Regulatory responses to excess zinc ingestion in growing rats

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Running head: Excess zinc in growing rats

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Abbreviations: BW: body weight; qRT-PCR, quantitative RT-PCR; Mt: Metallothionein; Igf-1: Insulin-like growth factor-1.

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Abstract

The growth of weaning piglets is effectively improved by feeding a high Zn diet (3,000 mg Zn/kg of diet). The present study examined whether feeding a diet supplemented with Zn (1,016–3,000 mg/kg) for 10 d induces growth benefits in rats. In addition, tissue weight, Zn content of tissues, and expression of Zn transporters were examined in these rats. Zn supplementation did not significantly increase body weight. Breaking line model analyses indicated that the weight of the pancreas, the organ most sensitive to excess Zn, significantly decreased with increasing Zn intake beyond 15.2 mg/d. Excess Zn has been suggested to accumulate in the liver, kidney, and bone in order to protect the pancreas. Zn concentrations in the plasma, liver, kidney, and femur increased with increasing Zn intake up to approximately 30 mg/d, whereas those in the pancreas increased up to 8.4 mg/d and decreased by Zn intake beyond 8.4 mg/d. The expression levels of the Zn transporters *Zip4* and *ZnT1* in the intestinal epithelium were significantly lower in rats fed a diet supplemented with 1,016 mg/kg Zn compared to those fed the basal diet. The present study reveals that (1) excess Zn intake does not accelerate growth in rats, but is detrimental to the pancreas, (2) the excess Zn is effectively accumulated in the liver, kidney, and bone, without sufficient protection of the pancreas, and (3) expression of the Zn transporters is down-regulated in response to excess Zn intake.

Introduction

Zn is an essential mineral that acts as a co-factor for numerous enzymes and transcription factors[1]. The physiological responses to Zn deficiency are well-characterised in mammals[2-5], whereas less information is available regarding the effects of excess Zn intake. The pancreas has been suggested as the tissue most sensitive to excess Zn[6]. The National Research Council (NRC)[7] proposes that Zn accumulates in tissues such as the liver, kidney, and bone, in order to protect other organs from failure induced by Zn accumulation. Zn concentrations were increased in the liver (6-fold) and in the kidney (11-fold) in pre-ruminant calves fed a diet supplemented with 500 to 700 mg Zn/kg, whereas the increases in Zn concentration in the heart and muscle were relatively smaller[8]; similar results were obtained in sheep fed a diet supplemented with 700 to 2,100 mg Zn/kg[9]. Furthermore, the Zn concentrations in the liver, kidney, and bone were higher in rats fed a diet supplemented with 2,438 mg Zn/kg compared with growing rats fed a diet containing 38 mg Zn/kg, and the Zn concentration of these tissues plateaued at supplementation levels of 2,438 to 7,238 mg Zn/kg[10].

Elevated Zn intake (3,000 mg/kg) for a period of 14 d surprisingly induces growth in weaning piglets[11-13]. Considering that the Zn requirement for growing pigs is 100 mg/kg[14], the Zn-induced growth promotion results from the pharmacological effects of excess Zn intake. The physiological basis for these pharmacological effects remains unclear. ZnO possesses antimicrobial properties[15], but several studies suggest that ZnO promotes growth in early-weaned and conventionally weaned pigs, regardless of diarrhoea prevalence or intestinal microbial numbers[16-18].

Zn homeostasis is primarily maintained by regulation of its absorption and secretion. Several Zn transporters of the Slc39 (Zip) and Slc30 (ZnT) families have been identified. Members of the Zip family have been shown to increase the cytosolic Zn concentration,

whereas those of the ZnT family decrease the cytosolic concentration[19-22]. Zip4 and ZnT1 are involved in Zn absorption in the small intestine[23, 24], whereas Zip5 is responsible for intestinal Zn secretion[25]. In addition, Zn is secreted from the pancreas into the gut by Zip5 and ZnT1[25, 26]. Zn transporter activities are modulated in response to Zn depletion through alteration of gene expression, transporter translocation, or both[22].

We hypothesised that growth promotion induced by excess Zn intake (3,000 mg/kg) is not limited to weaning piglets but is instead observed in other animals, including rats. In addition, it was hypothesised that each tissue grows proportionally in rats fed diets supplemented with excess Zn. In order to examine these points, we examined body and tissue growth, accumulation of Zn in tissues, and expression of Zn transporters in growing rats fed diets supplemented with excess Zn. We specifically examined (1) whether growth promotion induced by excess Zn is observed in growing rats, (2) whether the concept proposed by the NRC regarding the prevention from Zn toxicity is applicable, and (3) whether Zn transporter gene expression is altered in response to excess Zn ingestion. Our results indicate that excess Zn ingestion did not enhance the growth performance in growing rats, but in fact decreased pancreatic weight. Unexpectedly, the gene transcript levels of both the intestinal Zn transporters involved in Zn absorption and those involved in secretion were decreased in rats fed diets with higher Zn contents.

Materials and methods

Animals and diets

The experiments were approved by the Kyoto University Animal Experiment Committee (20-19). Twenty-eight male specific pathogen-free Sprague-Dawley rats

aged 4 wk were housed individually in stainless-steel cages under constant conditions (24°C, 50% humidity) with a fixed light-dark cycle (lights on from 0500 to 1900). Because excess Zn-induced growth promotion in weaning pigs is not necessarily due to the antimicrobial effects as described above, we used specific pathogen-free rats. After a 5 d acclimatisation period of feeding the basal diet (24 mg Zn/kg) shown in Table 1, rats were randomly assigned to receive diets with differing Zn concentrations. The requirement of Zn in growing rats is 12 mg/kg[27]; the basal diet contained twice as much Zn [27]. Zn content in the diet recommended for growing rats by AIN[28] is 30 mg/kg. All groups were allowed free access to food and distilled water for the 10 d study period. The diets were prepared by addition of ZnO to a Zn-deficient diet at the expense of glucose to provide a 24, 1,016, 2,008, or 3,000 mg Zn/kg diet, and the actual measured content was 23.8, 1,050, 2,090, or 3,200 mg Zn/kg diet, respectively. Since this study tested responses to dietary Zn status, i.e. excess intake as well as deficiency, egg white was used as a protein source. D-biotin was added to the basal diet due to the high avidin content of egg white in order to prevent biotin deficiency. Body weight (BW) and feed consumption were measured every day.

At the end of the 10-d experimental period, rats were killed by bleeding from the abdominal aorta under isoflurane anaesthesia. Tissues (the liver, kidney, pancreas, spleen, small intestine, testis, gastrocnemius muscle, femur, and perirenal fat pad) were collected and weighed. Blood collected with a heparinised syringe was centrifuged at 2,500 × *g* for 30 min at 4°C to obtain the plasma. The intestine was flushed with saline and scraped with slide glass to obtain the intestinal epithelium. Other tissues were rinsed in saline, immediately frozen in liquid nitrogen, and stored at -80°C until analysis.

