.0	10.0	-		-	
Rice bran	9.20	-	-		-	
Canola meal			1.04		1.04	
Glycerol	3.00	3.00	-		-	
Lard	-	-	1.64		1.61	
Poultry fat	1.90	4.50	-		-	
Calcium carbonate	1.40	1.78	1.65		1.28	
Monocalcium phosphate	0.10	0.46	1.15		-	
Sodium bicarbonate			0.30		0.30	
Salt	0.20	0.32	0.30		0.30	
L-Lysine-HCl	0.23	0.41	0.13		0.14	
DL-Methionine	-	-	0.03		0.03	
Choline	0.03	0.03	-		-	
L-Threonine	0.00	0.02	0.01		0.02	
Vit-Min Premix[1]			0.20		0.20	
Titanium Dioxide (TiO_2)	-	-	0.50		0.50	
Phytase (FTU/kg feed)	500	500	-	-	250	500
Analytical characteristics[2]						
Gross energy (GE), MJ/kg	-	-	16.5	16.9	16.6	16.7
Dry matter (DM), %	-	-	89.1	89.0	89.1	89.0
Ash, %	-	-	6.30	4.91	4.93	4.90
Crude protein (CP), %	-	-	16.9	17.2	16.9	16.7
NDF, %	-	-	11.4	12.0	11.0	11.9
ADF, %	-	-	4.19	4.40	4.14	4.40
Ether extract (EE), %	-	-	4.12	3.83	3.95	3.89
Macromineral, g/kg						
Ca	-	-	9.74	6.70	6.60	6.56
P	-	-	6.47	3.85	4.02	4.00
Phytic P, calculated			2.45		2.48	
Trace mineral, mg/kg						
Zn	-	-	120.7	81.0	75.5	82.4
Fe	-	-	98.2	79.8	102.4	115.9
Cu	-	-	28.1	33.2	40.4	44.0
Phytase activity, FTU/kg	-	-	< 50	< 50	298	469

Rec: recommended Ca and P in-feed levels (Ca=0.95% Total P total=0.65%, digestible P 0.31 %). **Low:** low Ca and P in-feed levels (Ca=0.6% Total P total=0.4%, digestible P 0.2%). **Low250:** Low diet supplemented with 250 FTU of Phytase/kg of feed. **Low500:** Low diet supplemented with 500 FTU of Phytase/kg of feed. [1]Supplied per kilogram of feed: 10,000 IU of vitamin A, 1,524 IU of vitamin D as cholecalciferol, 100 IU of vitamin D as 25-hydroxicholecalciferol, 60 mg of vitamin E, 3.48 mg of vitamin K3, 1.54 mg of vitamin B1, 5.03 mg of vitamin B2, 2.47 mg of vitamin B6, 0.03 mg of vitamin B12, 28.8 mg of Nicotinic acid, 17.0 mg of pantothenic acid, 0.20 mg of biotin, 2.21 mg of Folic acid, 4.00 mg of Fe as Iron sulphate, 3.90 mg of Cu as Copper sulphate, 55.0 mg of Zn as Zinc oxide, 11.5 mg of Mn as Manganese oxide, 1.00 mg of I, 0.01 mg of Se as organic Selenium, 0.30 mg of Se as sodium selenite, 1.01 mg of ethoxyquin.
[2]Analyzed nutrient composition expressed as feed basis. Analyzed according to the following procedures: DM, drying to constant weight at 103 °C; Ash, muffle furnace (550 °C, 8 h); CP, Kjeldahl method.

Experimental treatments

Two different lactation diets (with recommended Ca, P and Zn in-feed levels: Rec; or low Ca, P and Zn in-feed levels: Low) were formulated to meet the requirements for maintenance and milk production of lactating sows (FEDNA, 2006) except for Ca, P and Zn (Table 1). A commercial phytase with an analyzed activity of 41,700 FTU/g (modified *Escherichia coli* phytase, AB-Enzymes, Darmstadt, Germany) was supplemented to the Low diet in order to obtain the experimental treatments with 0 FTU of phytase/ kg of feed (Low), 250 FTU of phytase/ kg of feed (Low250) and 500 FTU of phytase/ kg of feed (Low500). Titanium dioxide (TiO_2) was added as indigestible marker at 0.5% inclusion rate. On d21 a standard creep-feed (Zn 2,560 mg/kg; Fe 133 mg/kg, Cu 135 mg/kg) was introduced to piglets until the end of the experimental period.

Controls and sample collection

Sow's BW, body condition scoring and P2 backfat thickness (ultrasound, Renco Lean-Meater® series 12, MN, USA) were measured on day 110 of gestation and 28 days postpartum. Sows feed intake was registered throughout the experiment. Health status of the sows and their litters were regularly assessed and recorded by the presence of any abnormal signs, such as reduced appetite, fever, mastitis and other signs caused by postpartum dysgalactia syndrome. Piglets were weighted at 1, 21 and 28 days of life and the number of weaned piglets and pre-weaning mortality was registered. Post-weaning estrus and its date were recorded when occurring.

Fecal samples (200 g) from each sow were daily collected (at 09:00 am) between day 21 and 25 of lactation. Samples of each sow were pooled, mixed and a representative sample was dried and grinded. Blood samples from each sow were obtained by caudal venipuncture into 10-mL vacutainer tubes (BD Vacutainer®, lithium heparin, BD-Plymouth, UK) free of detectable Zn within 48h postpartum and on day 21. Plasma was obtained by centrifugation (2,000 x *g*, 10 min, 15ºC) of blood samples. On day 21 of lactation, milk samples were individually collected (at 11:00 am) from the teats of one mammary chain (30-50 mL) from all sows. Fecal, plasma and milk samples were kept frozen at -20 ºC until analyzes were performed.

Chemical analysis

Feed, fecal, colostrum, milk and hair samples were digested with concentrated nitric acid (HNO_3, 68 %) in a microwave oven (model MARSXpress, CEM GmbH, Kamp-Lintfort, Germany). Feed samples (0.5 g) were digested with 5 mL of HNO_3 at 180 ºC for 45 minutes; fecal samples (0.25 g) were digested with 5 mL of HNO_3 and 2 mL of H_2O_2 at 190 ºC for 15 minutes followed by 205 ºC for 30 minutes. Colostrum and milk samples 0.7-1 g were digested with 3 mL of HNO_3 in polytetrafluoroethylene (PTFE) reactor at 90 ºC for 48 hours. Hair samples were washed with hexane and the aqueous solution of Triton X 0.1 %. Afterwards sub samples of 50-100 mg were digested with 3 mL of HNO_3 and 2 mL of H_2O_2 in PTFE reactor, heating at 90 ºC overnight. All digested samples were diluted with 33 mL of deionized water. Plasma samples were diluted with HNO_3 1 %.

Feed and fecal samples were analyzed for Ca, P, Zn, Fe and Cu whereas plasma, colostrum and milk samples were analyzed for Zn, Fe and Cu and hair samples were analyzed for Zn content. All analyzes were performed by inductive coupled plasma optical spectrophotometer (ICP-OES model Optima 4300DV, PerkinElmer Inc., Waltham, USA).

Ti content of feed and fecal samples from Trial 1 were colorimetrically determined after acid extraction of marker from ash, following the method proposed by Short et al., (1996). Apparent total tract digestibility was calculated for Ca, P and Zn following the marker (Ti) ratio method. In-feed phytase activities (FTU/kg) were determined by the internal, validated method of the producer (method B-074).

Statistical analysis

Data from experiment 1 were analyzed by ANOVA using SAS 9.2 and including treatment, parity and its interaction into the statistical model. Sows were categorized according to parity number (group 0= nulliparous and 1^{st} parity sows; group 1= 2^{nd}, 3^{rd} and 4^{th} parity sows; group 2= 5^{th}, 6^{th} and 7^{th} parity sows) before analyses to get a balanced distribution among treatments. Sow was used as the experimental unit. For experiment 2, each descriptive variable (sow morphological and reproductive parameters) and mineral composition of colostrum, milk and hair was analyzed by ANOVA (SAS Institute Inc., Cary, NC). Only the parity number was included in the model as a classification factor. Mineral component values were transformed to logarithmic values to reach normality. Multiple mean comparisons were done by Tukey's correction. The alpha level for significance was 0.05.

3.3. Results

Experiment 1

In Table 3.2. performance parameters of the sow are shown by parity number. Sows BW at 107 d of gestation increased gradually from the first to the seventh parity number. Sows of the first parity number showed lower BW than sows from the 5th, 7th, 8th and 9th parity number ($P < 0.05$). No differences were observed for backfat thickness across different parity numbers by remaining constant around 14-15 mm ($P > 0.10$).

Table 3.2. Average sow performance of sows according to their parity number. Experiment 1.

		n	Sow BW d107 gest.	Sow BF	Piglets born total /sow	Stillborn Piglets /sow	Piglets born alive /sow	Piglets nursed /sow
			kg	mm				
Parity number	**1**	15	235[b]	14.1	15.1	1.20[ab]	13.5	12.4[a]
	2	10	253[ab]	13.7	14.8	0.30[b]	14.2	12.0[a]
	3	10	260[ab]	14.1	15.0	0.20[b]	14.3	12.0[a]
	4	10	275[ab]	14.1	13.3	0.60[b]	12.5	12.2[a]
	5	10	294[a]	16.2	14.5	0.30[b]	14.1	11.1[b]
	6	10	279[ab]	14.2	16.2	1.60[ab]	14.3	10.0[c]
	7	10	297[a]	14.8	12.6	0.90[ab]	11.6	10.0[c]
	8	10	290[a]	16.4	14.6	3.10[a]	11.5	10.0[c]
	9	5	293[a]	15.6	12.2	1.20[ab]	10.2	10.0[c]
	10	5	289[ab]	11.0	15.0	3.20[a]	11.6	10.0[c]
SEM			16.1	2.1	1.4	0.77	1.3	0.12
P-value			<0.001	0.380	0.247	0.002	0.045	<0.001

[a,b,c] Means in the same row with different superscripts differ significantly ($P < 0.05$).

The number of piglets born alive was constant along the different parity numbers except for the oldest sows, which showed a lower number of piglets born alive ($P < 0.05$). The number of stillborn piglets was significantly greater ($P < 0.05$) in older sows of 8th and 10th but not in those of 9th parity number compared to sows at 2nd, 3rd, 4th and 5th parity number. In contrast, no differences were observed in the number of mummified piglets (data not shown) and total number of piglets among parity numbers. The number of piglets nursed by sow was different as a management rule of the farm. Sows from the first to the forth parity grew 12 piglets, sows at the fifth parity grew 11 piglets and sows from the sixth to the tenth parity grew 10 piglets.

In Table 3.3. the colostrum, milk and piglets hair trace mineral concentrations are presented by parity number of the sows. The Fe, Cu and Zn colostrum levels were highly variable and no differences ($P > 0.05$) were detected among different parity numbers. No differences ($P > 0.05$) were detected in Fe and Zn milk levels however, Zn concentration in milk tended ($P < 0.10$) to be higher in some older sows (5th vs. 8th parity number, 5.34 vs. 7.12 mg/kg, $P = 0.051$) and Zn and Fe milk concentration variability was much higher in sows of 6th, 7th, 8th 9th and 10th parity number due to some extreme high values (data not shown).

Table 3.3. Average content of trace minerals (Zn, Fe, Cu) in colostrum and milk and Zn in hair samples of piglets depending on the sows parity number. Experiment 1.

		Zn (mg/kg)			Fe (mg/kg)		Cu (mg/kg)	
		Colostrum	Milk	Piglet hair	Colostrum	Milk	Colostrum	Milk
	n	95	94	81	95	94	95	94
Parity number	1	15.8	6.22	206	1.63	1.03	4.77	1.23[b]
	2	14.7	6.02	212	1.40	0.79	3.39	1.21[b]
	3	16.3	5.89	205	1.79	0.80	3.36	1.24[ab]
	4	16.3	6.02	197	1.55	0.93	3.93	1.28[ab]
	5	14.8	5.34	217	1.55	0.73	3.44	1.20[b]
	6	15.1	5.57	203	1.03	1.19	3.82	1.34[ab]
	7	14.1	6.66	211	1.36	1.24	3.14	1.54[a]
	8	18.2	7.12	210	1.48	1.01	4.44	1.45[ab]
	9	16.6	5.87	212	1.36	1.14	4.11	1.48[ab]
	10	18.6	6.68	222	1.47	1.18	4.38	1.57[a]
	SEM	1.23	0.310	6.69	0.131	0.123	0.371	0.062
	P-value	0.729	0.071	0.677	0.065	0.198	0.284	0.001

[a,b] Means in the same row with different superscripts differ significantly ($P < 0.05$)

Milk Cu concentration was higher in sows of seventh and tenth parity as compared to those of the first, second and fifth parity number (1.54 and 1.57 vs. 1.23, 1.21 and 1.20 mg/kg, $P < 0.05$).

Hair Zn concentration was not different in piglets from sows of different parity numbers. A high individual variation of Zn in piglet hair was observed particularly in those coming from 10th parity sows.

Experiment 2

The effects of dietary mineral and phytase content on sow and piglet performance are presented in Table 3.4. Sows BW on day 110 of gestation and on day 28 of lactation were different due to their parity number ($P < 0.001$) with youngest sows (group 0) being lighter than sows from parity group 1 and 2 (232 vs 284 and 296 kg on day 110 of gestation; and 187 vs. 232 and 251 kg on d 28 of lactation). Lactation ADFI was not affected by dietary treatment however it was affected by parity number with lower ADFI in youngest sows compared to oldest (4.73 vs. 5.11 kg; $P = 0.031$). Weaning-to-estrus interval and piglet performance were not affected by either the treatment or the parity number but youngest sows (group 0) tended to have a longer interval compared to older sows ($P = 0.06$). The interaction between treatment and parity number was not significant in any of the reproductive performance parameter including sows body condition score and sows backfat thickness measured on d 110 of gestation and d 28 postpartum (data not shown).

Table 3.4. Reproductive performance of the sows and litter performance as affected by dietary mineral content and phytase supplementation. Experiment 2.

		n	Sow BW d110 gest.	Sow BW d28	ADFI 0-28d	Weaning to estrus	Pigs weaned /sow	Piglet BW d0	Piglet BW d28
			kg	kg	kg	d		kg	kg
Treatments	**Rec**	9	273	219	4.93	6.00	11.1	1.67	7.36
	Low	10	269	225	4.89	9.17	11.7	1.60	6.83
	Low 250	11	272	225	4.94	6.00	10.6	1.68	7.70
	Low 500	11	269	224	5.03	6.44	10.8	1.70	7.30
Parity groups	**0**	13	232^{B}	187^{B}	4.73^{B}	8.71	11.4	1.61	7.12
	1	27	284^{A}	232^{A}	5.01^{AB}	6.00	11.0	1.66	7.02
	2	11	296^{A}	251^{A}	5.11^{A}	6.00	10.8	1.72	7.74
SEM			9.6	8.7	0.111	1.107	0.40	0.122	0.341
P-values	**Treat**		0.989	0.954	0.821	0.089	0.283	0.943	0.335
	Parity		<0.001	<0.001	0.024	0.055	0.502	0.778	0.181
	T*P		0.919	0.676	0.508	0.067	0.741	0.903	0.890

Experimental treatments: Rec: recommended Ca and P in-feed levels (Ca=0.95% Total P total=0.65%, digestible P 0.31 %). **Low:** low Ca and P in-feed levels (Ca=0.6% Total P total=0.4%, digestible P 0.2%). **Low250:** Low diet supplemented with 250 FTU of Phytase/kg of feed. **Low500:** Low diet supplemented with 500 FTU of Phytase/kg of feed. **Parity Groups**: **0:** sows at 0 and 1st parity number; **1:** sows at 2nd, 3rd and 4th parity number; **2:** sows at 5th, 6th and 7th parity number. $^{abc, AB,}$ Values in the same column with different letter are significantly different ($P < 0.05$).

