Supplementary Material



Supplemental Figure 1. Effects of varying supplementation of Zn from ZnSO4 \* H2O on the average total analyzed diet Zn concentration in experimental diet batches (A) as well as the correlation of total analyzed diet Zn concentrations in 1control vs. 1treatment experimental dietary batches. (Supplemental Table 1 shows the statistical measures of respective models).

1Treatment diets received 200 mg GLDA / kg diet and control diets received 200 mg hydrated silica/kg diet as a filler, respectively, each in the presence of varying dosages of dietary Zn from ZnSO4 \* H2O (+0, +5, +10, +15, +20, +25, +45 and +75 mg/kg) yielding average total dietary Zn concentrations of 30.9, 35.7, 40.5, 45.3, 50.8, 55.6, 74.2 and 103 mg/kg. Diet Zn analysis was based on triplicate weighing of three independent diet samples per dose level, respectively, Values are arithmetic means ± SDs, n = 8. Error bars represent respective standard deviation from mean values. Presented regression curves express no significant difference in curve parameters (slopes, intercepts) at P ≤ 0.05 based on T-statistics. Diet zinc, dietary zinc; GLDA, L-glutamic acid N,N-diacetic acid, tetrasodium salt; Zn, Zn, zinc.

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| Supplemental Table 1. Average analysed concentrations of GLDA and Zn in non-supplemented and GLDA supplemented dietary batches receiving varying supplementation of Zn from ZnSO4 \* H2O. |
| Zn supplementation level, mg/kg | GLDA supplementation level, mg/kg | Analysed diet Zn,mg/kg | Analysed diet GLDA,mg/kg |
| 0 | 0 | 31.3 | <1 |
| 5 | 0 | 35.4 | <1 |
| 10 | 0 | 40.3 | <1 |
| 15 | 0 | 45.2 | <1 |
| 20 | 0 | 49.5 | <1 |
| 25 | 0 | 54.3 | <1 |
| 45 | 0 | 74.3 | <1 |
| 75 | 0 | 102.6 | <1 |
| 0 | 200 | 30.5 | 197 |
| 5 | 200 | 35.9 | 200 |
| 10 | 200 | 40.6 | 200 |
| 15 | 200 | 45.4 | 203 |
| 20 | 200 | 52.1 | 194 |
| 25 | 200 | 56.8 | 194 |
| 45 | 200 | 74.0 | 199 |
| 75 | 200 | 104.1 | 197 |
| 1Each result represents the average value determined in 3 independent feed samples, analysed in duplicate weighing; GLDA, L-glutamic acid N,N-diacetic acid, tetrasodium salt; Zn, zinc. |

Supplemental Table 2. PCR primer and PCR product specifications.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Gene | Accession number | Sequence | Position on template(bp, 5’🡪3’) | Product length(bp) |
| *ACTB* | XM\_003357928.1 | fw GACTCAGATCATGTTCGAGACCTT | 449 🡪 551 | 103 |
|  |   | rev CATGACAATGCCAGTGGTGC |  |  |
| *B2M* | NM\_213978.1 | fw CTTTTCACACCGCTCCAGTA | 35 🡪 154 | 120 |
|  |   | rev GGCGTGAGTAAACCTGAACC |  |  |
| *GAPDH* | NM\_001206359.1 | fw CACATGGCCTCCAAGGAGTAA | 1082 🡪 1210 | 129 |
|  |  | rev GGAGATGCTCGGTGTGTTGG |  |  |
| *GUSB* | NM\_001123121.1 | fw TCACGAGGATCCACCTCTCAT | 1647 🡪 1808 | 162 |
|  |  | rev CCTATGGCCCTCTGAGGTGA |  |  |
| *H3* | NM\_213930.1 | fw CTTTGCAGGAGGCAAGTGAG | 333 🡪 445 | 113 |
|  |  | rev GCGTGCTAGCTGGATGTCT |  |  |
| *HPRT1* | NM\_001032376.2 | fw CCCCAGCGTCGTGATTAGTG | 111 🡪 241 | 131 |
|  |   | rev GCCGTTCAGTCCTGTCCATA |  |  |
| *LDHA* |  NM\_001172363.2 | fw CACCCCCTAAGCTGTCATGG  | 649 🡪 797 | 149 |
|  |   | rev ACCGCTTTCCAGTGTTCCTT |  |  |
| *TFRC* | NM\_214001.1 | fw GGTTCTTTTGTGTGGCAGCT | 12 🡪 85 | 74 |
|  |  | rev GGTTCTCCGCCAAACAAACT |  |  |
| *UBC* | XM\_003483411.1 | fw AGTGATGGCCAGTGAAGCAA | 2306 🡪 2442 | 137 |
|  |  | rev GCAGGCCACTGAGAGCTAAT |  |  |
| *Mt1A* | NM\_001001266.2 | fw TCTCACCTGCCTCCACTCAT | 33 🡪 153 | 121 |
|  |  | rev AGCAGCAGCTCTTCTTGCAG |  |  |