Determination of mineral contents

After wet-ash digestion of diets, plasma, and tissues with trace-element grade nitric acid and hydrogen peroxide, Zn concentrations in diets, plasma, and tissues were measured by atomic absorption spectrometry (AA-6600F; Shimadzu, Kyoto, Japan). The analytical accuracy of the Zn determination was confirmed by analysis of a certified reference material from bovine liver (standard reference material 1577b, National Institute of Standards and Technology, Gaithersburg, MD, USA).

RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from the small intestine epithelium, pancreas, and liver using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Recovered RNA was used as a template for reverse transcriptase using random primers (ABI high capacity cDNA reverse transcription kit; Applied Biosystems). The qRT-PCR was carried out using a SYBR premix Ex Taq II kit (TaKaRa, Otsu, Japan) in a Roter-Gene 6000 instrument (Corbett Research, Mortlake, Australia). PCR was performed as follows: an initial denaturation step of 10 s at 95°C, followed by 40 cycles of 5 s at 95°C, and 20 s at 60°C. The dissociation (melting) curve of qRT-PCR products was subsequently examined by changing the ramp temperature from 60°C to 94°C. Each sample showed a single peak, suggesting that the expected PCR products were obtained. The PCR primers used to detect *Zip4*, *Zip5*, *ZnT1*, metallothionein-1a (*Mt-1a*), *Mt-2a*, insulin-like growth factor-1 (*Igf-1*), and hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) were as follows: 5′- AAC CCA CCA GGG AGG AGA - 3′ and 5′- TTC TGG AAA CCC CTG CTT C -3′ for *Zip4*; 5′- CCT CGG GCC TAG ACC TCT T - 3′ and 5′-AGC TGG GAA CCA TTC AGA CA-3′ for *Zip5*; 5′-AAC ACC AGC AAT TCC AAC G-3′ and 5′-CCA CTG GAT CAT CAC TTC TCA A-3′ for *ZnT1*; 5′-CAC CAG ATC TCG GAA TGG AC-3′ and 5′-GCA GCA GCT CTT CTT GCA G-3′ for *Mt-1a*; 5′-ACC TCC TGC AAG AAA AGC TG-3′ and 5′-ACT TGT CCG AAG CCT CTT TG-3′ for *Mt-2a*; 5′-GGA CGC TCT TCA ATT CGT GT-3′ and 5′-CTT CAG CGG

AGC ACA GTA CA-3′ for *Igf-1*; and 5′-GAC CGG TTC TGT CAT GTC G-3′ and 5′-ACC TGG TTC ATC ATC ACT AAT CAC-3′ for *Hprt1*. Gene transcript levels in each sample were determined using the relative standard curve method. The level of gene transcripts was expressed as a ratio relative to *Hprt1* mRNA, with the level in rats fed the basal diet set to 1.

Statistical analyses

Data are expressed as the least square mean ± SEM. All analyses were performed using SAS[29]. The data on BW and feed intake were subjected to the MIXED procedure. Each rat was determined an experimental unit and measurements of the same rat on different days were considered repeated measures. The statistical model included the effects of diet, experimental day, and the interaction between both. In addition, the effects of dietary Zn on tissue weight were analysed with the GLM procedure. Furthermore, when tissue Zn concentrations or weights were plotted against daily Zn intake, a breaking point of daily Zn intake indicating a plateau was explored using the NLIN procedure. A model with 1 breaking point and no limit of its slope value before or after the point was applied. When the model was significant, and when the slope was not significantly different from 0, the breaking point was further determined by application of the model with 1 breaking point with 0 as the slope after the point. Differences were considered significant at $P < 0.05$.

Results

Body and tissue growth

Time-course changes in BW showed an insignificant effect of diet, but the effect of the interaction between diet and experimental day was significant, suggesting that the effect of excess Zn on BW depended on the length of the treatment period (Fig. 1). This

indicates that, unlike in piglets, a diet supplemented with 3,000 mg Zn/kg does not have a beneficial effect on BW gain in rats. As for daily feed intake, both the diet effect and the interaction between diet and experimental day were not statistically significant (Fig. 2A). In addition, feed efficiency, i.e. weight gain per feed intake, was not significantly affected by the diet, the experimental day, or the interaction between both (Fig. 2B).

Typical symptoms of Zn toxicity are vomiting and gastrointestinal dysfunction[30], and the pancreas is the organ most sensitive to Zn toxicity[6]. Two rats fed a diet supplemented with 3,000 mg Zn/kg excreted soft stools for the last 3 days of the experimental period, although the other rats did not exhibit any symptoms throughout the study. The weight of the pancreas relative to BW was lower in rats fed a diet supplemented with 2,008 mg Zn/kg or 3,000 mg Zn/kg compared to those fed a diet supplemented with 24 mg Zn/kg or 1,016 mg Zn/kg, whereas no significant effects of the diet on the weight of the liver, kidney, spleen, testis, gastrocnemius muscle, femur, and perirenal fat were detected (Table 2). Plotting the pancreas weight against daily Zn intake revealed a decrease in rats that ingested Zn at levels above 15.2 mg/d (Fig. 3).

Plasma and tissue Zn concentration

Plasma and tissue Zn concentrations were higher in rats fed diets supplemented with more than 1,016 mg Zn/kg than in those fed the basal diet (data not shown). To examine the relationship between Zn intake and the Zn concentrations in detail, plasma and tissue Zn concentrations were plotted against daily Zn intake (Fig. 4). Zn concentrations in the plasma, liver, kidney, and femur increased linearly with increasing intake of Zn up to 31.3, 37.1, 28.3, and 35.1 mg/d, respectively, and eventually reached a plateau (Fig. 4A-D). These results suggest that Zn efficiently accumulates in the plasma, liver, kidney, and femur in response to increased Zn intake and that the capacity to retain Zn is limited. In view of the higher proportion of skeletal muscle weight relative to total

BW, approximately 60% of body Zn is stored in the muscle tissue[31]; however, the Zn concentration in the muscle was not significantly altered by increasing Zn intake (Fig. 4E).

The relationship between the Zn concentration in the pancreas and daily Zn intake also indicated that the breaking point was 8.4 mg/d; the pancreatic Zn concentration increased up to this point (Fig. 3F). In contrast to the plasma, liver, kidney, and femur, the Zn concentration in the pancreas linearly decreased in rats that ingested Zn above the breaking point.

Expression of Zn transporters, Mt, and Igf-1

The gene transcript levels of the transporters involved in intestinal absorption and secretion of Zn were examined by qRT-PCR (Fig. 5). Expression of *Zip4* in the intestinal epithelium, which is responsible for the uptake of Zn across the mucosal membrane[24], was significantly lower in rats fed a diet supplemented with 1,016 mg Zn/kg than in those fed the basal diet, with no differences among dietary groups with higher Zn contents (Fig. 5A). The expression of *ZnT1* in the intestinal epithelium, which promotes Zn transport from the cytosol of epithelial cells to the portal vein[23], was also down-regulated in rats fed diets supplemented with 1,016–3,000 mg Zn/kg (Fig. 5B). *Zip5* is located at the basolateral membrane of the intestinal epithelium and promotes Zn transport from the portal vein to epithelial cells[25]. The expression of *Zip5* was down-regulated in rats fed diets supplemented with 1,016–3,000 mg Zn/kg; the expression in rats fed a diet supplemented with 2,008 mg Zn/kg was higher than that in rats fed a diet containing 1,016 mg Zn/kg (Fig. 5C). No significant differences were detected in the expression of *ZnT1* and *Zip5*, transporters responsible for Zn secretion into the gut[25, 26], in the pancreas (Fig. 5D and E).