The effects of dietary mineral and phytase content on mineral intake, absorption and excretion are presented in Table 3.5. Sows fed diets with recommended mineral content (Rec) had a higher Ca, P and Zn intake and fecal excretion compared to treatments fed reduced mineral diets ($P < 0.001$). Digested P during the whole lactation was higher ($P < 0.001$) in sows of the Rec treatment (343 g) than in those of the other 3 dietary treatments (Low, Low250 and Low500; 194, 248 and 261 g, respectively). Sows of the phytase treatments digested more P than those fed the unsupplemented Low diet (248 and 261 vs. 194, $P < 0.05$). Sows fed Low500 diet showed a higher P digestibility compared to those fed Rec and Low diets (46.3 vs 38.4 and 36.9 %, respectively; $P < 0.05$) and sows of Low250 treatment showed an intermediate P digestibility compared to other treatments (44.6 %). None of the variables presented were affected by parity number or treatment and parity interaction ($P > 0.10$). No effects between treatments were measured in respect to sows Ca digestibility or total excreted Ca amount. The reduced Zn level of Low diets reduced the total fecal Zn excretion compared to that of sows fed Rec diet ($P < 0.05$) with lowest Zn excretion of sows fed Low250 diet.

Table 3.5. Effect of dietary Ca and P levels and phytase levels on the mineral intake (Int), fecal excretion (Exc), digestibility (Dig) and calculated amount of digested P, Ca and Zn of sows. Experiment 2.

		Ca				P				Zn			
		Int	**Exc**	**Dig**	**Dig Ca 0-28**	**Int**	**Exc**	**Dig**	**Dig P 0-28**	**Int**	**Exc**	**Dig**	**Dig Zn 0-28**
		g/kg	g/kg	%	g	g/kg	g/kg	%	g	g/kg	g/kg	%	g
Experimental treatments	**Rec**	9.74	8.15^{a}	16.4	222	6.47	3.98^{a}	38.4^{b}	343^{a}	121	106^{a}	12.2	2.03^{a}
	Low	6.70	5.56^{b}	17.4	196	3.85	2.43^{b}	36.9^{b}	194^{c}	81.0	73.1^{b}	9.39	1.04^{b}
	Low 250	6.60	5.48^{b}	17.0	187	4.02	2.22^{b}	44.6^{ab}	248^{b}	75.5	71.8^{b}	4.39	0.46^{c}
	Low 500	6.56	5.21^{b}	20.6	192	4.00	2.15^{b}	46.3^{a}	261^{b}	82.4	74.8^{b}	8.79	1.02^{b}
Parity groups	**0**		6.38	13.4	177		2.73	40.2	245		82.8	6.72	1.10
	1		5.94	19.9	212		2.72	41.1	259		80.4	9.48	1.14
	2		5.96	20.2	209		2.63	43.4	280		80.7	9.87	1.17
SEM			0.276	3.72	40.5		0.099	2.10	13.6		3.25	5.043	0.023
P-values	**Treat**		<0.001	0.827	0.868		<0.001	0.005	<0.001		<0.001	0.702	<0.001
	Parity		0.303	0.226	0.692		0.684	0.480	0.143		0.783	0.848	0.170
	T*P		0.651	0.573	0.779		0.288	0.207	0.146		0.632	0.780	0.716

Experimental treatments: Rec: recommended Ca and P in-feed levels (Ca=0.95% Total P total=0.65%, digestible P 0.31 %). **Low:** low Ca and P in-feed levels (Ca=0.6% Total P total=0.4%, digestible P 0.2%). **Low250:** Low diet supplemented with 250 FTU of Phytase/kg of feed. **Low500:** Low diet supplemented with 500 FTU of Phytase/kg of feed. **Parity Groups**: **0:** sows at 0 and 1st parity number; **1:** sows at 2nd, 3rd and 4th parity number; **2:** sows at 5th, 6th and 7th parity number. ^{abc} Values in the same column with different letter are significantly different (P <0.05).

The effects of dietary treatments on plasma and milk Zn, Fe and Cu concentrations are presented in Table 3.6. None of the dietary treatments changed Zn, Fe and Cu concentrations in plasma and milk. Parity number did not affect any trace mineral concentration in plasma and milk except for Cu that was higher in plasma directly after farrowing (d0) in youngest sows (group 0) compared to oldest sows (group 2; 2.00 vs.1.57 mg/L; P = 0.003).

Table 3.6. Effect of Ca, P and phytase levels on the plasma and milk concentration of trace minerals (Zn, Fe and Cu) at different days after farrowing. Experiment 2.

		Zn (mg/L)			Fe (mg/L)			Cu (mg/L)		
		Plasma d0	Plasma d21	Milk	Plasma d0	Plasma d21	Milk	Plasma d0	Plasma d21	Milk
Experimental treatments	Rec	0.68	0.79	7.50	2.25	1.11	1.03	1.81	1.72	1.43
	Low	0.68	0.81	6.96	2.77	1.10	0.86	1.64	1.60	1.43
	Low 250	0.69	0.80	6.99	2.04	1.27	1.29	1.83	1.57	1.33
	Low 500	0.66	0.69	7.56	2.50	1.03	1.11	1.82	1.67	1.44
Parity groups	0	0.66	0.82	8.15	2.11	1.17	1.21	2.00^{A}	1.76	1.31
	1	0.66	0.80	6.93	2.03	1.08	1.06	1.75^{AB}	1.66	1.46
	2	0.71	0.70	6.68	3.02	1.12	0.94	1.57^{B}	1.50	1.46
SEM		0.107	0.083	0.710	0.472	0.114	0.221	0.098	0.075	0.097
P-values	Treat	0.989	0.597	0.852	0.626	0.445	0.442	0.455	0.430	0.759
	Parity	0.862	0.461	0.138	0.096	0.662	0.598	0.004	0.063	0.222
	T*P	0.540	0.933	0.995	0.552	0.408	0.777	0.429	0.433	0.416

Experimental treatments: Rec: recommended Ca and P in-feed levels (Ca=0.95% Total P total=0.65%, digestible P 0.31 %). **Low:** low Ca and P in-feed levels (Ca=0.6% Total P total=0.4%, digestible P 0.2%). **Low250:** Low diet supplemented with 250 FTU of Phytase/kg of feed. **Low500:** Low diet supplemented with 500 FTU of Phytase/kg of feed. **Parity Group**: **0:** sows at 0 and 1st parity number; **1:** sows at 2nd, 3rd and 4th parity number; **2:** sows at 5th, 6th and 7th parity number. ^{AB,} Values in the same row with different letter are significantly different ($P < 0.05$).

3.4. Discussion

For the present studies we hypothesized that the dietary phytase application or the sow's parity number could affect the Zn concentration in colostrum and milk. However, we were not able to observe significant differences on these parameters. The results confirms that sows regulate the Zn secretion in colostrum and milk (McCormick et al., 2014), at least for the Zn concentrations of the diets tested herein.

For experiment 1 we hypothesized that parity number of sows could have an effect on the reproductive performance of the animals as well as on the trace mineral content in colostrum and milk. The evolution of the reproductive performance parameters along different parity numbers was as described by Andres et al., (2008) for a sow population and similar to further literature until the 6th parity (Peters and Mahan, 2008). Sow BW increased with advancing parity until the seventh parity and then remained fairly constant until the tenth parity. On average the number of stillborn piglets remained constant until the seventh parity and then increased. Older sows had increased probability of stillbirth. The association between higher parities and stillbirth risk has been attributed to a poor uterine muscle tone leading to less efficient expulsion and prolonged farrowing (Cozler et al., 2002; Vanderhaeghe et al., 2013).

The average concentrations of Fe, Cu and Zn in colostrum and milk were similar to those found by other authors (Park et al., 1994; Csapo et al., 1996; Peters et al., 2010). The results

of the present study did not show statistically differences in the trace mineral content of colostrum and milk among groups of sow with different parity number, except for the Cu concentration in milk. However, if the statistical analysis is performed between young (1^{st} – 5^{th} parity) and old (6^{th} – 10^{th} parity) sows, differences appeared. Milk Fe concentration (young vs. old, 4.37 vs. 6.02 mg/kg; $P = 0.012$) and also milk Zn concentration (young vs. old, 30.0 vs. 32.8 mg/kg; $P = 0.041$), then was found of being higher in old compared to young sows and the differences in the Cu content in milk were more distinct ($P < 0.001$) than in the more differentiated overall analysis. We are not aware of any study presenting a similar evolution in the content of Zn, Fe and Cu in sow milk at different parity number. A explanation of these changes could be that a higher Fe, Cu and Zn milk level likely reflect the presence of some subclinical mammary gland infection in some sows, especially the older ones (6^{th}-10^{th} parity). We have not found any articles assessing the trace mineral levels in milk of sows with any mastitis indicators (somatic cell count, lactoferrin), but a correlation has been reported for other species such as sheeps, cows or goats (Burriel and Heys, 1997; Chaneton et al., 2008; Cheng et al., 2008). Another possible explanation for higher trace mineral levels in old sows could be the different number of piglets kept with the mothers during lactation in young sows (12 piglets /sow) compared to older sows (10 piglets/ sow). The reason of this common practice is that older sows are known to have less milk production and poorer quality of the milk likely associated with a lower number of available teats.

Hair Zn concentration reflects the Zn status of the animals in the longterm and changes of dietary Zn levels (Miller et al., 1966; Reinhold et al., 1968; Miller, 1970; Deeming and Weber, 1977; Combs et al., 1983; Combs, 1987; Bobilya et al., 1994).The mean zinc concentration in piglets hair (209 ppm) was in accordance to other studies in piglets (Bobilya et al., 1994) or in humans (Barrie et al., 1987; Medeiros et al., 1987) and was not related to different parity numbers of the sows. These results appear to confirm no differences on the mineral status of suckling piglets as depending on the age or parity number of their mothers.

Results of experiment 2 showed that supplementing microbial phytase derived from a modified *E.coli* to low P and Ca lactation diets improved P digestibility in lactating sows. No phytase effect on Ca and Zn digestibility, the reproductive performance, or on the milk trace mineral contents were found among treatments. Other authors (Jongbloed et al., 2004; Grela et al., 2011) have shown higher Ca, P, K, Na, Cu and Zn digestibilities in lactating sows when animals were eating higher phytase levels during both, the gestation and lactation period and in studies with a greater number of animals. Interestingly, Jongbloed et al., (2004) found an improved fecal Zn digestibility at higher phytase levels (10,000 FTU/kg feed),

although lower digestibility values were registered compared to the present study. In the present study we decreased the dietary Ca levels in order to improve phytate solubility and facilitate the phytase activity in the digestive tract (Pond and Jones, 1964; Wood and Zheng, 1997). We hypothesized that hydrolyzing the phytate-mineral complex, phytase would be able to increase Zn absorption. In contrast, Miller et al., (2013) recently demonstrated that higher Ca levels may increase total Zn absorption when included in high phytate diets. The mechanism described by Miller et al., (2013) suggest that high levels of Ca in the gut may form mineral complexes with phytate in competition with Zn, thus increasing total Zn release and absorption. In our experiment, the Ca levels ranged from high (9.74 g/kg) to low Ca levels (6.56-6.7 g/kg) in diets containing a high content of phytate (2.45-2.48 g/kg) as associated with the high levels of wheat bran and rapeseed meal in the diet. It is remarkable that Zn digestibility values were very low (from 4.39 to 12.2%), but not significant differences were detected likely due to the low number of replicates.

The absence of differences on reproductive performance could be associated with the small changes observed on mineral digestibility but also to the short period of dietary administration. Grela et al. (2010) reported a higher number of live born piglets and higher piglets BW on d 21 and 28 of lactation in the phytase supplemented diets (500 FTU/kg diet) compared to the non-supplemented treatment when phytase was administered during the whole gestation and lactation period.

Dietary factors did not affect the mineral status of lactating sows, as measured by mineral plasma concentration. This is in contrast to previous work in which the content of minerals in sow blood, especially Ca, Cu, Fe, P and Zn, was clearly affected by a longer (gestation and lactation) dietary supplementation with microbial phytase (Czech and Grela, 2004). This observation allowed them to conclude that using microbial phytase (500 FTU/kg) in sow diets might increase not only the P availability but also release other minerals (Ca, Mg, Fe, Zn) from complexes with phytate that have reduced solubility under conditions of the small intestine (Xu et al., 1992). These results are in accordance to in-vitro results (Maenz et al., 1999).

Colostrum and milk Zn, Fe and Cu concentration were not affected by dietary phytase supplementation, that is in contrast to other trial results (Grela et al., 2010). It is remarkable that Zn milk content was high (7.25 mg/L) when compared to levels analyzed for Fe (1.07 mg/L) and Cu (1.41 mg/L). Suggesting that the mammary gland mobilizes a relevant amount of Zn from maternal body circulation then secreted into milk. Because the concentration of Fe, Cu and especially for Zn in plasma (0.77, 1.07 and 1.41 mg/L, respectively) was lower

than in milk, this suggest that the mammary gland provides active transporting and regulation mechanisms for some trace minerals. Recent results Matte et al., (2014) support this hypothesis. Currently, 24 Zn transporters were identified and many of them have been preliminary characterized as Zn transport across membranes (McMahon and Cousins, 1998 a; Kambe et al., 2004; Eide, 2006).

Moreover, Zn, Fe and Cu concentrations in human milk seem to be independent of the maternal mineral status (Domellöf et al., 2004) and milk Zn and Fe concentrations are not correlated to maternal dietary intake (Hannan et al., 2009). Milk Zn level is maintained over a wide range in dietary Zn intake and most studies performed in humans have failed to show a positive effect of Zn supplementation on milk Zn level despite increased plasma Zn levels indicating that the regulation of milk Zn secretion is tightly controlled by an active transport through the mammary gland epithelium (Kelleher and Lönnerdal, 2005). More interestingly, some studies showed an inverse relationship between milk and plasma Zn concentration in woman from countries with problems of Zn deficiency and in rats with marginal Zn intake (Kelleher and Lönnerdal, 2005) however, this tendency was not seen in the current experiment with sows.

Nutrient requirements of sows increase during late gestation and lactation due to additional accretion of fetus and milk production particularly in high-producing sows having large litter size (Mahan et al., 2009). During lactation the amount of Zn secreted into milk is almost twice the amount of Zn transferred daily across the placenta to the fetus during pregnancy, as assumed by King, (2002) based on the mammary gland Zn transport. The consequences of this large amount of Zn transferred may be a progressive decrease on total Zn pool/reserves of the sow. Mahan and Newton, (1995) showed that sows of high parity number and those weaning heavy litters may show a lower body mineral content as compared to non-gravid gilts, with this decline particularly evident for Ca, P and Zn. In our second experiment we estimated an average daily Zn secretion of 66.8 mg/d based on the daily milk production (average daily litter BW gain x 4) and the milk Zn content. This value was higher than the daily amount of Zn absorbed in the whole-digestive tract (41.1 mg/d) as calculated from the feed intake and Zn apparent total tract digestibility values, confirming that during lactation sows were exposed to a mobilization of their Zn reserves. However, considering the results obtained in our first experiment with high parity sows, it is possible that sows may be able to replete Zn reserves during pregnancy when Zn adequate diets are provided.

3.5. Conclusion

High-producing sows secrete large amounts of Zn through milk during lactation regardless the parity number of the sow. Phytase application to low P and Ca diets improved apparent total tract digestibility of P, but this result was not associated to a higher Zn digestibility or higher levels of Zn in milk.