Primers were designed with Primer Blast using published porcine sequence information (37). Position on template indicates first base at 5 and last base at 3’ side of the DNA strand to which the respective primer pair binds, yielding the product length by polymerase activity, presented in the next column. *ACTB*, *beta-actin*; *B2M*, *β2 microglobuline*; bp, base pair; fw, forward primer; *GAPDH*, *glycerinaldehyde-3-phosphatedehydrogenase*; *GUSB*, *beta-glucuronidase*; *H3*, *histone 3*; *HPRT1*, *hypoxanthine-guanine phosphoribosyltransferase*; *LDHA*, *lactate dehydrogenase A*; *Mt1A*, *Metallothionein 1A*; rev, reverse primer; *TFRC*, *transferrin receptor protein 1*; *UBC*, *ubiquitin C*.; 5’🡪3’; indicates direction of polymerase activity on respective DNA strands.

Supplemental Table 3. Zootechnical performance of animals in response to varying dietary Zn and dietary GLDA supplementation.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Dietary Zn supplementation (mg/kg) |  | Treatment effects2 (p-value) |
|  | 0(30.9)1 | 5(35.7) | 10(40.4) | 15(45.3) | 20(50.8) | 25(55.6) | 45(74.1) | 75(103.3) | SEM3 | Zn | GLDA-Na4 | Zn\*GLDA-Na4 |
| Body weight (kg) |  |  |  |  |  |  |  |  |  |  |  |
| Start acclimatization period | 7.7 | 8.1 | 7.9 | 8.0 | 8.2 | 7.8 | 8.1 | 7.8 | 0.34 | 0.96 | 0.84 | 1.00 |
| 4E1 | 12.4 | 12.7 | 12.8 | 12.7 | 13.0 | 12.7 | 13.2 | 12.7 | 0.50 | 0.97 | 0.68 | 1.00 |
| 5E8 | 15.6 | 16.2 | 16.3 | 16.1 | 16.4 | 16.1 | 16.8 | 16.2 | 0.46 | 0.75 | 0.39 | 0.94 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
| Daily weight gain (kg) | 0.403 | 0.444 | 0.435 | 0.417 | 0.428 | 0.423 | 0.455 | 0.433 | 0.019 | 0.63 | 0.24 | 0.38 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
| Daily feed intake (kg) | 0.438 | 0.445 | 0.448 | 0.448 | 0.448 | 0.448 | 0.448 | 0.444 | 0.003 | 0.13 | 0.73 | 0.70 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
| Feed:gain | 1.10 | 1.03 | 1.08 | 1.14 | 1.08 | 1.07 | 1.00 | 1.04 | 0.06 | 0.78 | 0.26 | 0.37 |
| 1Between brackets, the average analysed total Zn concentrations determined in the different experimental feeds. 2Treatment effects with p≤0.05 were considered statistically significant; 3SEM = standard error of means (pooled standard error of the linear model); 4E1 = 1stexperimental day; 5E8 = 8th (last) experimental day; GLDA, L-glutamic acid N,N-diacetic acid, tetrasodium salt; Zn, zinc. |