Mt is involved in Zn homeostasis, and Mt expression is induced by several metals including Zn[32, 33]. Expression of *Mt-1a* has been shown to change in parallel with that of *ZnT1* in response to Zn exposure in cultured hepatoma cells and fibroblasts[34]; we therefore evaluated *Mt* expression in this study. The expression levels of *Mt-1a* and *Mt-2a* in the intestinal epithelium were higher in rats fed diets supplemented with 2,008 or 3,000 mg Zn/kg than in those fed the basal diet (Fig. 6A and B); *Mt-1a* expression was higher in rats fed a diet supplemented with 2,008 mg Zn/kg than in those fed a diet containing 3,000 mg Zn/kg. The expression of *Mt-1a* and *Mt-2a* in the liver was higher in rats fed diets supplemented with 1,016–3,000 mg Zn/kg than in those fed the basal diet; the expression increased in a dose-dependent manner (Fig. 6C and D). The regulatory expression of hepatic *Mt-1a* and *Mt-2a* in response to excess Zn intake is similar to the changes in Zn accumulation in the liver (Fig. 4B); this reflects the fact that Zn accumulated in the liver is incorporated into Mt-1a and Mt-2a, which buffer excess Zn to provide protection from Zn toxicity[33]. No significant changes in *Mt* expression were detected in the pancreas (Fig. 6E and F).

A previous study suggested that the increased expression of *Igf-1* and its receptor are responsible for excess Zn-induced growth promotion in piglets[13]. We evaluated expression of *Igf-1* in the intestinal epithelium and in the liver, one of the major Igf-1-producing organs[35]. Expression of *Igf-1* was not significantly different among groups, irrespective of the tissues analysed (Fig. 7).

Discussion

The present study examined the short-term effects of extremely high Zn intake on body and tissue weight; tissue Zn concentration; and the expression of Zn transporters, Mt, and Igf-1 in growing rats. Our results revealed that, in contrast to studies in piglets,

excess intake of Zn did not have any beneficial effects on growth, but rather induced a decrease in weight of the pancreas. The NRC[7] proposed that excess Zn predominantly accumulates in organs such as the liver, kidney, and bone in order to protect the pancreas, the organ most sensitive to excess Zn. The present results basically support this model: Zn concentrations in the liver, kidney, and femur proportionally increased with increasing Zn intake up to 28–37 mg/d. However, the weight of the pancreas was lower in rats that ingested more than 15 mg/d Zn. This indicates that the amount of Zn intake required to induce atrophy of the pancreas is smaller than that required to reach a plateau in Zn accumulation in other tissues. Thus, the present results obtained in rats ingesting extremely high concentrations of Zn for a short period suggest the imperfect buffering capacity of the liver, kidney, and bone against excess Zn ingestion. Furthermore, our results suggest that Zn accumulation in the skeletal muscle, the tissue that stores the majority of Zn in the body, is not affected by dietary Zn intake.

Zn depletion-induced *Zip4* expression in the small intestine is well-characterised [36-38]. By contrast, less information is available regarding the expression of Zn transporters in animals fed excess Zn. Expression of *ZnT1* and *ZnT2* in the small intestine was significantly higher in rats fed a diet containing 180 mg Zn/kg for 2 wk than in those fed a diet containing 30 mg Zn/kg[39]. The present study indicates that excess Zn ingestion clearly down-regulates the mRNA expression of the Zn transporters involved in intestinal Zn absorption, *Zip4* and *ZnT1*. The decrease in *Zip4* expression was particularly evident; the gene transcript level of *Zip4* in rats fed a diet supplemented with 1,016 mg Zn/kg was only around 5% of that in rats fed the basal diet, whereas the *ZnT1* mRNA level of rats fed a diet containing 1,016 mg Zn/kg was around 35% of that in rats fed the basal diet. These results suggest that in addition to a system detecting Zn depletion, intestinal cells also have a system to sense excess Zn, and they partly regulate Zn absorption through transcriptional inhibition of Zn transporters.

ZnT1, *Mt-1a*, and *Mt-2a* are transcriptionally regulated by MTF-1, a Zn-sensing transcription factor[40], and the expression of both genes increases in response to Zn exposure in cultured cells[34, 41, 42]; however, excess Zn ingestion (>2,008 mg Zn/kg) caused the down-regulation of *ZnT1* mRNA expression in the intestinal epithelium but up-regulation of *Mt-1a* and *Mt-2a* mRNA expression. An unidentified additional regulatory mechanism of *ZnT1* expression is likely involved in rats fed a diet with extremely high Zn contents.

The capacity for Zn accumulation in the liver, kidney, and bone in response to excess Zn ingestion was limited. Rats that ingested 28–37 mg Zn/d could not accumulate additional Zn in these tissues; this Zn level corresponded to ingestion of a diet supplemented with 2,008 mg Zn/kg. These results suggest stimulation of Zn secretion by feeding diets containing more than 2,008 mg Zn/kg, or inhibition of Zn absorption, or both. Although Zip5 plays a role in Zn secretion across the intestinal mucosa[25], the gene transcript level of *Zip5* in the intestinal epithelium was not elevated but instead decreased in rats fed a diet supplemented with 1,016 mg Zn/kg compared to those fed the basal diet. Thus, it is unlikely that the Zip5-mediated Zn secretion is increased in rats fed diets with high Zn content. The pancreas is the major organ for endogenous Zn secretion into the gut[43]. Expression levels of pancreatic *Zip5* and *ZnT1*, which are expressed predominantly in the acinar cells of the pancreas[25, 26], were not increased in response to the ingestion of diets supplemented with excess Zn. These results suggest that the increased Zn secretion from the pancreas is not responsible for the limited Zn accumulation in the liver, kidney, and bone.

Intestinal Mt levels are inversely related to the rate of Zn absorption[44], and it has been suggested that Mt inhibits Zn absorption[45]. Thus, the present finding that expression of

Mt-1a and *Mt-2a* was up-regulated in the intestinal epithelium of rats fed diets supplemented with 2,008 or 3,000 mg Zn/kg may reflect an inhibition of intestinal Zn absorption in these rats. However, the expression of intestinal *Mt-1a* and *Mt-2a* was not significantly higher in rats fed the diet supplemented with 3,000 mg Zn/kg than in those fed the diet containing 2,008 mg Zn/kg. Thus, the limit of tissue Zn accumulation in rats fed the diet supplemented with 2,008 mg Zn/kg could not be explained by the Mt-induced inhibition of Zn absorption. Although gene transcript levels of intestinal *Zip4* were not further decreased in rats fed diets containing more than 1,016 mg Zn/kg in this study, processing and translocation of Zip4 are modified in response to changes in Zn status[46, 47]. Thus, post-translational modifications may be responsible for the defence against Zn ingestion in diets containing more than 2,008 mg Zn/kg.