3.6. Acknowledgements

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Chapter 4

Effect of weaning and in-feed high doses of zinc oxide on zinc levels in different body compartments of piglets

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Accepted

Abstract

High doses of Zn are widely used for prevention and treatment of diarrhoea in weaning piglets; however, the mechanism of action of Zn against diarrhoea is still not well understood. The objective of this study was to evaluate whether weaning induces Zn deficiency in piglets. Eight litters of primiparous sows were selected for the experiment, and 3 piglets presenting similar weights were selected within each litter. Two of the three selected piglets from each litter were weaned at 21d of age and fed two different diets: a commercial control diet (WCt) and the same diet plus 2,000 ppm of Zn as ZnO (WZn). The third selected pig from each litter was kept unweaned (Uw) with the sow and the rest of the litter. All 24 selected animals were killed at 28 d of age, and blood, gastrointestinal content, liver, pancreas and spleen were sampled for Zn, Fe and Cu analysis (mg/kg or L of sample). Data were analysed using ANOVA including treatment as a fixed factor. Weaned pigs fed WCt diet presented a lower Zn concentration in plasma than Uw animals (0.76 ± 0.091 vs. 1.10 ± 0.099 mg/L, $p = 0.05$). Zinc levels in liver, pancreas and spleen were not affected by weaning. Total concentration of Zn was higher in gastrointestinal contents of weaned animals fed WCt diet than in Uw pigs ($p < 0.001$ for stomach, jejunum, ileum, caecum and colon). Supplementation with high doses of ZnO increased levels of Zn in gastrointestinal content ($p < 0.001$), liver ($p < 0.001$) and pancreas ($p < 0.001$) compared to WCt diet. It also increased plasma Zn to non-deficient levels (1.32 ± 0.086), but the increase was not as marked as in other locations and final concentration was not different than that in Uw animals ($p = 0.231$). Weaning creates a Zn deficiency situation in weaned pigs as observed by plasma Zn concentrations. ZnO supplementation counteracts Zn deficiency.

4.1. Introduction

Use of in-feed pharmacological levels of zinc (Zn, 2,000–2,500 ppm) as zinc oxide (ZnO) is widely accepted in pig industry for prevention and treatment of diarrhoea in early weaned pigs, especially in some EU countries after the antibiotic growth promoter ban in 2006 (European Commission, 2010). However, this practice has raised concerns about its environmental impact and it has to be eventually reduced (Monteiro et al., 2010). The use of Zn for diarrhoea control is mainly empirical, and understanding its mode of action would provide important information to find alternatives or reduce the high doses currently used. For many years, high in-feed doses of ZnO were perceived as an antimicrobial treatment that could decrease enterotoxigenic E. coli colonization and bacterial population in the gastrointestinal tract (Fairbrother et al., 2005). However, some studies indicate that 2,500–3,000 mg/kg of dietary ZnO does not always modify or even may increase E. coli population in the GIT (Hojberg et al., 2005; Molist et al., 2011). Alternatively, other hypotheses have proposed that Zn may improve the intestinal barrier function (Li et al., 2001), avoid bacterial adhesion to the epithelium (Roselli et al., 2003) and modulate the expression of genes encoding various stress response proteins (heat, inflammation, infection, oxidative stress) in enterocytes (Sargeant et al., 2011). These new hypothesis raise the role of Zn as a nutrient involved in many and encourage the search for dietary alternatives to provide effective levels of Zn at the target tissues. Zinc, usually provided as Zn sulphate, is also the only treatment recommended by the WHO for diarrhea in children in developing countries in combination with oral rehydration solution (WHO/UNICEF, 2004). Zn deficiency is so far one of the main hypotheses considered in humans (WHO, 2005), and the same hypothesis has been brought up recently for dogs (Panda et al., 2009). However, this hypothesis has not been explored in piglets. Transitory Zn deficiency, likely associated with anorexia, and major losses of Zn during the first days after weaning are hypothesized to occur to different extent after weaning. In this study, we hypothesize that weaning will induce lower Zn levels in piglets compared to not weaned littermates, and high in-feed doses of Zn will be required to counteract this lower Zn level. Thus, the objective of this experiment was to study how Zn concentrations are modified in plasma and different organs in littermates when unweaned or weaned to nutritional or therapeutic doses of dietary ZnO one week after weaning. Concentrations of iron (Fe) and copper (Cu) in plasma and different organs were also studied to assess whether similar decreases are to be expected in other microminerals after weaning or whether interactions (Hill et al., 1983 a) among different minerals apply at different dietary doses of Zn.

4.2. Materials and methods

This experiment received prior approval from the Animal Protocol Review Committee of the institution. The treatment, management, housing, husbandry and slaughtering conditions conformed to the European Union Guidelines (Council of the European Communities, 1986).

Animals and experimental design

Eight litters of primiparous sows were selected for the experiment from a commercial farm (Les Feixes S.A., Les Masies de Voltregà, Spain), and three piglets (Large White x Landrace) presenting similar weights (7.02 ± 0.693 kg) were selected within each litter at 21 days of age (total of 24 piglets mixed males and females). Two of the three selected piglets from each litter were weaned and transported to the Animal Facilities of the Universitat Autònoma de Barcelona and were allocated, based on sow origin and body weight, into eight pens (two animals/pen). Pens were allotted to two dietary treatments (four replicates/ treatment): a commercial control diet (WCt; Table 4.1) formulated to meet or exceed weaning pig nutrient requirements (NRC, 1998) and the same control diet with 2,000 ppm of Zn as ZnO added (WZn).

Table 4.1. Composition and characteristics of the basal diet (as-fed basis) Composition of the basal diet on an as-fed basis and analysed nutrient content of the diet on a DM basis.

Ingredients	g/kg
Corn Flakes	360
Wheat Flakes	240
Full fat extruded soybeans	110
Egg meal	92.7
Sweet whey powder	150
Soybean oil	16.5
Calcium carbonate	5.6
Dicalcium Phosphate	8.1
L-Lysine-HCl	4.6
DL-Methionine	0.5
L-Threonine	1.1
L-Tryptophan	0.9
Salt	4.0
Vitamin-Mineral Premix (*)	6.0
Analytical characteristics (†)	%
Dry matter (DM)	91.1
Ash	5.12
Crude protein (CP)	19.6
Lysine, calculated value	1.52

* Supplied per kilogram of feed: 10,200 IU of vitamin A, 2,100 IU of vitamin D as cholecalciferol, 39,9 mg of vitamin E, 3 mg of vitamin K_3, 2 mg of vitamin B_1, 3 mg of vitamin B_2, 3 mg of vitamin B_6, 0.025 mg of vitamin B_{12}, 20 mg of Pantotenate calcic, 60 mg of Nicotinic acid, 0.1 mg of biotin, 0.5 mg of Folic acid, 150 mg of Fe as iron sulphate, 156 mg of Cu as copper sulphate, 0.5 mg of Co, 120 mg of Zn as zinc oxide, 49.8 mg of Mn as manganese oxide, 2 mg of I, 0.3 mg of Se as sodium selenite. † Analysed according to the following procedures: DM, drying to constant weight at 103ºC; Ash, muffle furnace (550ºC, 8h); CP, Kjeldahl method.

The third selected pig from each litter was kept unweaned (Uw) with the sow and the rest of the litter. No antibiotics were added to the experimental diets. The experimental period lasted one week. Weaned animals had ad libitum access to experimental feed and water. Unweaned animals had ad libitum access to water but not feed. Feed intake and presence of diarrhoea, described as watery faeces, were recorded daily in weaned animals. Animals were individually weighted at the beginning and at the end of the experimental period (28 days of age). Room temperature was maintained at 25 ± 3 °C. At the end of the experimental period, pigs were slaughtered by an overdose of sodium pentobarbital (Dolethal, Vetoquinol, S.A., Lure, France) and exsanguination.

Sample collection and analysis

Just before slaughter, blood samples from each pig were obtained by puncture of the jugular vein into 10-ml heparinized vacutainer tubes (BD Vacutainer_, lithium heparin, BD-Plymouth, UK) free of detectable Zn. Plasma was obtained by centrifugation (2,000 x *g*, 10 min) of blood samples and was frozen at -20 °C. The remainder red and white blood cells were also kept at -20 °C until analysis. At slaughter, the gastrointestinal tract (GIT), liver, pancreas and spleen were excised from the animal and weighted (not pancreas). Then, a sample of stomach, jejunum, ileum, caecum and colon contents and a portion of liver, pancreas and spleen from each pig were sampled and stored at -20 °C until analysis was performed. Feed, GIT content, blood cells, liver, pancreas and spleen samples were digested with concentrated nitric acid (HNO_3, 68%) in a microwave oven (model MARSXpress, CEM GmbH, Kamp-Lintfort, Germany). For feed samples, 0.5 g was digested with 5 ml of HNO_3 at 180 °C for 45 minutes; for GIT samples, 0.25 g was digested with 5 ml of HNO_3 and 2 ml of H_2O_2 at 190 °C for 15 minutes and 205 °C for 30 minutes; for liver samples, 0.25 g was digested with 7 ml of HNO_3 and 2 ml of milli-Q H_2O at 175 °C for 40 minutes; and for pancreas and spleen, 0.25 g was digested with 10 ml of HNO_3 at 190 °C for 45 minutes. Plasma samples were diluted with HNO_3 1%. All samples were analysed for Zn, Fe and Cu by inductively coupled plasma optical spectrophotometer (ICP-OES model Optima 4300DV, PerkinElmer Inc., Waltham, MA, USA) except spleen and pancreas samples that were analysed only for Zn content.

Statistical analysis

Data were analysed by ANOVA using SAS 9.2 including treatment as a fixed factor. Animal was used as the experimental unit to account for the litter effect. Concentrations of Zn in intestinal tract were transformed to logarithm before analysis to reach normality. Significance was determined using an alpha level of 0.05. Trend was discussed using an alpha level of 0.10. Multiple mean separations were performed using Tukey′s correction.

4.3. Results

As expected, a higher average daily gain was reached in unweaned animals compared to weaned animals (254 vs. 136 g/pig/day, p = 0.029). No differences due to the diet were found in average daily gain or average daily feed intake (189 g/pig/day) between weaned animals. None of the animals presented diarrhoea. Concentration of Zn in diets was 380 and 2270 mg/kg of DM for control and ZnO diets respectively. Concentration of Fe and Cu in diets was 320 and 140 mg/kg of DM. Table 4.2 presents Zn, Fe and Cu concentrations in plasma and blood cells.

Table 4.2. Zinc, iron, and copper concentrations (mg/L) in plasma and blood cells.

	Unweaned	Weaned	Weaned + ZnO	SEM*	P value
Plasma					
Zn	1.10[a]	0.76[b]	1.32[a]	0.099	0.001
Fe	2.04	1.60	1.38	0.385	0.438
Cu	1.97	1.79	1.86	0.127	0.578
Blood Cells					
Zn	3.28[b]	3.53[ab]	4.13[a]	0.234	0.034
Fe	501	529	549	26.8	0.417
Cu	1.16[a]	0.97[b]	1.00[ab]	0.050	0.029

Values are LSmeans, n = 8 piglets in each experimental group. Values in the same row not followed by the same letter differ significantly.
*SEM= standard error of the mean.

Weaned pigs fed WCt diet presented a lower Zn concentration in plasma than unweaned animals (p = 0.05). Weaned animals fed in-feed high doses of Zn had a higher plasma Zn concentration than control animals (p = 0.001) and similar to those in unweaned animals. No differences were observed for Fe and Cu plasma concentrations between treatments. Weaned animals presented Zn blood cell levels similar to unweaned animals (p = 0.726). However, the addition of high doses of ZnO increased blood cell Zn concentration (p = 0.036) compared to unweaned animals. Unweaned animals also showed higher levels of

blood cell Cu than weaned controls ($p = 0.033$) and tended to have higher levels of Cu than ZnO-treated animals ($p = 0.068$). Concentrations of Zn, Fe and Cu in liver and concentrations of Zn in pancreas and spleen are presented in Table 4.3.

Table 4.3. Zinc, iron, and copper concentrations (mg/kg) in liver, and zinc concentration in pancreas, and spleen.

	Unweaned	Weaned	Weaned + ZnO	SEM*	P value
Liver					
Zn	67.0[b]	69.5[b]	330.8[a]	18.4	<0.001
Fe	153	173	156	35.7	0.896
Cu	50.6	48.5	40.1	7.79	0.545
Pancreas Zn	48.7[b]	35.2[b]	130.9[a]	15.54	<0.001
Spleen Zn	22.1	22.7	22.7	0.738	0.801

Values are LSmeans, n = 8 piglets in each experimental group. Values in the same row not followed by the same letter differ significantly.
*SEM= standard error of the mean.

No differences were found in the fresh weight of these organs among treatment groups (data not shown). Levels of Zn in liver, pancreas and spleen were not affected by weaning. However, weaned animals supplemented with high doses of ZnO showed higher liver Zn concentrations compared to control ($p < 0.001$) and unweaned animals ($p < 0.001$) and higher pancreas Zn concentration compared to control diet ($p < 0.001$) and unweaned animals ($p = 0.002$). Zn concentration in spleen was not affected by in-feed high Zn levels. No differences due to the treatments were found in Fe and Cu concentrations in liver. Concentration of Zn, Fe and Cu in stomach, jejunum, ileum, caecum and colon sections were higher in many cases in weaned control animals than in unweaned animals: Zn was higher in stomach ($p < 0.001$), jejunum ($p < 0.001$), ileum ($p < 0.017$), caecum ($p = 0.001$) and colon ($p < 0.001$); Fe was also higher in stomach ($p < 0.001$) and jejunum ($p = 0.029$); and Cu was increased in stomach ($p < 0.001$), jejunum ($p = 0.016$), ileum ($p = 0.015$) and colon ($p = 0.004$) compared to unweaned animals (Table 4.4).

Table 4.4. Zinc, iron, and copper concentrations (mg/kg) in stomach, jejunum, ileum, caecum and colon contents.

	Unweaned	Weaned	Weaned + ZnO	SEM*	P value
Stomach					
Zn	11.2^{c}	59.8^{b}	304.3^{a}	42.07	<0.001
Fe	24.0^{b}	69.5^{a}	66.6^{a}	5.51	<0.001
Cu	4.20^{b}	25.4^{a}	23.7^{a}	2.51	<0.001
Jejunum					
Zn	7.49^{c}	74.0^{b}	330.0^{a}	35.99	<0.001
Fe	11.9^{b}	51.6^{a}	48.1^{a}	9.05	0.007
Cu	4.6^{b}	22.3^{a}	17.6^{ab}	3.801	0.007
Ileum					
Zn	42^{c}	165^{b}	598^{a}	114.6	<0.001
Fe	125	92	74	32.8	0.502
Cu	18.0^{b}	51.0^{a}	34.1^{ab}	8.02	0.019
Caecum					
Zn	57^{c}	253^{b}	1012^{a}	121.2	<0.001
Fe	199	214	176	49.5	0.530
Cu	43.5	97.7	74.6	13.92	0.073
Colon					
Zn	92^{c}	471^{b}	2141^{a}	266.2	<0.001
Fe	337	220	209	76.6	0.919
Cu	63^{b}	195^{a}	162^{a}	27.6	0.007

Values are LSmeans, n = 8 piglets in each experimental group. Values in the same row not followed by the same letter differ significantly.
*SEM= standard error of the mean.

Animals supplemented with high doses of ZnO also presented higher levels of Fe and Cu compared to unweaned animals in stomach ($p < 0.001$ and $p < 0.001$, respectively, for Fe and Cu), jejunum ($p = 0.048$ for Fe) and colon ($p = 0.030$ for Cu). Concerning Zn, animals treated with high doses of ZnO had higher levels compared to both control and unweaned animals in stomach, jejunum, ileum, caecum and colon ($p < 0.001$ in all cases). No significant differences were observed for the Fe and Cu concentration in digesta between both weaning treatments.