In piglets, short-term feeding of a diet supplemented with 3,000 mg Zn/kg effectively enhanced BW gain[11-13], and this feeding regimen is applied in practice on pig farms. Li *et al.*[13] suggested that the excess Zn ingestion stimulates Igf-1-mediated signalling, which enhances the villous height of the small intestinal mucosa resulting in growth promotion of piglets. Considering that *Igf-1* expression in the small intestine and in the liver was not significantly increased in response to excess Zn ingestion, the inability to stimulate the Igf-1 axis may be one of the reasons why body growth was not accelerated in rats fed a diet supplemented with 3,000 mg Zn/kg.

The present study clarified that growing rats have defence mechanisms against excess Zn ingestion; in addition to the effective accumulation of excess Zn in the liver, kidney, and bone, the down-regulated mRNA expression of Zn transporters involved in the intestinal absorption contributes to the protection of the pancreas against excess Zn-mediated adverse effects. Furthermore, up-regulation of Mt expression in the small intestine results in the inhibition of Zn absorption. These multiple defences may

contribute to the relative tolerance to excess Zn ingestion.

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Table 1. Ingredients of the basal diet

	g/kg diet
Glucose	634.96
Egg white powder	200
Corn oil	100
Cellulose powder	20
Vitamin mixture[1]	10
D-biotin	0.01
Mineral mixture[2]	35
ZnO (77.3% Zn)	0.03

[1]The vitamin mixture (g/kg) contains: nicotinic acid, 3.000; Ca pantothenate, 1.600; pyridoxine-HCl, 0.700; thiamine-HCl, 0.600; riboflavin, 0.600; folic acid, 0.20; D-biotin, 0.020; vitamin B_{12} (cyanocobalamin) (0.1% in mannitol), 2.500; vitamin E (all-*rac*-α-tocopheryl acetate) (500 IU/g), 15.00; vitamin A (all-*trans*-retinyl palmitate)(500,000 IU/g), 0.800; vitamin D_3 (cholecalciferol) (400,000 IU/g), 0.250; vitamin K (phylloquinone), 0.075; powdered sucrose, 974.655.

[2]The mineral mixture (g/kg) contains: $CaCO_3$, 357.00; KH_2PO_4, 196.00; $K_3(C_6H_5O_7)\cdot H_2O$, 70.78; NaCl, 74.00; K_2SO_4, 46.60; MgO, 24.00; $Fe(C_6H_5O_7)$, 6.06; $MnCO_3$, 0.63; $CuCO_3$, 0.30; KIO_3, 0.01; Na_2O_3Se, 0.01025; $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 0.00795; $NaSiO_2\cdot 9H_2O$, 1.45; $CrK(SO_4)_2\cdot 12H_2O$, 0.275; LiCl, 0174; H_3BO_3, 0.0815; NaF, 0.0635; $NiCO_3$, 0.0318; NH_4VO_3, 0.0066; powdered sucrose 222.676.

Table 2. Relative tissue weight of rats fed the diets supplemented with excess Zn

(Mean values with standard errors, $n = 7$)

Dietary Zn, *mg/kg diet*:	24	1,016	2,008	3,000	SEM	$P <$
Tissue weight, mg/g BW						
Liver	45.9	45.0	47.8	48.5	1.4	NS
Kidney*	4.57	4.51	4.86	4.77	0.15	NS
Pancreas	6.19 [a]	6.57 [a]	5.18 [b]	5.19 [b]	0.28	0.003
Spleen	2.51	2.47	2.42	2.80	0.10	NS
Testis*	5.16	5.30	5.56	5.42	0.23	NS
Gastrocnemius muscle *	0.98	0.96	0.95	0.90	0.04	NS
Femur*	3.81	3.75	3.99	3.74	0.09	NS
Perirenal fat*	4.93	4.24	3.50	3.43	0.51	NS

Values represent means and SEM ($n = 7$).

*Average weight of tissues from both left and right parts.

Means without a common letter in superscripts differ significantly ($P < 0.05$).

Figure legends

Fig. 1. Time-course changes in BW in growing rats. Rats were fed diets containing various concentrations of Zn for 10 days. BW was plotted against experimental days. Values represent the means ± SEM (n = 7). ○: 24 mg Zn/kg, △: 1,016 mg Zn/kg, ◇: 2,008 mg Zn/kg, □: 3,000 mg Zn/kg.

Fig. 2. Time-course changes in daily feed intake and feed efficiency in growing rats. Rats were fed diets containing various concentrations of Zn for 10 days. Daily feed intake (A) and feed efficiency (B) were plotted against experimental days. Values represent the means ± SEM (n = 7). ○: 24 mg Zn/kg, △: 1,016 mg Zn/kg, ◇: 2,008 mg Zn/kg, □: 3,000 mg Zn/kg.

Fig. 3. Effects of excess Zn intake on the weight of the pancreas in growing rats. Rats were fed diets with various concentrations of Zn for 10 days. The weight of the pancreas relative to BW was plotted against average daily intake of Zn. The breaking point of daily Zn intake on the pancreas weight was calculated, and shown in the figure by an arrow. ○: 24 mg Zn/kg, △: 1,016 mg Zn/kg, ◇: 2,008 mg Zn/kg, □: 3,000 mg Zn/kg.

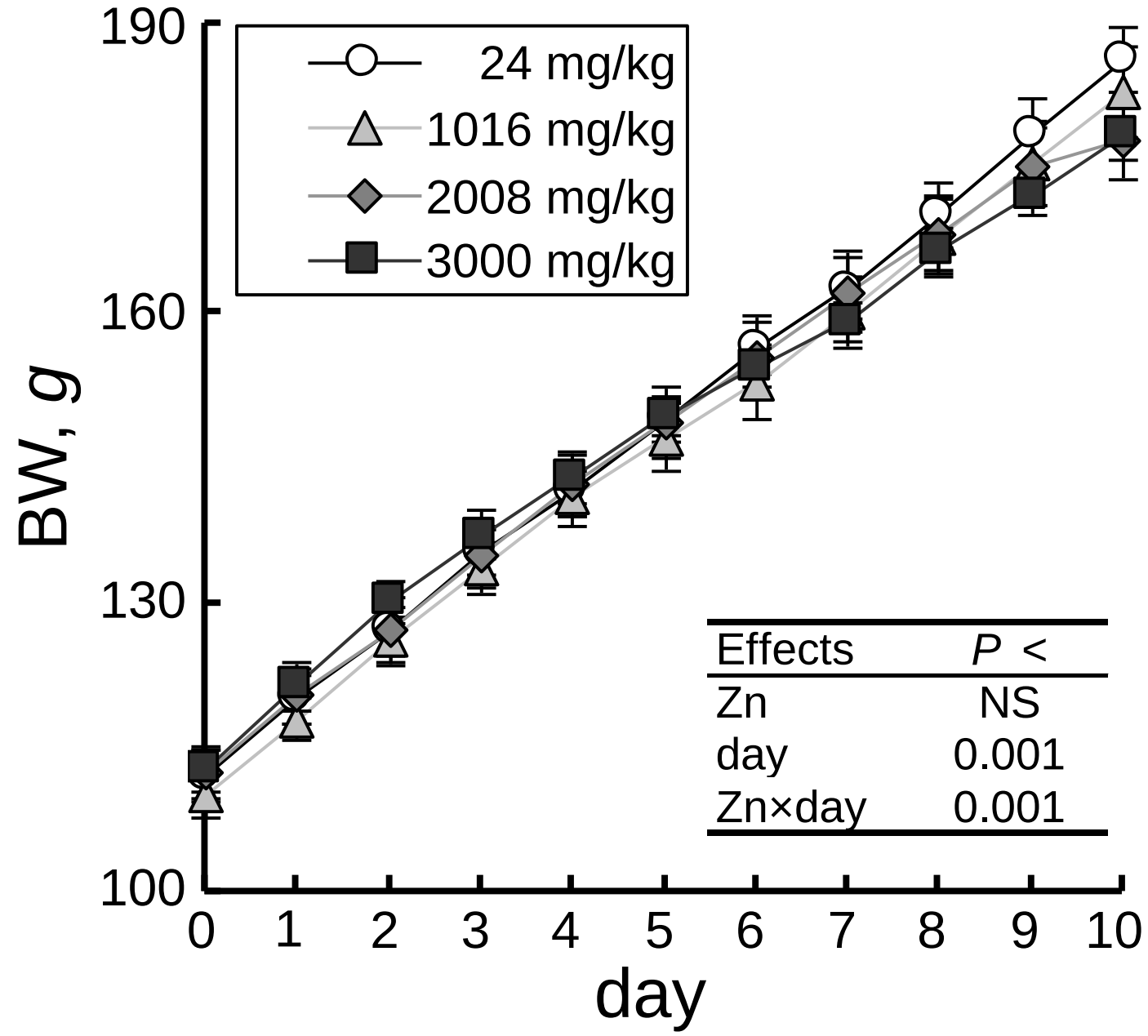
Fig. 4. Relationship between Zn intake and plasma and tissue concentrations of Zn in growing rats. Rats were fed diets with various concentrations of Zn for 10 days. Zn concentrations in the plasma (A), liver (B), kidney (C), femur (D), gastrocnemial muscle (E), and pancreas (F) were plotted against average daily intake of Zn. The breaking point of daily Zn intake on plasma and tissue concentrations of Zn was calculated, and indicated in the figure by an arrow. ○: 24 mg Zn/kg, △: 1,016 mg Zn/kg, ◇: 2,008 mg Zn/kg, □: 3,000 mg Zn/kg. Note that there was no break point

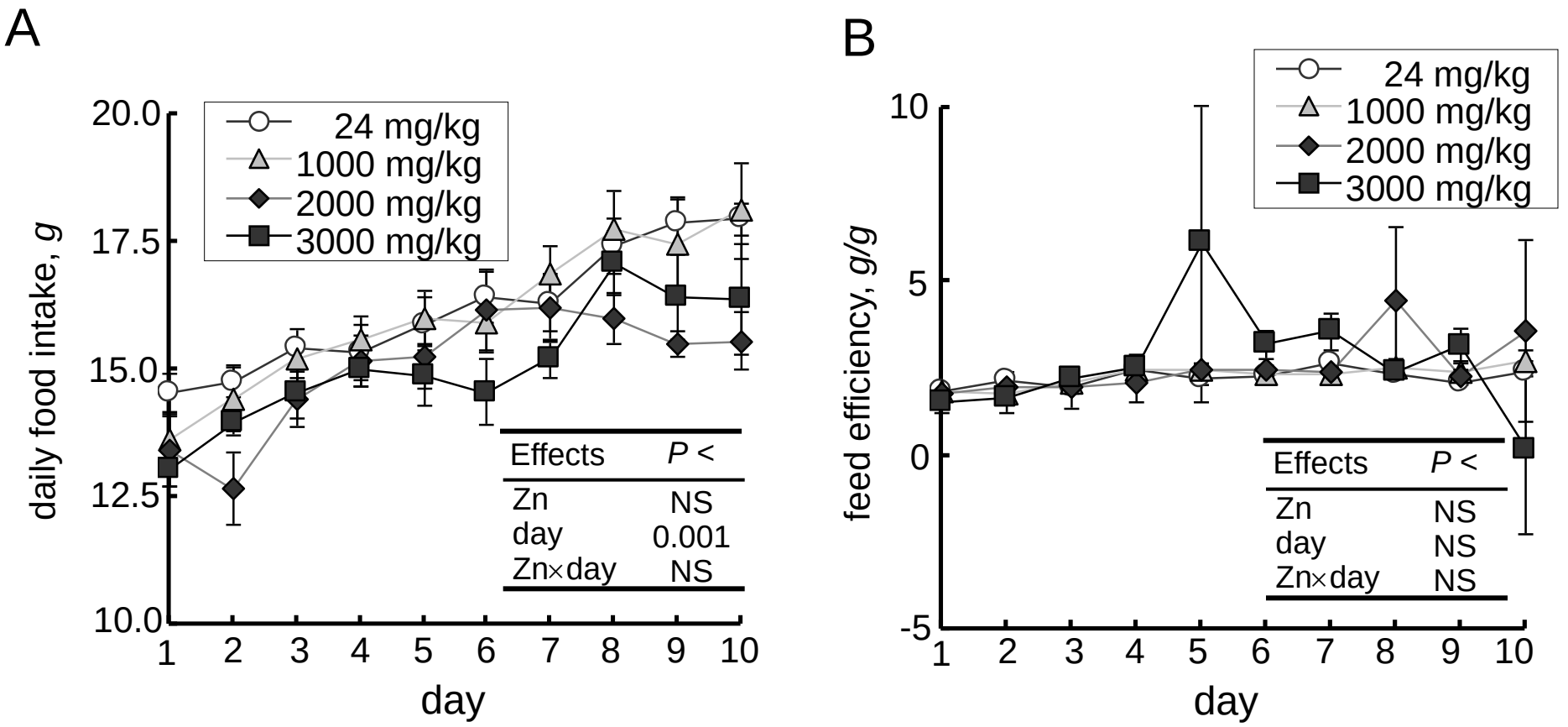
on Zn concentration in the gastrocnemial muscle.