4.4. Discussion

In the present article, we explored the hypothesis that a transitory Zn deficiency may occur in early weaning pigs. This possibility has been previously explored by (Carlson et al., 2007 a) comparing pre- and post-weaning Zn concentrations in plasma and mucosa, and no differences were found. However, this hypothesis has not been explored comparing weaned and unweaned piglets of the same age. In the present study, we found that Zn concentration in plasma was lower in weaned animals fed an adequate Zn diet as compared to unweaned littermates or weaned with therapeutic doses of ZnO. No similar decreases were observed for

other microminerals, such as Fe and Cu. Assessment of Zn status is not easy because Zn is involved in many vital functions and very little is known about its metabolism. No single measure is suitable to accurately assess the Zn status of an individual (Wood, 2000). Plasma Zn has limitations when applied to individuals in some situations, for example, when different pathologies are present (Arsenault et al., 2011). Parameters like Zn in different organs, alkaline phosphatase or metallothionein in different tissues may be more adequate or complementary to plasma Zn levels (Lowe et al., 2009). However, plasma Zn is considered a useful biomarker for Zn status in human populations (Brown et al., 2004; Hess et al., 2007; Lowe et al., 2009) and it has been used to relate diarrhoea to Zn deficiency (Bahl et al., 1998; Walker et al., 2009). Human plasma Zn concentrations under 0.65–0.70 mg/L are usually considered deficient (Maret and Sandstead, 2006). In the present experiment, plasma Zn concentrations were 0.76 mg/L in weaned pigs compared to 1.10 mg/L in unweaned littermates, but no diarrhoea was observed, which confirms that a lower plasma Zn concentration may be observed after weaning independently of diarrhoea. A lower level may be expected in more unfavourable conditions. In fact, plasma levels of 0.65 mg/L or less can be easily found in literature for weaning pigs (Carlson et al., 1999; Revy et al., 2006; Pieper et al., 2012) and are often observed in our laboratory in pigs fed non-deficient diets. The occurrence of diarrhea in relation to plasma levels of Zn should be studied at a population level in pigs as it is done for humans to check whether plasma Zn levels are related to diarrhoea occurrence. Therapeutic ZnO treatment did restore plasma Zn levels to those observed in unweaned animals. It is interesting to remark that plasma Zn concentrations above 1 mg/L are not reached with supplementation of diets with levels up to 1,000 ppm, and it is necessary to use levels of 2,000–2,500 ppm of Zn as ZnO to reach Zn plasma levels above 1 mg/L (Carlson et al., 2007 a). On the other hand, plasma Zn is clearly affected by early weaning and it drops to deficient levels on day 3 after weaning even with therapeutic doses of in-feed ZnO (Davin et al., unpublished data). This drop may be related to the lower Zn input induced by anorexia. However, pig anorexia has been also related to metabolic acidosis (Cersosimo et al., 1987), which is a metabolic condition related to a temporary increase in the urinary excretion of cations (Adeva and Souto, 2011). Thus, high in-feed levels of ZnO may be necessary to counteract this situation. The drop in Zn concentrations observed in plasma due to weaning was not paralleled in blood cells, liver, pancreas or spleen. In fact, liver and pancreas results showed the importance of these organs as Zn reservoirs. Literature in how Zn deficiency affects Zn concentrations in tissues normally refers to long deficiency periods with severe Zn deficiency (Prasad et al., 1969; Burch et al.,

1975). However, which tissue would be first affected under acute deficiency is not well known. Intracellular concentrations of Zn may be prioritized given the important functions Zn play on normal function of cells. On the other hand, Zn concentration in liver and pancreas of pigs fed on the high in-feed doses of ZnO was increased 4- to 5-fold compared to non-treated animals. Pancreas, along with liver and kidney, are characterized by high Zn turnover when high doses are administered (Sheline et al., 1943). The movement of Zn between plasma and liver compartments is known to be fast, and complete exchange of tracer 65-Zn occurs in less than 2 days in humans and in rats (Grungreiff, 2002). Thus, it seems that the liver is able to manage and store high amounts of dietary Zn, while the pancreas is considered the main way of excretion of Zn back to GIT as a way to regulate Zn homoeostasis (De Lisle et al., 1996). This huge increase in Zn in liver and pancreas also indicates that high doses of dietary Zn clearly increase absorption. Hess et al., (2007) indicated that body Zn can be divided into 2 metabolic pools: a rapid exchangeable pool with fast turnover and a slow pool with a slower turnover. The rapid pool, composed of the most metabolically active forms of Zn, includes plasma and extracellular fluid in liver, pancreatic, kidney and intestinal tissue (Ballatori, 1991; Faa et al., 2008). How overloading this rapid pool could affect diarrhoea and how an overload of the slow pool could midterm be released to the blood should be better studied. On the other hand, the convenience of such long-term high Zn dose may be discussed because liver accumulation may be even higher for longer periods and such levels of Zn may be considered toxic (Brink et al., 1959). Concerning intestinal content, it is relevant that the concentration of all three studied minerals was very low in unweaned animals when compared to the weaned pigs. These differences make sense because weaned animals are fed on supplemented inorganic mineral diets compared to the milk where Zn, Fe and Cu are mainly present in the milk lipid fraction bound to fat globule membrane or to xanthine oxidoreductase, associated with whey proteins (mainly lactoferrin or albumin), bound to casein or associated with a low-molecular-weight compound (Hunt and Nielsen, 2009). Concentrations of Zn, Fe and Cu in sow's milk can be found in literature (Peters et al., 2010) and have been measured by our research group in different trials with the following mean values: 6 mg Zn/kg, 1 mg Fe/kg and 1.2 mg Cu/kg of fresh milk with 20% DM, at the fourth week of lactation. If we assume that lactating piglets could be consuming around 1 kg of milk the last day of experiment (Marshall et al., 2006; Thodberg and Sørensen, 2006), we can calculate that Uw animals consumed over 6 mg Zn/d compared to 72 and 429 mg Zn/d for treatments Wct and WZn respectively. Thus, it is relevant that weaning on adequate Zn diets (Wct) induced remarkable increases in the mineral Zn content

in digesta, but these changes were not able to prevent a decrease in the plasma Zn and did not affect Zn concentration in different organs. An overload of dietary Zn promoted a Zn concentration up to 600 mg/L in ileum digesta and up to 2140 mg/L in colonic digesta, which may have effects on microbial populations (Sawai, 2003). Previous reports have suggested that therapeutic doses of Zn decrease Cu bioavailability as an enhanced Zn uptake by mucosal cells stimulates the synthesis of metallothioneins, which bind Cu in the intestinal wall (Revy et al., 2004). However, in the present study, we did not show any interaction between Zn and Cu in digesta or in any of the analysed main organs at doses is compatible with previously described interactions (Hill et al., 1983 a). We also showed no interactions between Zn and Fe in digesta or in any of the analysed main organs. In conclusion, in the present experiment, we show that early weaning of pigs induces a drop in plasma Zn concentrations, which was independent of diarrhea and close to the deficient levels referred for other species, such as children. This drop can be counteracted by high doses of dietary ZnO. Moreover, different concentrations of Zn found in liver, spleen and pancreas of supplemented animals indicate that pigs may accumulate Zn in some organs at high levels. Unknown effects at this concentration or a midterm release to blood should be considered in further studies. Plasma Zn concentrations in diarrhoea should be also studied at a population level to clarify what happens in practical piglet barns.

4.5. Acknowledgements

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Chapter 5

Evolution of zinc, iron, and copper concentrations along the gastrointestinal tract of piglets weaned with or without in-feed high doses of zinc oxide compared to unweaned littermates

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Abstract

High doses of Zn are used to treat diarrhea in weaning pigs but are also an environmental concern. Mechanism of action of Zn against diarrhea is still not well understood. The amount of solubilized Zn, the relation of Zn with Fe and Cu, and the concentration of these elements in the gastrointestinal tract (GIT) are key data to understand its mechanism of action and optimize its use. Eight litters were used and 3 piglets were selected within each litter. Two piglets from each litter were weaned at 21d of age and fed 2 different diets; a commercial control diet (WCt), and the same diet + 2,000ppm of Zn as ZnO (WZn). The third pig was kept unweaned (Uw) with the rest of the litter. All 24 selected pigs were killed at 28d of age and GIT contents were sampled. Soluble and insoluble factions of the GIT content were separated, and Zn, Fe, and Cu concentrations were analyzed. Soluble fraction of the GIT content represented 20 to 50% less of the GIT content in Uw pigs than in weaned pigs. Concentration of Zn increased 3-5 fold along the GIT for weaned pigs fed WZn compared to WCt and Uw pigs ($P < 0.01$ in all cases). Percentage of solubilized Zn was 4-10 folds higher in jejunum, ileum, and cecum of Uw pigs than in those weaned ($P < 0.01$ in all cases). Concentration of Fe in the soluble fraction was higher for Uw pigs compared to weaned ones along the GIT ($P < 0.05$ in all cases) even when concentration in total content was lower for Uw pigs in stomach ($P = 0.001$) and jejunum ($P = 0.029$). Concentrations of Cu were lower in Uw pigs than in weaned ones along the GIT ($P < 0.05$ in all cases). Surprisingly, animals on WZn showed a 5-10 fold increase of Cu solubilized in distal parts of the GIT (cecum and colon, $P < 0.001$) compared to other groups. Differences in Zn, Fe, and Cu concentrations found among treatments will be very useful in future studies understanding ZnO mechanism of action and optimizing its use in order to avoid contamination.

5.1. Introduction

High doses of ZnO in the diet are used for prevention and treatment of diarrhea in weaning pigs but the mechanism of action of Zn against diarrhea is still not well understood. Different hypothesis have been proposed, such as protective effects on enterocytes against bacteria (Roselli et al., 2003), or modulation of genes encoding for stress response proteins in enterocytes (Sargeant et al., 2011). In vitro studies of the mechanism involved in the anti-diarrhea effect of Zn have been very informative so far. However, detailed information about real concentrations obtained with ZnO treatments in the gastrointestinal tract (GIT) of the pigs, especially in the soluble fraction, is lacking. There is no either detailed information about the concentrations of Zn in the GIT of unweaned pigs. In the present study, we performed a detailed description of the Zn, Fe, and Cu concentrations in the GIT content of unweaned and weaned pigs, the later treated or not with therapeutic doses of ZnO.

5.2. Materials and methods

This experiment received approval from the Animal Protocol Review Committee of the Universitat Autònoma de Barcelona. The treatment, management, husbandry, and slaughtering conformed to the European Union Guidelines (Council of the European Communities, 1986).

Experimental Design and Analyses

Eight litters were selected from a commercial farm (Les Feixes S.A., Les Masies de Voltregà, Spain) and three piglets (Large White × Landrace) were selected within each litter at 21 days of age (7.02 ± 0.693 kg). Two of the three selected piglets from each litter were weaned and transported to the experimental farm at the Universitat Autònoma de Barcelona and were allocated into eight pens (two animals/pen) on the basis of the sow origin. Pens were allotted to two dietary treatments (4 replicates/treatment): a commercial control diet (WCt) containing 19.6% CP and 1.52% Lys (cereal, 60%; extruded soy, 11%; egg meal, 9%; sweet whey powder, 15%; as fed basis) and WCt with 2,000ppm of Zn as ZnO added (WZn). Analyzed concentrations of Zn, Fe, and Cu in WCt were 120.0 mg of Zn, 150.0 mg of Fe, and 15.6 mg of Cu per kg of feed, as-fed basis. Pigs had *ad libitum* access to feed and water and no antimicrobial substance was added to the diets. The third selected pig from each litter was kept unweaned (Uw) with the sow and the rest of the litter and had *ad libitum* access to water. Room temperature was maintained at 25 ± 3 ºC. At the end of the experimental period pigs were slaughtered by an overdose of sodium pentobarbital (Dolethal, Vetoquinol, S.A., Lure, France). Stomach, jejunum, ileum, cecum, and colon contents were sampled and stored at -20

ºC. Samples were ultra-centrifuged (18,000 x *g*, 1 h, 20 ºC). The supernatant was filtered (45mm) to obtain the soluble fraction. Soluble and insoluble fractions were digested with concentrated HNO_3 in a microwave oven (MARSXpress, CEM GmbH, Kamp-Lintfort, Germany) and were analyzed for Zn, Fe, and Cu by inductive coupled plasma optical spectrophotometer (Optima 4300DV, PerkinElmer Inc., Waltham, MA).

Calculations and Statistical Analysis

Concentrations of Zn, Fe, and Cu in soluble and insoluble fractions were summed in order to obtain concentration in total GIT content. The percentage of total Zn, Fe, and Cu present in the soluble fraction was also calculated. Data was analyzed by ANOVA using SAS 9.2 including treatment as a fixed factor. Concentrations were logarithm transformed before analysis when required in order to reach normality. Alpha level for determination of significance was 0.05 and trend was discussed with an alpha of 0.10. Multiple mean comparisons were done by Tukey´s correction.

5.3. Results

Weaning increased soluble fraction (fresh matter) in stomach (39.8 vs. 70.6 %, SEM = 6.35; $P = 0.004$), jejunum (67.1 vs. 81.4 %, SEM = 4.44; $P = 0.045$), cecum (35.8 vs. 60.2 %, SEM = 6.27; $P = 0.022$), and colon digesta (7.28 vs. 41.9%, SEM = 6.86; $P = 0.003$), but not in ileum digesta (59.1 ± 23.76 %). Therapeutic doses of ZnO did not affect percentage of soluble fraction. Data concerning Zn concentrations in total GIT content are shown in Table 5.1. Data concerning Fe and Cu concentrations in total GIT content is shown in this section. Weaning increased Zn concentrations along the GIT ($P < 0.001$ in all cases) and therapeutic doses of ZnO increased Zn concentrations along GIT compared to non-treated pigs ($P < 0.001$ in all cases except for ileum where $P = 0.02$). Weaning also increased Fe concentrations in stomach (26.1 vs. 69.5 SEM = 6.08; $P < 0.001$), and jejunum (10.9 vs. 51.6 SEM = 11.70; $P = 0.029$), but not in ileum (94.5 ± 80.40), cecum (196 ± 121.3), and colon (244 ± 171.2). Weaning increased Cu concentrations in stomach (3.93 vs. 25.4 SEM = 2.82; $P < 0.001$), jejunum (3.59 vs. 22.3 SEM = 4.90; $P = 0.016$), ileum (18.0 vs. 51.0 SEM = 8.02; $P = 0.022$), cecum (43.5 vs. 97.7 SEM = 13.92; $P = 0.073$), and colon (62.8 vs. 195.1 SEM = 27.59; $P = 0.007$). Therapeutic doses of ZnO did not affect Fe or Cu concentrations in total GIT content compared to pigs on diet WCt.

Weaning increased soluble Zn concentration in stomach ($P = 0.002$) but decreased percentage of Zn solubilized in jejunum, ileum, and cecum ($P < 0.01$ in all cases). Therapeutic ZnO

increased soluble Zn concentrations along the GIT ($P < 0.001$ in all cases) with no modification of the percentage of Zn solubilized. Weaning also decreased soluble Fe concentrations in stomach ($P = 0.017$), ileum ($P = 0.046$), cecum ($P = 0.038$), and colon ($P = 0.008$) and percentage of Fe solubilized in stomach ($P = 0.040$) and jejunum ($P = 0.001$). Therapeutic doses of ZnO did not affect soluble Fe concentrations compared to pigs on diet WCt. Soluble Cu concentration was also increased by weaning in stomach ($P = 0.025$), and ileum ($P = 0.007$). Therapeutic doses of ZnO did not affect Cu soluble concentrations in these locations but increased soluble Cu concentration and percentage of solubilized Cu in cecum ($P = 0.007$ and < 0.001) and colon ($P = 0.020$ and <0.001) compared to WCt.

Table 5.1. Zinc concentration in total gastrointestinal content and Zn, Fe, and Cu concentration (and percentage) in the soluble fraction of gastrointestinal content.