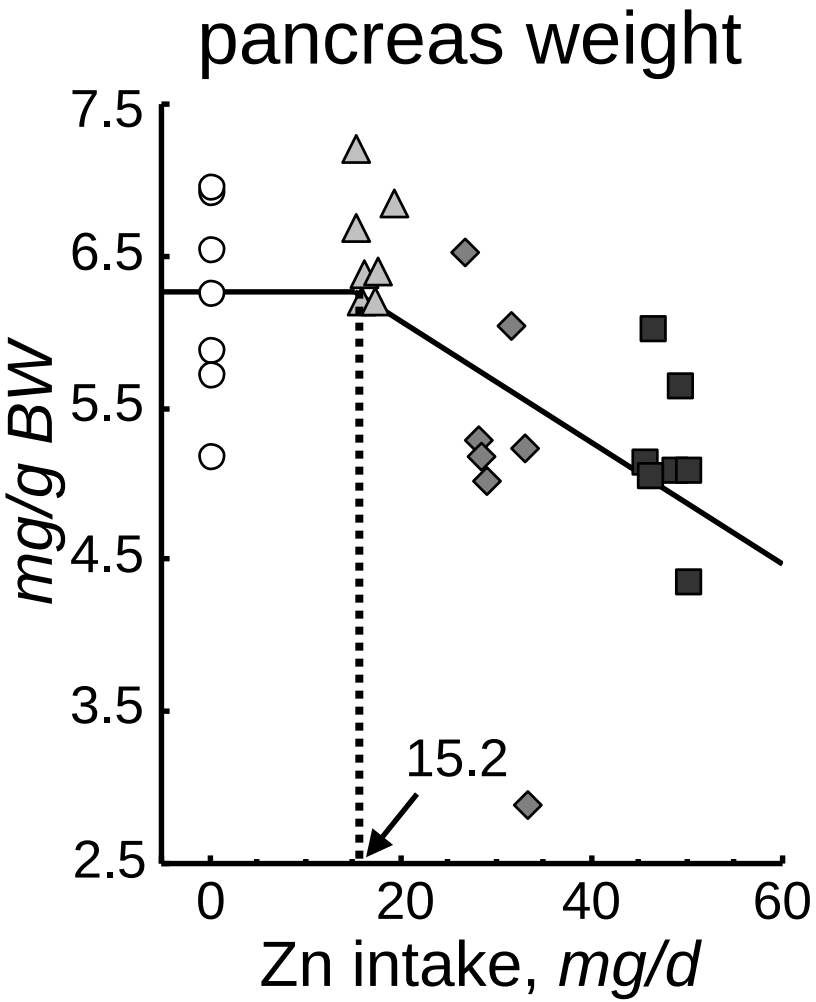
Fig. 5. Gene expression of Zn transporters in the small intestinal epithelium and the pancreas of rats. Rats were fed diets supplemented with various concentrations of Zn for 10 days. Gene expression of *Zip4* (A), *ZnT1* (B), and *Zip5* (C) in the small intestine, and *ZnT1* (D) and *Zip5* (E) in the pancreas was examined by qRT-PCR. The transcription levels were expressed as ratios to *Hprt1* with the level in rats fed the basal diet set to 1. Values represent the means + SEM (n = 7). Means that do not have a common letter above the bars differ significantly ($P < 0.05$).

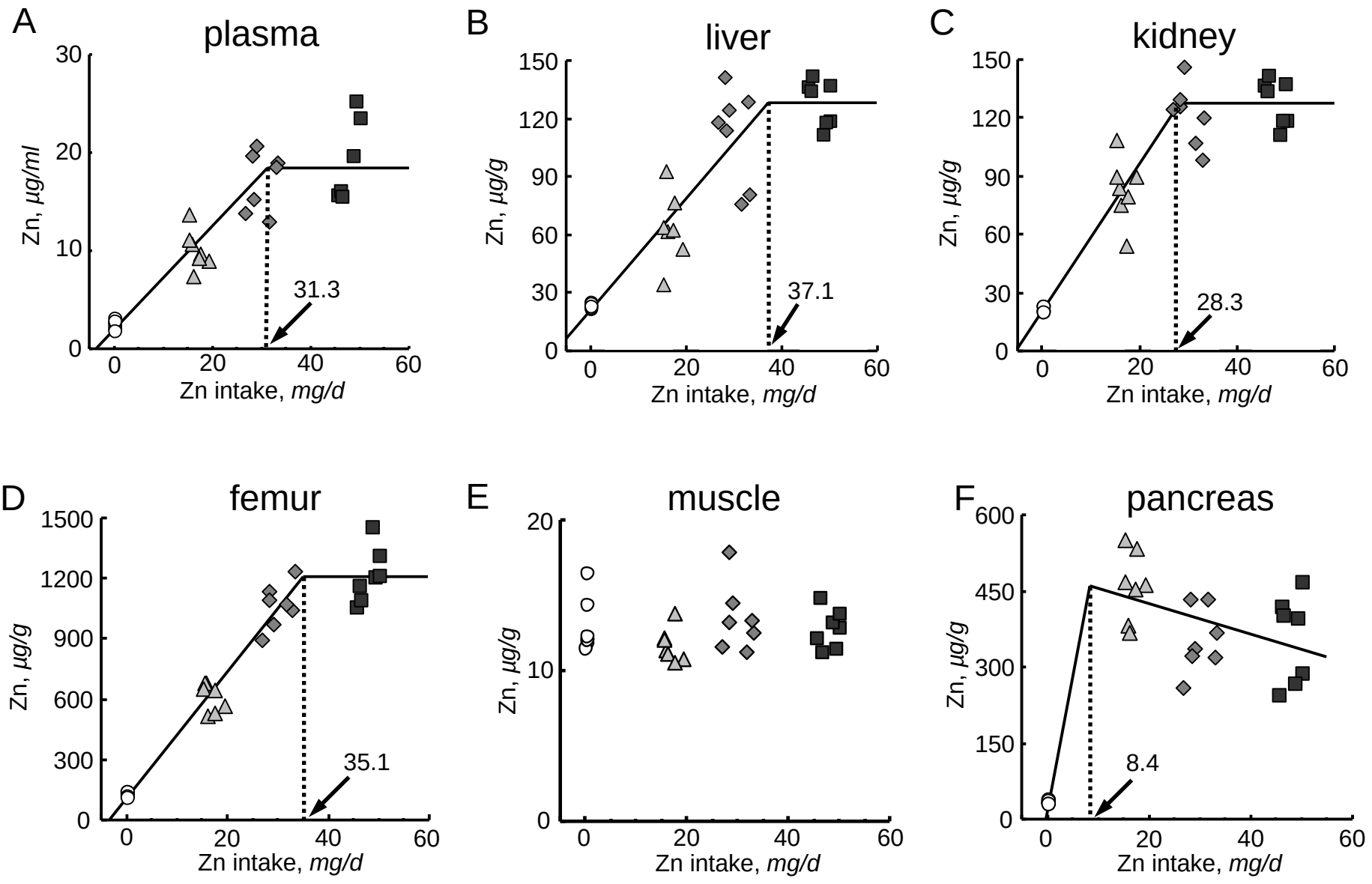
Fig. 6. Gene expression of Mt in the small intestinal epithelium, liver, and pancreas of rats. Rats were fed diets supplemented with various concentrations of Zn for 10 days. Gene expression of *Mt-1a* (A, C, and E) and *Mt-2a* (B, D, and F) in the small intestine (A and B), liver (C and D), and pancreas (E and F) was examined by qRT-PCR. The transcription levels were expressed as ratios to *Hprt1* with the level in rats fed the basal diet set to 1. Values represent the means + SEM (n = 7). Means that do not have a common letter above the bars differ significantly ($P < 0.05$).

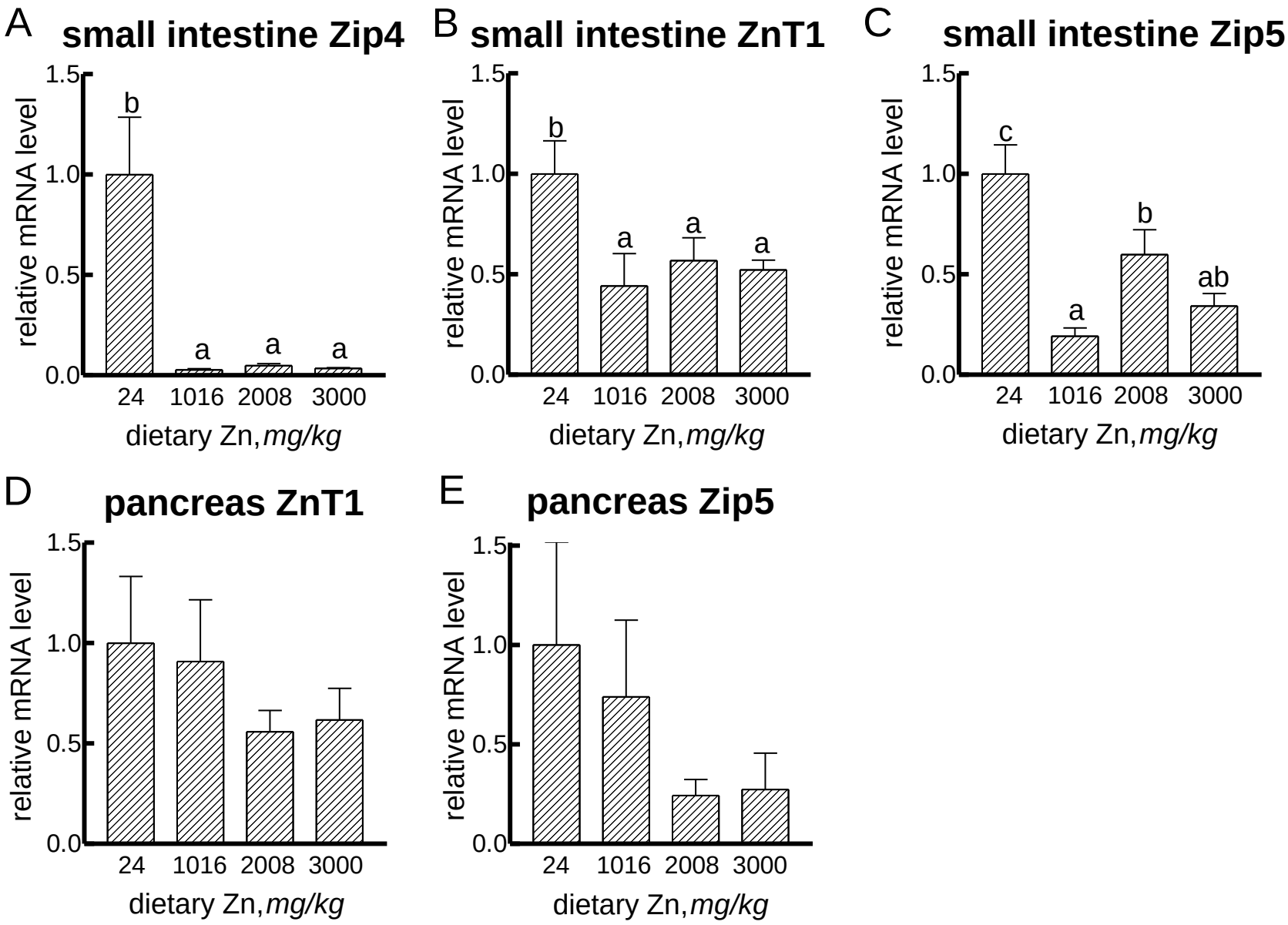
Fig. 7. Gene expression of Igf-1 in the small intestinal epithelium and liver of rats. Rats were fed diets supplemented with various concentrations of Zn for 10 days. Gene expression of *Igf-1* in the small intestine (A) and liver (B) was examined by qRT-PCR. The transcription levels were expressed as ratios to *Hprt1* with the level in rats fed the basal diet set to 1. Values represent the means + SEM (n = 7). Means that do not have a common letter above the bars differ significantly ($P < 0.05$).