Item	Unweaned	Weaned	Weaned+ZnO	SEM*	P value
Zn total content, mg/kg					
Stomach	11.3c	59.8b	304a	47.34	<0.001
Jejunum	7.62c	74.0b	329a	46.60	<0.001
Ileum	42.4c	165b	598a	114.6	<0.001
Cecum	56.9c	253b	1012a	121.2	<0.001
Colon	92.7c	471b	2141a	266.2	<0.001
Zn soluble fraction, mg/L					
Stomach	10.6^{c} (38.7)	28.5^{b} (37.0)	143.2^{a} (43.9)	12.89 (10.69)	< 0.001 (0.840)
Jejunum	5.5^{b} (44.5^{a})	4.1^{b} (6.5^{b})	10.2^{a} (3.2^{b})	1.59 (3.36)	0.003 (< 0.001)
Ileum	20.0^{b} (27.6^{a})	6.6^{b} (2.9^{b})	56.8^{a} (10.9^{ab})	12.00 (5.08)	0.002 (0.006)
Cecum	6.4^{ab} (6.25^{a})	2.5^{b} (0.61^{b})	11.1^{a} (0.68^{b})	2.31 (1.054)	0.005 (< 0.001)
Colon	4.3^{b} (0.35)	3.4^{b} (0.38)	13.1^{a} (0.34)	2.12 (0.112)	0.002 (0.954)
Fe soluble fraction, mg/L					
Stomach	4.06^{a} (7.25^{a})	1.43^{b} (1.44^{b})	2.06^{ab} (2.22^{ab})	0.673 (1.708)	0.020 (0.039)
Jejunum	3.03 (21.8^{a})	1.81 (2.72^{b})	1.14 (2.92^{b})	0.604 (3.527)	0.063 (< 0.001)
Ileum	14.4^{a} (11.9)	5.56^{b} (4.35)	3.43^{b} (11.30)	2.570 (3.900)	0.013 (0.256)
Cecum	16.12^{a} (3.96)	3.63^{b} (1.05)	2.55^{b} (1.12)	3.538 (0.923)	0.018 (0.050)
Colon	20.01^{a} (1.10)	8.84^{b} (1.68)	6.24^{b} (1.45)	2.549 (0.350)	0.002 (0.442)
Cu soluble fraction, mg/L					
Stomach	1.00^{b} (11.0)	4.35^{a} (13.1)	3.98^{a} (15.4)	0.907 (6.072)	0.023 (0.851)
Jejunum	1.88 (33.1)	9.91 (31.8)	6.04 (31.4)	3.006 (7.728)	0.114 (0.984)
Ileum	10.8^{b} (35.4)	36.4^{a} (42.4)	22.1^{ab} (53.7)	5.561 (8.591)	0.008 (0.279)
Cecum	2.77^{b} (3.65^{b})	5.86^{b} (3.63^{b})	20.01^{a} (16.31^{a})	3.317 (2.279)	0.001 (< 0.001)
Colon	5.74^{b} (0.65^{b})	8.14^{b} (1.57^{b})	50.22^{a} (13.31^{a})	12.521 (1.447)	0.011 (< 0.001)

^{abc} Within a row, means without a common superscript differ ($P < 0.05$).

[1] Zn, Fe, and Cu concentration was measured in soluble and insoluble fractions of the intestinal content as wet basis. Total concentration of Zn, Fe, and Cu was obtained by adding concentrations obtained for each fraction. Percentage of Zn, Fe, and Cu solubilized was obtained as the proportion of the total concentration found in the soluble fraction. Percentages are shown in parentheses. Values are LSmeans, n=8 piglets in each experimental group.

5.4. Discussion

There is a large body of literature on ZnO use in weaning pig diarrhea. However, some important gaps were found while unraveling its mechanisms of action. In particular, no detailed information was found about concentrations of Zn in soluble fractions of each intestinal compartment and about concentrations in unweaned pigs. Concentrations in the GIT due to ZnO treatment ranged from 300 to 2,000 mg/L. Thus intestinal bacteria and cells are exposed to big variations that may greatly affect their metabolism. Soluble fraction is thought to be the most important for mineral absorption and antimicrobial effects. Concentrations of minerals in this fraction were very low, maximum 10 mg/L, except for stomach, where low pH solubilizes Zn at a large extent, and for ileum in pigs fed WZn. Authors cannot provide any explanation for the higher concentration found in ileum. It is of special interest the effect that ZnO treatment produced in Cu solubility in the hindgut. Data from organ mineral content of the same pigs published in Davin et al. (2012) actually showed a slight increase in Cu in some cellular compartments, such as blood cells.

In conclusion, data shows detailed information on how Zn, Fe, and Cu concentrations in the GIT tract change between weaned and unweaned, and treated and untreated pigs in order to be used in future studies clarifying ZnO effects on diarrhea.

5.5. Acknowledgments

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Chapter 6

Effect of different Zn sources and posologies on serum Zn concentration in weaning pigs

Abstract

Zinc is used to prevent and treat diarrhea in piglets and children. However, while Zn is administered in children as sulfate at relatively low levels and deficiency is the main hypothesis, high levels of Zn are fed in piglets as Zn oxide and different hypothesis are considered. Two experiments were designed to assess how Zn plasma/serum concentration changes when different levels, sources and posologies of Zn are administered to piglets at weaning. In experiment 1, two hundred and eighty piglets presenting similar weights were selected at 28 d of age and distributed to seven treatments: a commercial control diet (Ct) and the same diet plus 100 ppm of Zn as ZnO (100ZnO) or $ZnSO_4$ (100ZnS), 250 ppm of $ZnSO_4$ (250ZnS), 700 ppm of $ZnSO_4$ (700ZnS), and 2,500 ppm of Zn as ZnO (2500ZnO). An extra treatment with 100ZnO diet and fasting for 24h after weaning was included (FAST). In experiment 2, one hundred and sixty piglets were selected at the same age and distributed to six treatments: $ZnSO_4$ tablet (20 mg Zn/day) given to previously fasted (fTb) or non-fasted (Tb) pigs, in-feed 250 ppm of $ZnSO_4$ (ZnS), in-feed 2,500 ppm of Zn as ZnO (ZnO), and 2 negative controls, one fasted (fCt) and one non-fasted (Ct). Blood samples from four animals per treatment were obtained pre-weaning and on d 1, 2, 3 and 5 post-weaning. Liver and blood was also obtained on d 7 from four animals per treatment in experiment 2. All samples were analyzed for Zn, Fe and Cu. In both experiments Zn serum concentrations after weaning decreased to levels considered deficient in humans (< 0.65 mg/L) and in both experiments 2,500 ppm ZnO was the only treatment able to increase Zn serum concentration back to physiological levels as soon as d 3 or d 7 post-weaning, respectively. Liver Zn concentration was also greater with 2,500 ppm of ZnO than with the rest of Zn supplemented or fortified treatments. Weaning creates a Zn deficiency measured by serum Zn concentrations and none of the Zn treatments in this study were effective to restore Zn serum levels in weaned piglets except for pharmacological levels of ZnO.

6.1. Introduction

Zn is essential for normal growth and development in mammals due to its critical role in catalytic, structural and regulatory processes (Suttle, 2010). It is a component of more than 300 enzymes and an even greater number of other proteins, which emphasizes its indispensable role for health (Plum et al., 2010).

The recommended dietary Zn level for weaned pigs is 100 mg/kg of feed or 26.6 mg/day (between 5-7 kg of BW) and 46.8 mg/day (between 7-11 kg of BW) (NRC, 2012) and although the upper EU limit is 150 mg/kg when used as an in-feed additive, levels up to 3,000 mg/kg of zinc oxide (ZnO) are widely used as a medicine to prevent and treat post-weaning diarrhea (Hahn and Baker, 1993; Case and Carlson, 2002; Hu et al., 2012) and improve performance (Sales, 2013). The mechanism of action of Zn against diarrhea in pigs is still not well understood and different authors have suggested different hypotheses: influence on the development of the gut microbiota (Hojberg et al., 2005; Vahjen et al., 2010, 2011; Pieper et al., 2012; Starke et al., 2013, 2014), improvement of the intestinal barrier function (Li et al., 2001; Sturniolo et al., 2002; Canani et al., 2005; Carlson et al., 2008; Liu et al., 2011), modulation of the inflammatory response (Sargeant et al., 2011) and more recently, reversion of Zn deficiency situation (Davin et al., 2013).

In humans, Zn (as $ZnSO_4$) is the only treatment recommended by the WHO for diarrhea in children in developing countries (WHO/UNICEF, 2004). In contrast to pigs, a single daily dose of 10-20 mg of Zn as $ZnSO_4$ dispersed with oral rehydration solution (ORS) is administered to children in fasting conditions and Zn deficiency is so far the main hypothesis considered to explain the effects of Zn on treating and preventing diarrhea (WHO, 2005).

Plasma or serum Zn concentration is the only biochemical indicator recommended by the WHO, UNICEF, International Atomic Energy Agency and the International Zinc Nutrition Consultative Group (IZiNCG) for the assessment of population Zn status (Hess et al., 2007) and likewise the European Micronutrient Recommendations Aligned Network of Excellence concluded that plasma/serum Zn concentration is the only valid biomarker to measure Zn status in both Zn-supplemented and Zn-depleted individuals (Lowe et al., 2009). Zn in serum represents <0.2% of total Zn in the body and together with Zn in the liver, extracellular fluid, pancreas, kidney and intestinal tissue, compose the rapid exchangeable metabolic pool (approximately 10% of whole-body Zn) representing the most metabolically active Zn compartment. In contrast, skeletal muscle and bone are the primary components of the slow pool, which represents nearly 90% of the whole-body Zn (Hess et al., 2007).

In a recent article, Davin et al. (2013) showed that weaning provokes a decrease in Zn status when un-weaned pigs were compared to weaned littermates one week after weaning but no change was observed in animals that were receiving high amounts of Zn as ZnO, moreover Fe and Cu status were not decreased and was not different between different treatments. The use of different Zn levels or sources, apart from in-feed pharmacological ZnO concentrations, to prevent and treat post-weaning diarrhea in pigs has rarely been discussed.

The objective of the experiments presented was to assess how Zn plasma/serum concentration changes depending on different Zn levels and sources at weaning with special interest on different posologies of $ZnSO_4$. We hypothesized that feeding piglets with nutritional levels of $ZnSO_4$ in alternative posologies will bring serum Zn levels back to pre-weaning levels after weaning. Concentrations of iron (Fe) and copper (Cu) in plasma/serum and liver were also studied to assess whether similar decreases are to be expected in other microminerals after weaning or whether interactions among different minerals apply at different dietary doses and posologies of Zn.

6.2. Material and methods

The two experiments received approval from the Animal Protocol Review Committee of the Universitat Autònoma de Barcelona. The treatment, management, housing, and husbandry conformed to the European Union Guidelines (European Parliament, 2010).

Facilities and diets

The experiments here presented were carried out in a commercial lactation-weaning facility (Collsuri, Manlleu, Spain) with reported cases of diarrhea when therapeutic ZnO was removed from the diet. A commercial basal diet minimizing Zn content (Ct) was formulated to meet or exceed weaning pig nutrient requirements (NRC, 2012) except for Zn. The same ingredients normally used in this farm were used (cereal, 61%; extruded soy, 19%; sweet whey powder, 8%; fishmeal, 5%; as fed basis). Calculated composition of the diet was 19% CP, 1.4% Lys, and 33 mg Zn, 140 mg Fe and 20 mg Cu per kg of feed, as-fed basis. The calculated phytate/Zn molar ratio of basal diet was 3.54. No antimicrobial substance was added to the experimental diets (Table 6.1.).

Table 6.1. Composition of the basal diet on an as-fed basis and analyzed nutrient content of the diet on a DM basis

Ingredient composition	**g/kg**
Corn	282.0
Barley, 2 rows	170.0
Wheat grain soft	160.0
Full fat extruded soybean	154.0
Soybean meal 44% CP	32.7
Sweet whey powder	75.0
Fish meal	50.0
Soybean oil	20.0
Animal plasma 80% CP	15.0
Monocalcium Phosphate	9.50
Calcium carbonate	9.20
L-Lysine HCl, 78 %	4.90
DL-Methionine, 99 %	2.30
L-Threonine	3.30
L-Tryptophan	6.40
Salt	3.60
Vitamin-Mineral Premix(*)	2.00
Nutrient composition (**)	**%**
Dry matter (DM)	90.3
Ash	5.65
Crude protein (CP)	19.7
Lysine, calculated value	1.46
Zn, mg/kg	140

(*)Supplied per kilogram of feed: 13,000 IU of vitamin A, 1.800 IU of vitamin D as cholecalciferol, 45 IU of vitamin E, 2.1 mg of vitamin K_3, 1.7 mg of vitamin B1, 5 mg of vitamin B2, 2.5 mg of vitamin B6, 0.028 mg of vitamin B12, 15 mg of pantothenate calcium, 27.5 mg of nicotinic acid, 0.11 mg of biotin, 0.6 mg of folic acid, 10 mg of Cu as copper sulphate, 45 of Mn as manganese oxide, 0.7 mg of I, 0.3 mg of Se as sodium selenite.
(**) Analyzed according to the following procedures: DM, drying to constant weight at 103ºC; Ash, muffle furnace (550ºC, 8h); CP, Kjeldahl method.

Experiment 1

Thirty-one litters were used for this experiment. One pig (Large White x Landrace) was selected randomly from each litter and was bled 1 d before weaning. Two-hundred and eighty pigs were randomly selected at weaning (28 ± 1 d of age; BW = 7.52 ± 0.973 kg) and allocated to 28 pens (10 animals/pen). Pens were allotted to 7 treatments (4 pens/treatment). Treatments used in the experiment were diet Ct as it was or supplemented based on calculations to reach: the NRC recommended level of Zn for post-weaning pigs from 5 to 11 kg live weight (100mg/kg of feed) as either ZnO (100ZnO) or $ZnSO_4$ (100ZnS); the dose of $ZnSO_4$ recommended by the WHO for diarrhea treatment in humans (20 mg Zn/day, assuming a daily consumption of 100 g of feed; 250 mg/kg of feed, 250ZnS); the maximum

dose of $ZnSO_4$ (700 mg/kg of feed) not producing rejection in pigs (700ZnS); and the therapeutic dose of Zn (2,500mg/kg of feed) as ZnO (2,500ZnO) normally used in pig production to prevent diarrhea. An extra treatment was added using diet 100ZnO but animals were fasted for 24h immediately after weaning (FAST). Animals had *ad libitum* access to feed and water. Room temperature was maintained at 25 ± 4 ºC.

One animal from each pen was bled on d 1, 2, 3 and 5 after weaning. Each animal was bled only once during the experimental period. Blood samples from each pig were obtained by puncture of the jugular vein into 10-mL vacutainer® tubes (BD Vacutainer®, Z, BD-Plymouth, UK) free of detectable Zn.

Experiment 2

Twenty-four litters from the same commercial lactation-weaning facility (Collsuri, Manlleu, Spain) were used for this experiment. One pig (Large White x Landrace) was selected randomly from each litter and was bled 1 d before weaning. One-hundred and sixty pigs were randomly selected at weaning (28 ± 1 d of age; BW= 7.96 ± 1.089) and randomly allocated to 16 pens (10 animals/pen).

Treatments used in this experiment were the $ZnSO_4$ tablets recommended by WHO in humans for diarrhea prevention (ZinCfant, Nutriset, Malaunay, France) given to previously fasted (fTb) or not fasted (Tb) pigs, the equivalent theoretical tablet dose of $ZnSO_4$ but included in the feed (ZnS), the therapeutic in-feed ZnO treatment normally used in piglets (ZnO), and 2 negative controls, one fasted (fCt) and one not fasted (Ct).Treatments were distributed to pens as follows. Treatments ZnS and ZnO were assigned to 4 replicates each. Treatments fTb and Tb and treatments fCt and Ct were assigned in pairs to 4 replicates. Thus, there were 4 pens with fasted pigs including 5 pigs in treatment fTb and 5 pigs in treatment fCt, and 4 pens with pigs fed as libitum including 5 pigs in treatment Tb and 5 pigs in treatment Ct each.