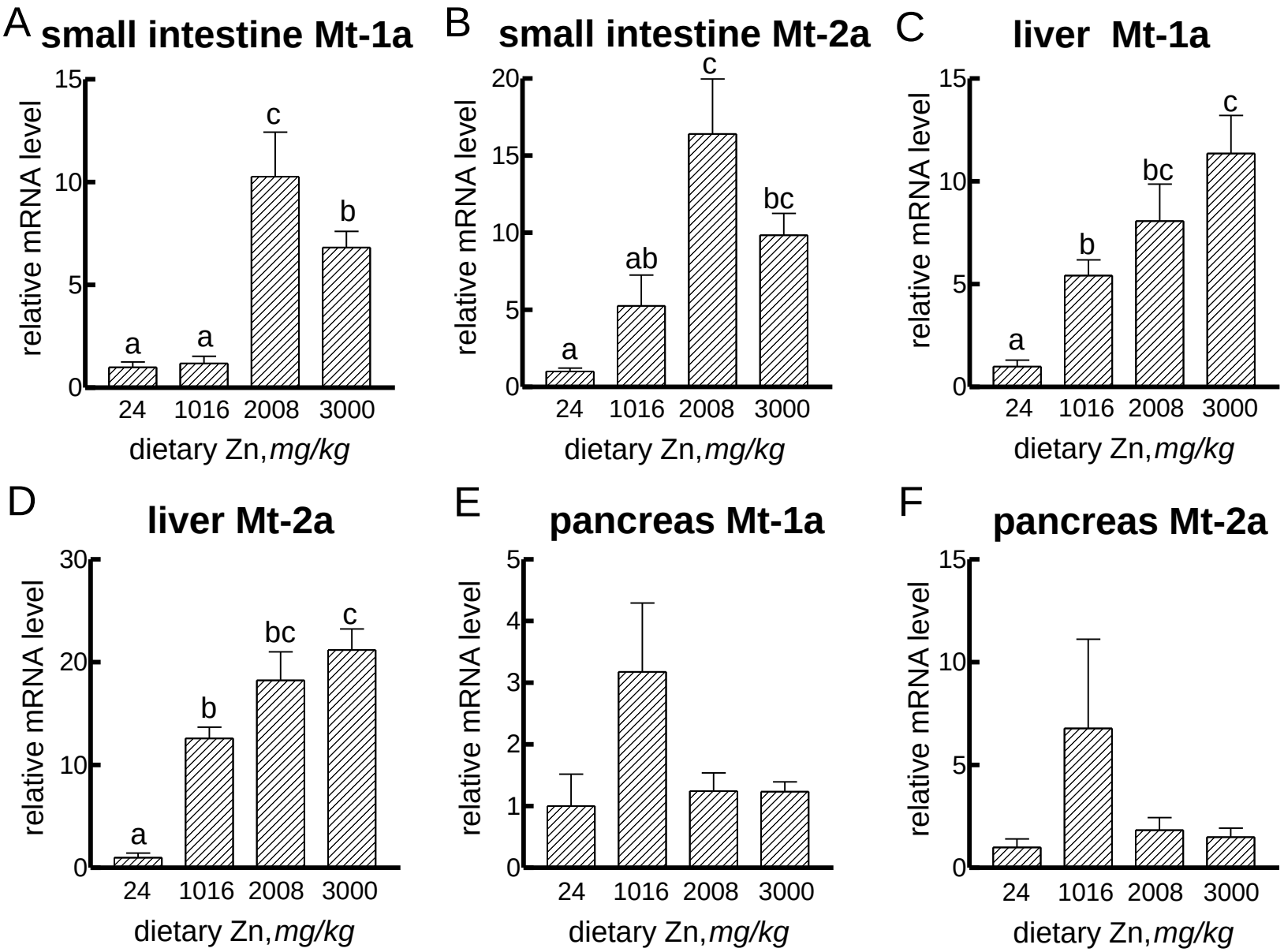

24 mg/kg
1016 mg/kg
2008 mg/kg
3000 mg/kg
BW, g
190
160
130
100
0
1
2
3
4
5
6
7
8
9
10
day
Effects
P <
Zn
NS
day
0.001
Zn×day
0.001

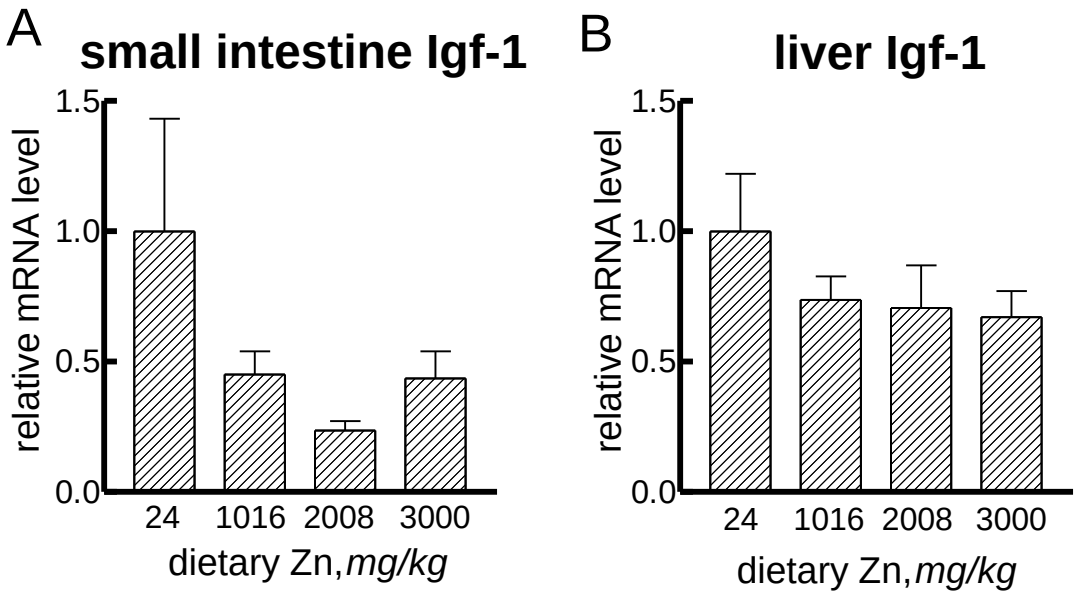

A
daily food intake, g
20.0
17.5
15.0
12.5
10.0
1
2
3
4
5
6
7
8
9
10
day
24 mg/kg
1000 mg/kg
2000 mg/kg
3000 mg/kg
Effects
P <
Zn
NS
day
0.001
Zn×day
NS
B
feed efficiency, g/g
10
5
0
-5
1
2
3
4
5
6
7
8
9
10
day
24 mg/kg
1000 mg/kg
2000 mg/kg
3000 mg/kg
Effects
P <
Zn
NS
day
NS
Zn×day
NS


pancreas weight
7.5
6.5
5.5
4.5
3.5
2.5
mg/g BW
15.2
0
20
40
60
Zn intake, mg/d


A
plasma
Zn, μg/ml
Zn intake, mg/d
31.3
B
liver
Zn, μg/g
Zn intake, mg/d
37.1
C
kidney
Zn, μg/g
Zn intake, mg/d
28.3
D
femur
Zn, μg/g
Zn intake, mg/d
35.1
E
muscle
Zn, μg/g
Zn intake, mg/d
F
pancreas
Zn, μg/g
Zn intake, mg/d
8.4


A
small intestine Zip4
relative mRNA level
1.5
1.0
0.5
0.0
b
a
a
a
24
1016
2008
3000
dietary Zn, mg/kg
B
small intestine ZnT1
relative mRNA level
1.5
1.0
0.5
0.0
b
a
a
a
24
1016
2008
3000
dietary Zn, mg/kg
C
small intestine Zip5
relative mRNA level
1.5
1.0
0.5
0.0
c
a
b
ab
24
1016
2008
3000
dietary Zn, mg/kg
D
pancreas ZnT1
relative mRNA level
1.5
1.0
0.5
0.0
24
1016
2008
3000
dietary Zn, mg/kg
E
pancreas Zip5
relative mRNA level
1.5
1.0
0.5
0.0
24
1016
2008
3000
dietary Zn, mg/kg


A small intestine Mt-1a
relative mRNA level
15
10
5
0
a
a
c
b
24
1016
2008
3000
dietary Zn, mg/kg
B small intestine Mt-2a
relative mRNA level
20
15
10
5
0
a
ab
c
bc
24
1016
2008
3000
dietary Zn, mg/kg
C liver Mt-1a
relative mRNA level
15
10
5
0
a
b
bc
c
24
1016
2008
3000
dietary Zn, mg/kg
D liver Mt-2a
relative mRNA level
30
20
10
0
a
b
bc
c
24
1016
2008
3000
dietary Zn, mg/kg
E pancreas Mt-1a
relative mRNA level
5
4
3
2
1
0
24
1016
2008
3000
dietary Zn, mg/kg
F pancreas Mt-2a
relative mRNA level
15
10
5
0
24
1016
2008
3000
dietary Zn, mg/kg


A
small intestine Igf-1
relative mRNA level
1.5
1.0
0.5
0.0
24
1016
2008
3000
dietary Zn, mg/kg
B
liver Igf-1
relative mRNA level
1.5
1.0
0.5
0.0
24
1016
2008
3000
dietary Zn, mg/kg