Fasting was applied by removing the feeder each day at 6:00am, and ZinCfant® tablets were administered to the corresponding pigs at 10:00am by individual oral administration. $ZnSO_4$ tablet dose used was the same as that of a 6-months old child dose for diarrhea treatment considering similar BW (20 mg Zn/day) resulting in a daily oral dose of 4 $ZnSO_4$ tablets per pig, each containing 5 mg of Zn as $ZnSO_4$. All four tables were diluted in 10 mL of saline (B. Braun Melsungen AG, Melsungen, Germany). The solution was then administered by a plastic syringe with an oral cannula to each pig. Controls received an oral dose of 10 mL of saline

through the same method. Saline was administered to controls with a different plastic syringe to avoid cross-contamination.

One animal per pen per treatment (n=4) was bled on d 1, 2, 3, 5 and 7 after weaning. Each animal was bled only once during the experimental period. Blood samples from each pig were obtained by puncture of the jugular vein into 10-ml vacutainer® tubes (BD Vacutainer®, Z, BD-Plymouth, UK) free of detectable Zn. At the end of the experimental period, one animal per pen per treatment (n=4) was slaughtered by an overdose of sodium pentobarbital (Dolethal, Vetoquinol, S.A., Lure, France) and exsanguination. Liver was excised from the animal, sampled and stored at -20ºC until analysis was performed.

Sample analysis.

Serum was obtained by centrifugation (2,000 x *g*, 10 min, 15ºC) of blood samples, frozen at -20ºC and kept at this temperature until analyses were performed. Serum samples were diluted with HNO_3 1%. Liver samples were digested with concentrated nitric acid (HNO_3 68%) at 175ºC for 40 minutes in a microwave oven (model MARSXpress, CEM GmbH, Kamp-Lintfort, Germany). All samples were analyzed for Cu, Fe, and Zn content by inductively coupled plasma optical spectrophotometer (ICP-OES model Optima 4300DV, PerkinElmer Inc., Waltham, MA, USA).

Statistical analysis

Data from each experiment were analyzed separately using SAS 9.2 (SAS Institute, Cary, NC). Serum Zn, Fe, and Cu concentrations after weaning were analyzed by ANOVA including treatment, day of extraction and their interaction as fixed factors whereas for liver Zn, Fe and Cu, treatment was included as fixed factor. Serum Zn, Fe, and Cu concentrations on d 1, 2, 3, 5, (and 7 in exp. 2) after weaning from Ct diet were compared to pre-weaning concentrations by ANOVA using only day of extraction as fixed factor. Tukey's correction was used for multiple mean comparisons. Pen was used as the experimental unit in all analyses. Alpha used for determination of significance was 0.05. Interactions presenting p-values below 0.15 where further studied for multiple mean comparisons as recommended by Lowry (1992) for this kind of arrangements.

6.3. Results

Analyzed concentrations of Zn, Fe, and Cu in the Ct diet were 140 mg of Zn, 230 mg of Fe and 26 mg of Cu per kg of feed, as-fed basis.

Experiment 1

Serum Zn concentration before weaning was 0.819 ± 0.2119 mg/L and was reduced on d 1 (0.673 mg/L; $P = 0.005$), d 2 (0.526 mg/L; $P < 0.001$), and d 5 (0.596 mg/L; $P < 0.001$). Serum Fe and Cu concentrations before weaning (1.43 ± 0.656 and 1.69 ± 0.319 mg/L, respectively) were not different to post-weaning levels. Zn concentration after weaning showed an interaction effect of treatment depending on the day ($P < 0.001$; Table 6.2.). On d 1 and d 2, no differences among the dietary treatments were recorded. On days 3 and 5, pigs fed diet 2500ZnO showed higher serum Zn level than all other treatments (d 3, $P < 0.05$, d 5, $P < 0.01$), except for 100ZnS on d 3. Serum Fe (1.32 ± 0.565 mg/L) concentrations were not affected by treatment but a positive trend ($P = 0.07$) was reported depending on age from d 1 to d 5 (1.07, 1.31, 1.43 and 1.46 mg/L). Serum Cu (1.66 ± 0.292 mg/L) concentrations were affected by extraction day ($P = 0.024$) being higher on d 1 compared to d 2 (1.75 vs. 1.52 mg/L, $P = 0.015$) but not compared to d 3 and d 5 (1.68 and 1.67 mg/L, respectively; $P > 0.05$).

Table 6.2. Serum Zn concentrations after weaning (mg/L). Exp 1. (n=4).

	Day 1	Day 2	Day 3	Day 5
Ct	0.63 ^{cd}	0.43 ^{d}	0.61 ^{cd}	0.60 ^{cd}
FAST	0.75 ^{abc}	0.60 ^{cd}	0.66 ^{cd}	0.54 ^{cd}
100ZnS	0.56 ^{cd}	0.45 ^{d}	0.69 ^{bcd}	0.65 ^{cd}
250ZnS	0.77 ^{abc}	0.56 ^{cd}	0.68 ^{cd}	0.62 ^{cd}
700ZnS	0.67 ^{cd}	0.53 ^{cd}	0.53 ^{cd}	0.63 ^{cd}
100ZnO	0.65 ^{cd}	0.59 ^{cd}	0.55 ^{cd}	0.50 ^{cd}
2500ZnO	0.67 ^{cd}	0.70 ^{bcd}	1.02 ^{ab}	1.07 ^{a}
Mean	0.67	0.55	0.68	0.66
SEM = 0.081	p-values:			
	Treatment < 0.001	Day < 0.001	Interaction < 0.001	

^{abcd} Within a row or column means without a common superscript differ ($P < 0.05$).

[1]Treatments: basal diet with no Zn added (Ct); basal diet supplemented to: NRC recommendation of Zn (100ppm) as either ZnO (100ZnO) or $ZnSO_4$ (100ZnS), the dose of Zn as $ZnSO_4$ recommended by the WHO (250ZnS), the maximum dose of $ZnSO_4$ not producing rejection in pigs (700ZnS), the antidiarrheal dose of Zn as ZnO used in pig production (2500ZnO); and 100ZnO diet with animals fasted for 24h hours after weaning (FAST).

Experiment 2

Serum Zn concentration before weaning was 0.744 ± 0.164 mg/L and was reduced on d 5 (0.617 mg/L; $P < 0.05$) and on d 7 (0.561 mg/L; $P < 0.001$). Serum Fe concentration before weaning was 1.79 ± 0.482 mg/L and tended to be lower on d 1 (0.978 mg/L; $P < 0.07$). Serum Cu concentration before weaning (1.68 ± 0.279 mg/L) was not different to post weaning levels (1.65 ± 0.235 mg/L).

Table 6.3. presents post-weaning Zn concentrations in serum. There was a treatment effect ($P < 0.010$) with mean serum Zn levels being higher for animals fed high doses of ZnO than for other treatments. The serum Zn concentration in animals fed ZnO diet on day 7 (0.985 mg/L) was higher ($P < 0.02$) than Zn concentrations for animals on treatments Tb ($P = 0.043$), ZnS ($P = 0.029$), Ct ($P = 0.086$) and fCt ($P = 0.007$). Serum Fe (1.50 ± 0.722 mg/mL) and Cu (1.65 ± 0.237 mg/mL) concentrations were not affected by treatment. However, animals receiving in-feed high ZnO supplemented diet tended to show lower Cu concentrations compared to Ct and fCt (1.51 vs. 1.72 and 1.72 mg/L; $P = 0.074$ and 0.063, respectively). Serum Cu was not affected by extraction day ($P = 0.713$) but Fe was higher on d 3 and 7 compared to d 1 (1.64 and 1.86 vs. 1.07 mg/L; $P = 0.035$ and 0.002, respectively).

Table 6.3. Serum Zn concentration after weaning (mg/L). Exp. 2. (n = 4)

		Day 1	Day 2	Day 3	Day 5	Day 7
Control	No-fasting (Ct)	0.67 [ab]	0.71 [ab]	0.64 [ab]	0.67 [ab]	0.55 [b]
	Fasting (fCt)	0.73 [ab]	0.62 [ab]	0.61 [b]	0.55 [b]	0.54 [b]
Tablet	No-fasting (Tb)	0.69 [ab]	0.66 [ab]	0.66 [ab]	0.59 [b]	0.58 [b]
	Fasting (fTb)	0.75 [ab]	0.72 [ab]	0.65 [ab]	0.64 [ab]	0.56 [b]
ZnS		0.66 [ab]	0.64 [ab]	0.64 [ab]	0.64 [ab]	0.58 [b]
ZnO		0.66 [ab]	0.66 [ab]	0.73 [ab]	0.78 [ab]	0.99 [a]
	Mean	0.69	0.67	0.65	0.64	0.63
	SEM = 0.077	p-values:				
		Treatment < 0.010	Day < 0.605	Interaction = 0.107		

[ab] Within a row or column means without a common superscript differ ($P < 0.05$).

[1]Treatments: basal diet with no Zn added, non-fasting (Ct); basal diet with no Zn added, fasting (fCt); $ZnSO_4$ tablet, non-fasting (Tb); $ZnSO_4$ tablet, fasting (fTb); basal diet supplemented to the dose of Zn as $ZnSO_4$ recommended by the WHO (ZnS); basal diet supplemented to the antidiarrheal dose of Zn as ZnO (ZnO).

Concentrations of Zn, Fe and Cu in liver are presented in Table 6.4. Levels of Zn in liver were affected by treatment ($P = 0.001$) as in-feed high ZnO supplemented animals showed higher Zn levels compared to all the other treatments ($P < 0.05$ in all cases). Liver Fe (101 ± 58.3 mg/kg) and Cu (32.8 ± 12.51 mg/kg) concentration were not affected by treatment.

Table 6.4. Zinc, iron and copper concentrations (mg/kg) in liver from pigs 7 days after weaning (Exp. 2; n=4).

	Control		Tablet		ZnS	ZnO		p-value
	No-fasting	Fasting	No-fasting	Fasting			SEM	Treat
Zn	59.5[b]	58.8[b]	68.3[b]	72.8[b]	51.3[b]	151.5[a]	14.25	0.001
Fe	100.0	97.8	152.0	103.5	92.8	62.8	29.25	0.462
Cu	27.8	37.0	33.5	28.3	33.8	36.5	6.75	0.878

[ab] Within a row or column means without a common superscript differ ($P < 0.05$).

[1]Treatments: basal diet with no Zn added, non-fasting (Ct); basal diet with no Zn added, fasting (fCt); $ZnSO_4$ tablet, non-fasting (Tb); $ZnSO_4$ tablet, fasting (fTb); basal diet supplemented to the dose of Zn as $ZnSO_4$ recommended by the WHO (ZnS); basal diet supplemented to the antidiarrheal dose of Zn as ZnO (ZnO).

6.4. Discussion

The basal diet used in this experiment was the same commercial diet normally used in the farm but without any Zn source or antimicrobial substance added. However, analyzed Zn and Fe levels in Ct diets were higher than calculated ones (140 vs. 33 and 230 vs. 140 mg/kg, respectively). These differences were probably due to differences among theoretical and actual composition of feed ingredients. This situation may be common in commercial practice because Zn is not routinely analyzed in feeds or feed ingredients and its addition to diets is based in theoretical values.

Zn requirements for growing pigs are presented as per kg of diet or as amount per day by NRC, (2012) and change according to BW of the animals; pigs between 5-7 kg require 100 mg/kg of diet or 26.6 mg/day and pigs between 7-11 kg require 100 mg/kg of diet or 46.8 mg/day. In-feed Zn recommended level is maintained at different BW because of different estimated feed intakes (280 and 493 g/day for 5-7 and 7-11 kg pigs, respectively). On the other hand, Zn requirements in humans are presented as daily RDA (recommended dietary allowance) by IOM, (2006) and change according to the age of individuals; 0-1 year: 2.5 mg/day; and 1-3 years: 3.0 mg/day. Thus, daily Zn requirements are much higher in piglets than in children, probably due to much higher growing rate.

The piglets used in both experiments had an average BW of 7.52 and 7.96 kg, and a daily feed intake below 140 g (980 g/week). Feed intake was not affected by treatments (data not shown). A feed intake below or around 1 kg during the 1st week post-weaning is common in European countries. With this feed intake, an in-feed Zn concentration of 334 mg/kg would be necessary to reach the NRC recommended daily Zn intake (46.8 mg/d). Otherwise, the daily feed intake should be around 312 g to reach the recommended daily Zn (46.8 mg) if the

upper Zn in-feed limit in the EU (150 mg/kg) is considered (Regulation(EC), 2003), an elusive goal in the first days after weaning in European commercial conditions. The high estimated feed intake of NRC is probably due to early-weaning management practice in the US, which is not the case in the EU. In the present work, NRC daily Zn requirements were only reached with 700ZnS and 2500ZnO. Even higher Zn intake might be advisable in individuals receiving a vegetable-based diet (like weaned pigs and children) as some of the Zn, and also Fe and Ca, present in the ingredients could be not readily available due to interactions with other feed components as may be phytates (Lonnerdal, 2000; Gibson et al., 2010). Although Zn deficiency is normally not considered as a main explanation for anti-diarrhea effects of ZnO in pigs, pigs do have a low ingestion of Zn after weaning and weaning induces a fast drop of plasma Zn concentrations in weaned pigs compared to un-weaned littermates at the same age (Davin et al., 2013) and therapeutic levels of in-feed ZnO are required to re-establish pre-weaning levels of plasma Zn. Previous studies (Poulsen and Larsen, 1995; Carlson et al., 2007 a) found no difference when comparing pre- and post-weaning Zn concentrations in plasma, because pre-weaning concentration was relatively low compared to Davin et al. (2013) and experiments here presented (0.6 vs. 0.82 and 0.74 mg/L, respectively). Normal serum Zn concentrations are reported to be within the range of 0.7 and 1.5 mg/L and serum Zn concentrations associated with marginal status are within the range of 0.4 and 0.8 mg/L (Puls, 1994). In humans a concentration of less than about 0.65 mg/L suggests deficiency (Crook, 2011). Other authors found serum Zn concentrations as low as 0.33 mg/L in zinc-deficient piglets (Johanning et al., 1990).The two experiments presented here showed a drop in serum Zn concentrations after weaning to levels lower than 0.65 mg/L. This decrease in serum Zn may be due to temporary deficiency promoted by post weaning anorexia and by inflammatory reactions induced by stress after weaning. In humans it is well established that serum Zn concentration can fall due to factors unrelated to Zn status or dietary Zn intake such as infection inflammation, exercise, stress or trauma (King, 2011). The serum Zn pool is a minor pool, but highly mobile and immunologically important. Pro-inflammatory cytokines mediate changes in hepatic Zn homeostasis during infections leading to sequestration of Zn into liver cells and subsequently to hypozincaemia (Liuzzi et al., 2005) similar to the hypoferremia suggested as a host defense process to restrict Fe from pathogens (Weinberg, 1984 Armitage et al., 2011). Several transporters are known to regulate Zn transport into and out of cells and organelles and research advances have increased recently (Lichten and Cousins, 2009). For instance, Zn homeostasis is regulated by metallothioneins (MT) during an inflammatory response (Mocchegiani et al., 2000) by trafficking Zn through

the cell and releasing it to Zn-requiring proteins. MT regulates Zn absorption during demanding episodes and MT synthesis is stimulated by dietary Zn supplementation and by inflammation and the acute phase response (Roohani et al., 2013).

Diets Ct and FAST in exp 1 were both used in order to promote a low Zn intake after weaning and a more pronounced decrease in serum Zn than in supplemented diets. However this effect was not observed. This fact may indicate that weaning itself is the main cause of the serum Zn levels drop. Zn serum level maybe be kept from being lower by tissue catabolism during starvation which can release Zn into the circulation to some extent (Moran et al., 2012). None of the treatments including $ZnSO_4$ in exp. 1 increased serum levels of Zn compared to 100ZnO diet. On the other hand, the treatment including therapeutic levels of Zn as ZnO, 2500ZnO, clearly restored pre-weaning serum levels of Zn on d 3, 5 and 7 post-weaning. Previous studies found that dietary Zn concentrations, as ZnO, need to be higher than 1,000 ppm to elicit increased serum Zn concentrations in weaning pigs (Hahn and Baker, 1993; Poulsen and Larsen, 1995; Hill et al., 2001; Carlson et al., 2007 a) however, to our knowledge, the effect of dietary Zn concentrations between 100-1,000ppm as $ZnSO_4$ in serological Zn of weaning pigs has never been tested. Hahn and Baker (1993) assessed Zn serum concentration when 3,000 mg/kg of Zn were added to pig diet either as ZnO or $ZnSO_4$. Fortification with 3,000 mg/kg of Zn as ZnO resulted in plasma Zn levels that were only half the Zn plasma levels observed in pigs fed 3,000 mg/kg of Zn as $ZnSO_4$ suggesting that pigs, like chickens (Wedekind and Baker, 1990; Wedekind et al., 1992), utilize Zn from ZnO far less efficiently than Zn from $ZnSO_4$. Schell and Kornegay (1996) recorded results that confirm this hypothesis in weanling pigs however dietary Zn concentrations should be as high as 2,000 and 3,000 ppm to exert differences between ZnO and $ZnSO_4$ one week after weaning.

In contrast, in children much lower Zn doses administered with tablets (supplementation), 10-20 mg Zn/day as $ZnSO_4$, equivalent to in-feed (fortification) treatments 250ZnS (exp. 1) and $ZnSO_4$ (exp. 2), produced an increase of serum Zn concentrations within 2-5 d of starting supplementation (Wessells et al., 2010), and an increase of 9% of serum Zn concentration was registered by doubling Zn intake (Moran et al., 2012). More interestingly, an increase of serum Zn levels was only seen among those children who received Zn in the form of an aqueous supplement but not in those who received the zinc-fortified food either as $ZnSO_4$ (Brown et al., 2007) or ZnO (Lo et al., 2011). Zn absorption in humans was not different between Zn sources (ZnO and $ZnSO_4$) when added to fortified food (López de Romaña et al., 2003; Hotz et al., 2005; Rosado et al., 2012). We hypothesized that a similar effect could be

seen between the two different approaches of $ZnSO_4$ administration (supplementation and fortification) in piglets in exp 2. However, Zn serum concentrations were not increased as expected in animals that received Tb and ZnS treatments in exp 2. A possible explanation for Tb results may be attributable to relatively low bioavailability of the tableted form of Zn compared to a reference dose of aqueous $ZnSO_4$ (Solomons et al., 2011) however, this difference in bioavailability was not found in a short-term Zn supplementations trial also in humans (Wessells et al., 2012). A higher in-feed $ZnSO_4$ concentration might be needed to observe an increase on serum Zn. Another interesting approach might be to assess the effect of more soluble Zn products like nanoparticles (Hilty et al., 2009), supplemented and fortified, on serum Zn concentration of pigs.

Fasting was also considered an important factor in exp. 2 regarding the administration of Zn tablets. In many intervention Zn trials in humans like Brown et al. (2007) a fasting period (≈ 2 hours) is advised before Zn supplementation to avoid negative interactions with other nutrients and to reach a better absorption of Zn. We were not able to detect differences due to fasting in exp 2 between Ct and fCt or Tb and fTb. Probably large discrepancies should not be expected as the CVs between fasting and non-fasted samples are similar (Brown et al., 2004), specifically Hotz et al., (2003) pointed that the difference introduced by fasting state is lower (7.3%) than for example the difference introduced by time of day (morning compared to afternoon, 9.5%).Serum Cu concentrations were also lower on d 2 and then increased again on d 3 and 5 in exp.1 but no differences were found in exp. 2; Carlson et al. (2007 a) showed that plasma Cu concentrations were reduced in a time-related manner from d 1-2 until d 14-15 after weaning but this is not in agreement with Poulsen (1995), who found that plasma Cu concentration increased during the first 7 d after weaning and thereafter decreased. Serum Fe showed a positive trend after weaning in exp.1 and exp. 2.

Liver plays a central role in Zn homeostasis (Stamoulis et al., 2007) and together with pancreas and kidney are characterized by a high Zn turnover when high doses are administered (Sheline et al., 1943). High levels of dietary Zn increase Zn concentration and induction of MT in liver, kidney and intestinal mucosa (Carlson et al., 1999; Martinez et al., 2004, 2005; Martin et al., 2013 b). In exp 2 we observed an accumulation of Zn in liver (152 mg/kg) only after feeding pharmacological levels of Zn as ZnO for one week. Interestingly, Schell and Kornegay (1996) reported similar concentrations (155-156 mg/kg) feeding 1,000 ppm of Zn as either ZnO or $ZnSO_4$ for two weeks, but higher liver Zn concentrations were reached with 2,000 ppm of dietary Zn being different depending on Zn source (372 mg/kg in ZnO treatment and 949 mg/kg in $ZnSO_4$ treatment). Lower Zn levels comparing different Zn

sources have not been found on the literature but it might be that a minimum amount of dietary Zn is necessary to observe increased liver Zn concentrations. This could be the reason why we only observed an accumulation of Zn in liver after feeding pharmacological levels of Zn as ZnO and that might also mean that is the only treatment able to increase Zn absorption.

To our knowledge this is the first time Zn serum concentration is analyzed after weaning in weaned pigs using $ZnSO_4$ soluble tablet. We showed how weaning reduces serum Zn concentrations acutely in pigs and this decrease is not solved by $ZnSO_4$ supplementation neither fortification combined or not with fasting. The only treatment able to bring serum Zn concentrations back to pre-weaning levels was 3,000ppm of ZnO.

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Chapter 7

General discussion

Zn is an essential nutrient for all forms of life that becomes especially critical in periods of high metabolic demand like growth and lactation. During these periods, Zn deficiency may occur, being the responses well-characterized in mammals (Giugliano and Millward, 1984; Park et al., 1986; Fairweather-Tait and Hurrell, 1996; Sandstead et al., 2000).

In children, the prevalence of Zn deficiency is high in developing countries as it is also high the incidence of illness (e.g. diarrhea, pneumonia) and death from infectious disease (Black, 2003). A transient period of Zn deficiency is also common during post-weaning anorexia in piglets as we have observed in Chapter 4 (Davin et al., 2013). To our knowledge this is the first time that this hypothesis is proposed in weaned pigs using the plasma/serum Zn concentration as an indicator of Zn status.

The present work focuses the study of how Zn status of piglets change around weaning and to what extent it is possible to modify it afterwards.

Firstly (7.1.), we wanted to confirm how far piglet's pre-weaning Zn status can be affected by gestation and lactation (Chapter 3).

Then (7.2.), we wanted to study the effects of weaning and pharmacological levels of Zn as ZnO on Zn status by analyzing Zn concentration in key organs (Chapter 4) considering the Zn solubility and concentration in different sections of the GIT (Chapter 4 and 5).

Finally (7.3.), we assessed Zn status around weaning and explored the likely effects of different Zn sources and methods of application (Chapter 6).

7.1. The pre-weaning Zn status

The Zn status at the starting point (i.e. pre-weaning Zn concentration in plasma) is critical to confirm this hypothesis (Carlson et al., 2007 a). Pre-weaning plasma/serum Zn concentrations of piglets can be affected by (a) piglet's age, (b) creep feed consumption and (c) sow's transfer during gestation and lactation.

Chapter 3 confirmed that colostrum and milk Zn concentrations are above Fe and Cu concentrations and well above sow's plasma Zn concentration. In our work we were not able to find differences in colostrum and milk Zn concentrations according to sow's age or to changes in sow's diet. These findings confirm that the positive Zn transfer from maternal circulation to colostrum and milk is tightly regulated (Kelleher and Lönnerdal, 2005; Kelleher et al., 2009; McCormick et al., 2014).

Sows older than 6th parity number have not been usually considered on studies of mineral milk concentration (Peters and Mahan, 2008; Peters et al., 2010) probably due to the lack of representation of this age groups in commercial farms and/or to avoid variability associated with clinical or subclinical pathologies that are common with age (e.g. mastitis, lameness). Mahan and Newton, (1995) suggested that sow Zn status, among other minerals, is reduced by consecutive reproductive stages with high demanding mineral secretion. In our work, we estimated that an average 0.86 g of Zn was absorbed by each sow during 21 days of lactation whereas an average1.4 g of Zn was exported through milk during the same period.

Sow's diet is the other factor studied in our work. The dietary levels of Ca, P, Zn and phytase were modified however no effects on Zn status and milk concentration were observed (Chapter 3). Some authors have suggested that dietary Zn concentration needs to be very extreme to display any effects on milk Zn (Hill et al., 1983 b). There are few other articles studying the effect of phytase on Zn digestibility and colostrum and milk Zn concentrations (Jongbloed et al., 2004; Grela et al., 2010, 2011). They obtained positive responses of phytase supplementation on the parameters previously mentioned largely due to the following factors: phytase was provided during both pregnancy and lactation phase, in some cases in higher dose (from 750 to 10,000 PU/Kg at Jongbloed et al., 2004) and a larger number of animals were used than in our study.

Another parameter that could have been interesting to study in Chapter 3 is Zn status of sows, before and after farrowing, to know how parturition affects Zn status depending on sows age/productive weariness and if this could have any implication on Zn colostrum and milk.

Recently, Matte et al., (2014) did something similar and collected blood from late pregnant sows and their offspring on d 0 and 3 of life to estimate the transfer of different micronutrients from sow to their offspring. They concluded that there is an active pre-natal and post-natal transfer for Zn, among other micronutrients, from sow to piglet and that sow milk is a poor source of these micronutrients and piglet may possibly experience nutritional deficits other than Fe. Unfortunately sow's age was not included as an experimental factor.

Definitely it would have been interesting to include this factor and to follow piglet's micronutrient status throughout the lactation to check the previous mentioned "starting point" at weaning and to study how piglet's age at weaning (a) and creep feed consumption (b) affect it.

7.2. Effects of weaning and pharmacological ZnO

Plasma Zn concentration is the best indicator of Zn status. In Chapter 4, weaning produced a drop on plasma Zn concentrations and pharmacological levels of ZnO restored it back to physiological levels. However, no effect of weaning was registered in Zn concentration in other tissues like liver and pancreas in contrast to pharmacological ZnO that induced a quick accumulation of Zn in liver and pancreas, in accordance to other authors (Martin et al., 2013 b). Zn concentration at all different portions of the GIT was higher in weaned compared to unweaned piglets but interestingly more available (solubilized) Zn was observed in unweaned animals.

In Chapter 6, it was again observed that weaning produces a drop on plasma Zn concentrations as 5 and 7 days after weaning, concentrations were lower than pre-weaning concentrations (0.16 = 0.66 vs. 0.82 and 0.11= 0.63 vs. 0.74, mg/L) in accordance to the comparison in Chapter 4 between 7-day-weaned piglets and unweaned littermates (0.34 = 0.76 vs. 1.10, mg/L). Plasma Zn concentrations seem to be lowest 2 days after weaning and then seem to remain fairly constant. These effects are probably due to the low Zn intake meaning that there is either a low feed intake and/or a low dietary Zn level. Individual housing, like metabolic cages, would have been a good option to find out this question and to obtain individual fecal samples for digestibility calculations.

Other explanations for the Zn drop in plasma include:

- reduced Zn absorption due to the observed alterations in the small intestine structure and digestive function immediately after weaning (Hopwood and Hampson, 2003), or due to negative interactions with phytate and Ca.
- increased endogenous Zn excretion in the digestive tract, previously described in Chapter 1.2.2.5.
- increased hepatic Zn concentration during acute response to infection and inflammatory redistribution of Zn from plasma to liver through MT. Further explanation is found in Chapter 1.2.2.4.

7.2.1. What are the effects of high ZnO over time?

Feeding high levels of Zn has positive short-term effects on weaned pigs. Improved performance, reduction of the incidence and severity of diarrhea and improved fecal consistency (Mavromichalis et al., 2001; Zhang and Guo, 2009), reduced intestinal damage, reduced intestinal permeability, improved mucosal repair are some of the positive effects

(Roy et al., 1992; Mavromichalis et al., 2000; Sturniolo et al., 2002; Tran et al., 2003; Zhang and Guo, 2009).

According to the meta-analysis performed by Sales, (2013) there is no significant influence of the duration of pharmacological ZnO supplementation on piglets growth. However, there are more evidences that long-term supplementation can exert non- or negative effects in the animals. For example, Martin et al., 2013 b; Walk et al., 2013 found no growth-promoting effects after two weeks of supplementation. The reasons for that are unclear but could be related to the disappearance of the immunologic protection against pathogens (Janczyk et al., 2013) or to the excessive accumulation of Zn in organs. High levels of dietary Zn as ZnO clearly outbalance Zn homeostasis with increased accumulation of Zn in various organs including the small intestine in piglets (Martin et al., 2013 a; b). Thus results are in accordance to Chapter 4, as pharmacological ZnO was able to increase liver and pancreas Zn concentration in just one week, and continue to increase the longer the supplementation period (Schell and Kornegay, 1996; Martin et al., 2013 b). Liver, pancreas and kidney are characterized by high Zn turnover when high doses are administered (Sheline et al., 1943).

High accumulation of Zn in organs activates the multiple defense mechanisms (Fujimura et al., 2012) to avoid Zn absorption (down-regulation of Zn transporters) and to increase Zn excretion (up-regulation of MT expression) and can lead to the activation of stress response (Bondzio et al., 2013 b). All these responses could negatively affect the performance of the animals, however the risk/benefit balance of withdraw pharmacological ZnO after two weeks of weaning should be weighted by the sanitary status of the facilities.

7.3. Zn status around weaning and different Zn applications

7.3.1. Why $ZnSO_4$ was selected?

In Chapter 6, $ZnSO_4$ was selected because its great use in the food industry and against childhood diarrhea. Another reason was its common use in pig diets either as an additive or pharmacological levels (Hahn and Baker, 1993).

During the present PhD thesis we previously explored the differences on palatability and their in-vitro solubilities in GIT among Zn sources ($ZnSO_4$, ZnO). The results of these preliminary studies are reported in Annex 1 and Annex 2. In a double choice-test both, ZnO and $ZnSO_4$ showed a lower preference than the control diet at doses of 3,000ppm for ZnO (25.1% of the total intake) and 600 ppm and 900 ppm for $ZnSO_4$ (32.8% and 27.1%), but not with 300 ppm $ZnSO_4$ (equivalent to human therapeutic level). In the in vitro trial mean solubility was two

times higher in stomach than jejunum (13.3 vs 6.08 %) except for 3000ZnO that was 5 times higher in stomach than in jejunum (3.90 and 0.78 %). Zn solubility in stomach was lower in ZnO (6.37 %) than $ZnSO_4$ powder (10.8 %), ZnCl (10.6 %) and Zn chelates (10.6 %). Solubility was not different for 3000ZnO (3.90 %) compared to ZnO. Zn solubility in jejunum was no different among different treatments, all treatments showed a solubility below 5 %. 3000ZnO showed the lowest solubility both in stomach and jejunum. For further details about the experimental designs and results see Annex 1 and 2.

The results confirm that piglets are able to detect and reject the presence of high levels of Zn in the diets in a double choice test, especially with high soluble sources (Zn sulfate). However, despite the low palatability, Zn supplementation has been described to increase feed intake and performance when only one diet is provided, which confirm the inherent potential of Zn to enhance feed intake mechanisms in piglets. It appears interesting to perform further double-choice tests trying to explore procedures to mask the undesirable taste of Zn sources with flavors or using encapsulation techniques. In children $ZnSO_4$ tablets and highly sweetened and vanilla-flavored syrup have demonstrated to effectively hide the taste of Zn (Larson et al., 2005).

The differences observed on solubility among sources are expected to influence on feed palatability but also on the Zn absorption. However, it could be suggested that changes in the GIT content composition (Ca, phytate, phytase) could affect the Zn solubility of different sources and dietary regimes in vivo. Cell cultures, and specifically IPEC-J2 cell line, may provide a good in vitro model for swine to study the effect of feedstuffs on the inflammatory response caused by specific porcine pathogens or to further investigate the ZnO therapeutic mechanism. However, it is difficult to design the procedures about how solubilize high concentrations of ZnO without adding too much acid that could damage the cell line.

Feeding pharmacological levels of ZnO for one week seems a good option to increase plasma ZnO levels back to pre-weaning levels. Results from Chapter 6 (0.25 = 0.74 vs. 0.99 and 0.25 = 0.82 vs. 1.07) are in accordance to results in Chapter 4 (0.22 = 1.10 vs. 1.32). However, despite showing a greater solubility in vitro, none $ZnSO_4$ treatments were able to increase Zn plasma/serum as 3,000 ppm of ZnO, so at the in vivo conditions tested $ZnSO_4$ does not seem a good alternative to increase Zn plasma/serum. However, as previously mentioned its effects on performance and as antidiarrheal treatment should be assessed with more replicates.

We planned to include a Zn injectable treatment during the experimental design of Chapter 6 trials in order to evaluate its effect on Zn body pools. We didn't find an appropriate chemical

formula to administer to the animals regardless the few number of publications found (Schell and Kornegay, 1994). Injectable Zn would have clarified whether Zn deficiency in piglets is just due to post-weaning anorexia or to other processes caused by weaning.

Another interesting option that has already been used with success to improve serum Zn concentration is gastric nutriment-intubation of piglets with organic Zn (Zn methionine) however a lot of handling of the animals is probably required (Caine et al., 2009; Metzler-Zebeli et al., 2010).

7.4. Zn level in control diets

In Chapters 4, 5 and 6, control diets were formulated to contain nutritional Zn levels (100 mg/kg) or none Zn (33 mg/kg). However when analyzed they contained 380 and 140 mg Zn/kg, respectively. In all cases we used a commercial diet containing a premix without any added Zn source. Thus, this unexpected increase is coming from the ingredients of the diet. When we analyzed the Zn content of the limestone used in our experiments we obtained a Zn concentration of 4.78 g/kg.

7.5. Could the experimental facilities affect the experimental output?

High Zn concentrations are used as a preventive and therapeutic tool for diarrhea as well as growth-promoter in piglets as short-term feeding (commonly for 2 weeks) of a diet supplemented with 3,000 mg ZnO/kg of diet, and in humans 10-20 mg Zn /day for 2 weeks is also used as preventive and therapeutic. Chapter 4 and 6 were not conceived to show any effect of treatments on piglet's performance or antidiarrheal effect. For that purpose more replicates would be probably needed. However, in other recent studies, such as Martin et al., 2013 a; b, the lack of differences on performance and diarrheal episodes between treatments were attributed to the high sanitary status of the experimental facilities used. Animals in Chapter 4 remained in the experimental facilities whereas piglets in Chapter 6 stayed under commercial conditions. To our knowledge there is no scientific report on the use of ZnO in swine commercial farms according to their health status, it would be interesting to know if a correlation between health status and therapeutic ZnO utilization (concentration, duration) is correlated.

Health status of the farm and its relation to Zn status could be an interesting point to be further studied. In humans, a subclinical disorder that occurs among inhabitants of environments with poor sanitation and hygiene, like those in developing countries, has been

linked to a poor response after nutritional therapies (including vitamin A or Zn interventions), Zn deficiency and child stunting (Humphrey, 2009; Korpe and Petri, 2012; Black et al., 2013; Lindenmayer et al., 2014). Human EE (environmental enteropathy) causes decreased absorptive capacity due to fundamental changes in both small intestinal structure (villus atrophy, crypt hyperplasia, inflammatory cell infiltrate), increased intestinal permeability and impaired immune function (Humphrey, 2009; Korpe and Petri, 2012), that can resemble the symptoms of weaned pigs.

This interesting hypothesis could partially explain the lack of response to all Zn treatments in Chapter 6, except for pharmacological ZnO, and could also relate the farm health status factor previously mentioned. The precise mechanisms underlying EE and the cause/effect between Zn deficiency and EE are not fully understood. Manary et al., (2002) suggested that EE was impairing the ability to reabsorb endogenously secreted Zn in children consuming a high-phytate and low-animal protein diet. EE is critical for developing combined interventions to improve child health (Lindenmayer et al., 2014) but probably is not exactly the same environment that piglets found just after weaning.

It would be of interest to consider this relation in weaned pigs and/or growing pigs, for example by performing a population-based study in pig farms to confirm Zn deficiency hypothesis and correlate Zn status of pigs at different age to sanitary status and enteropathy hypothesis and the in-feed levels of Zn, Ca, P and phytate.

On May 5th 2014 the EFSA's FEEDAP published a "Scientific Opinion on the potential reduction of the currently authorized maximum Zn content in complete feed" for all animal species. The original request of this document came from the European Commission, which asked a scientific opinion on the potential reduction of the currently authorized maximum Zn content in complete feed. According to the document main conclusions, the newly proposed total maximum contents of total Zn ensure health, welfare and productivity of the target species and do not affect consumer safety; as well it will reduce the amount of Zn in manure released in the environment by about 20 %. The newly proposed total maximum contents of total Zn in complete feed for piglets (150 mg/kg) is the same as the stands the Regulation (EC) No 1334/2003. However the FEEDAP Scientific opinion includes a new section which indicates that *"The use of phytase, either from endogenous source or from a feed additive, in feeding piglets, pigs for fattening and sows would allow a further reduction of the NPMC by*

30 % (from 150 to 110 mg Zn/kg feed for piglets and sows and from 100 to 70 mg Zn/kg feed for pigs for fattening)".

The pressure of the European authorities on the reduction of Zn utilization in animal feeds has increased the interest of companies on searching for new Zn products. In piglets, most of the new Zn products are more soluble and available than ZnO and aim to have the same therapeutic effect against post-weaning diarrhea than pharmacological ZnO but at a much lower dose. It is important at that point to remind that Zn can act as a nutrient (purpose of additive Zn) or as a therapeutic (purpose of pharmacological levels of ZnO).

From our expertise it has been impossible to counteract plasma Zn drop after weaning with lower levels and a more soluble source of Zn than 2,500 mg/kg of feed of ZnO. In high sanitary conditions reduced levels of alternative Zn sources and new Zn products could be used without any concern in combination of phytase and other additives like organic acids. However, it could be risky to use nutritional levels of Zn in poor sanitary conditions and much more attention should be paid to detect any health problem.

Chapter 8

Conclusions

The results obtained in this thesis allow us to conclude that in our experimental conditions:

1- Zinc concentration in colostrum and milk was fairly constant in sows of different age or after consuming diets with different mineral content or supplemented with phytase, which suggest that Zn secretion into colostrum and milk is a highly regulated process and piglets Zn status seems to be not affected by sow's factors.

2- Weaning produces a drop in Zn plasma concentration compared to unweaned littermates. The lowest Zn plasma levels are reached 2-3 days post-weaning and pharmacological levels of ZnO rapidly increased Zn plasma levels. The drop is independent of a 24h fasting period which suggests a direct effect of weaning on the plasma values, likely by increasing endogenous Zn losses in the feces or after sequestration by internal organs.

3- Zinc oxide is less soluble than other Zn sources, such as zinc sulfate, zinc chloride, zinc acetate or zinc chelates. The lowest solubilities were observed for zinc oxide at therapeutic doses and in the high pH milieu of the small intestine digesta.

4- Pharmacological levels of zinc oxide in piglet's feed produce a large increase of the amount of Zn in the soluble fraction of the stomach and an increased concentration along the GIT one week after weaning. The amount of Zn absorbed increase to the point that highly metabolic organs like liver and pancreas become loaded and Zn is excreted through feces.

5- Zinc sulfate was not able to increase serum Zn levels in piglets, in contrast to children in which it is extensively used as antidiarrheal treatment. In piglets zinc sulfate has palatability concerns at lower doses compared to zinc oxide.

6- Zinc sulfate administered as either in suspension or mixed in piglet's feed was not able to increase plasma Zn concentrations, regardless of the fasting situation of the animals.

Chapter 9

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Annexes

Annex 1

Palatability problems with zinc oxide and zinc sulphate as diarrhea treatments

Davin et al., 2011 Abstract presented at the ESPHM 2011

Therapeutic doses of in-feed ZnO (3,000ppm) have become a common practice in some EU countries to prevent or treat diarrhea in weaning pigs. However, high doses Zn are not retained by the animals, provoking a high Zn excretion in the slurry and environmental concerns. Alternative more soluble sources of Zn, such as Zn sulphate ($ZnSO_4$), may allow for a likely reduction of in-feed Zn doses. However, the presence of soluble Zn in the mouth may provoke palatability problems and feed refusal. In the present trial we evaluated the palatability of a range dose of ZnO and $ZnSO_4$ in feed by double-choice feeding trials. Two-hundred and forty 28 days old piglets were weaned and allocated by sex and weight in twelve pens (20 pigs / pen). Pens were initially distributed to six different treatments at random: 1,000, 2,000 and 3,000 ppm of ZnO or 300, 600 and 900 ppm of $ZnSO_4$, and rotated in three periods following a crossover design for each Zn source. The lower $ZnSO_4$ level (300 $ZnSO_4$) was selected considering the WHO therapeutic level of Zn (10-20 mg/day), assuming a feed consumption of 300 g of feed7day.The six treatments were compared with a control diet without added zinc. Both, ZnO and $ZnSO_4$ showed a lower preference than the control diet at doses of 3,000ppm for ZnO (25.09% of the total intake, p-value=0.0005) and 600 ppm and 900 ppm for $ZnSO_4$ (32.83% and 27.07%, p-values=0.0259 and 0.0261, respectively). On a fourth period 3,000 ppm of ZnO was compared vs 900 ppm of $ZnSO_4$ in all pens. Animals fed previously on $ZnSO_4$ showed a preference for the $ZnSO_4$ (62%), animals fed on ZnO did not show any preference.

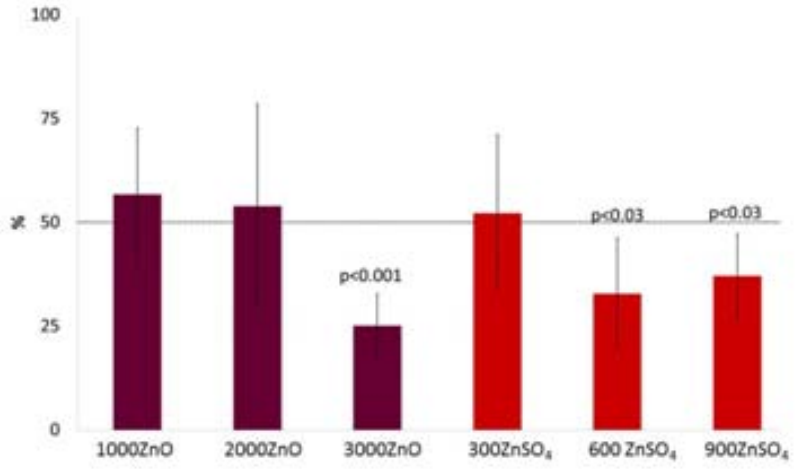

Figure Annex 1. Preference of different Zn treatments compared to a control diet containing no added Zn.

Annex 2

In vitro solubility of different Zn sources using GIT content

Davin et al., 2014 Abstract presented at TEMA 2014

High in-feed levels of Zn (2,500ppm) as ZnO are used to treat post-weaning diarrhea in early-weaned pigs although its mode of action is still hypothetical. New more soluble in-feed Zn products are sought to obtain the antidiarrheal effect, and to prevent negative effects on feed appetence and environmental contamination with a lower Zn dose. WHO recommends $ZnSO_4$ tablets to prevent and treat childhood diarrhea. Other commercial Zn sources include chelates, chlorides, and nanoparticles, mainly claiming a better solubility and bioavailability in the gastrointestinal tract (GIT). We assessed the Zn concentration in the soluble fraction and calculated solubility of different commercial Zn products on GIT content (stomach and jejunum) from early-weaned pigs.

Gastric and jejunal content was collected from 35 d old piglets fed a diet (based in cereals and soy, 5.26 g/kg phytate) with no added Zn. Incubation tubes were filled with 30 mL of gastric or jejunal content and the necessary amount of each Zn source was added to reach a final concentration of 150 ppm of Zn. Treatments were as follows: commercial ZnO (Apsamix®), nanoZnO (Sigma), $ZnSO_4$ powder (Sigma), $ZnSO_4$ tablets (Nutriset, France), Zn acetate (ZnA; Sigma), Zn chloride (ZnCl; Sigma), and Zn chelates. A negative (basal amount of Zn in stomach = 59.8 and in jejunum = 74.0 mg/kg) and a positive control (3,000 ppm ZnO added) were also included. All tubes in duplicate were incubated at 37 °C, 60 rpm, for 30 minutes, and then 10 mL of content was centrifuged at 18,000 x *g* at 37 °C for 30 minutes to obtain the soluble fraction that were analyzed for Zn by ICP. Solubility of added Zn was calculated.

Mean Zn concentration in the soluble fraction was two and a half times higher in stomach than in jejunum (29.8 vs. 11.8 mg/kg) except for 3000ZnO that was 7 times higher in stomach than in jejunum (212 vs. 30.3 mg/kg). Zn concentration in stomach soluble fraction was lower in ZnO (20.8 mg/kg) compared to $ZnSO_4$ powder (32.4 mg/kg; $P = 0.02$), ZnCl (32.9 mg/kg; $P = 0.01$), Zn chelates (31.8 mg/kg; $P = 0.01$) and 3000ZnO (212 mg/kg; $P < 0.001$). 3000ZnO showed higher Zn concentration than all other treatments ($P < 0.001$). Zn concentration in soluble jejunum fraction was lower in ZnO treatment (5.35 mg/kg) than $ZnSO_4$ powder (13.6; $P = 0.07$), ZnCl (13.3; $P = 0.055$), Zn chelates (12.6; $P = 0.04$) and 3000ZnO (30.3 mg/kg; $P < 0.01$).

Mean solubility was two times higher in stomach than jejunum (13.3 vs 6.08 %) except for 3000ZnO that was 5 times higher in stomach than in jejunum (3.90 and 0.78%). Zn solubility in stomach was lower in ZnO (6.37 %) than $ZnSO_4$ powder (10.8 %; $P = 0.03$), ZnCl (10.6 %; $P = 0.03$) and Zn chelates (10.6 %; $P = 0.01$). Solubility was not different for 3000ZnO (3.90 %) compared to ZnO. Zn solubility in jejunum was no different among different

treatments, all treatments showed a solubility below 5%. 3000ZnO showed the lowest solubility both in stomach and jejunum.

ZnO is less soluble in gastrointestinal content of piglets than the rest of the products here tested. Zn concentration in soluble fraction is higher when high levels of ZnO are included. Previous results obtained in our lab showed that a high in-feed level of ZnO was able to increase Zn concentrations in serum and liver of weaned piglets in contrast to more soluble sources. This can suggest that a high in-feed ZnO may be transitory needed to provide more Zn is available for the young piglets.