Supplemental Table 4. Broken-line regression analyses of the response of 1apparently absorbed diet zinc (mg/kg diet intake) in weaned piglets fed 2control and 2treatment diets for 8d

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | 3Regression models | Parameter estimates | *P* values | *R²* |
| Control | *y* = a1 + *b1x* for *x* ≤ *XB* | *XB*, 54.3 ± 4.93 | <0.0001 | 0.97 |
|  | *y* = a2 + *b2X* for *x* > *XB* | *YB*, 2.25 ± 0.71 | 0.01 |  |
|  |  | *a1*, -8.40 ± 1.40 | 0.004 |  |
|  |  | *a2*, -1.75 ± 0.18 | 0.01 |  |
|  |  | *b1*, 0.20 ± 0.03 | 0.0001 |  |
|  |  | *b2*, 0.07 ± 0.01 | 0.0006 |  |
| GLDA-Na4 | *y* = a1 + *b1x* for *x* ≤ *XB* | *XB*, 53.2 ± 3.57 | <0.0001 | 0.96 |
|  | *y* = a2 + *b2X* for *x* > *XB* | *YB*, 3.69 ± 0.52 | <0.0001 |  |
|  |  | *a1,* -6.94 ± 1.35 | 0.007 |  |
|  |  | *a2*, 1.33\* ± 0.12 | 0.06 |  |
|  |  | *b1*, 0.20 ± 0.03 | <0.0001 |  |
|  |  | *b2*, 0.04 ± 0.01 | 0.008 |  |

1Apparently absorbed feed Zn was calculated on the basis of respective ratios of Zn and TiO2 concentrations in feed and feces, respectively, and is expressed as mg/kg feed intake;2Treatment diets received 200 mg GLDA- Na4/kg and control diets received 200 mg hydrated silica/kg diet as a filler, respectively, each in the presence of varying dosages of dietary Zn from ZnSO4 \* H2O (+0, +5, +10, +15, +20, +25, +45 and +75 mg/kg) yielding average total dietary Zn concentrations of 30.9, 35.7, 40.5, 45.3, 50.8, 55.6, 74.2 and 103 mg/kg. 3Broken-line regression models were estimated on the basis of independent arithmetic group means relative to dietary zinc concentration (n = 8). Parameter estimates are presented as means ± SEs to indicate the precision of estimation. *P ≤ 0.05* was considered to be significant. *b1*, slope of the broken-line regression curves over dietary zinc doses ≤ *XB*; *b2*, slope of the broken-line regression curves over dietary zinc doses >*XB*; GLDA-Na4,L-glutamic acid N,N- diacetic acid, tetrasodium salt; *XB*, *X* intercept of the breakpoint in the parameter response; *YB*, *Y* intercept of the breakpoint in the parameter response; Zn, zinc.

Supplemental Table 5. Linear regression of the response of zinc (mg/L), relative 1zinc-binding capacity (%) and alkaline phosphatase activity (U/L) in blood plasma as well as 2bone zinc (mg/kg ash) in weaned piglets fed control and treatment diets for 8d.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | 3Regression models | Parameter estimates | *P* values | *R2* |
| Plasma Zn | Control | y = a + bx | *a*, 0.05 ± 0.04 | 0.30 | 0.90 |
|  |  |  | *b*, 0.005 ± 0.0007 | 0.0003 |  |
|  | GLDA-Na4 | y = a + bx | *a*, 0.09 ± 0.04 | 0.07 | 0.92 |
|  |  |  | *b*, 0.005 ± 0.0007 | 0.0002 |  |
| Plasma ZBC | Control | y = a + bx | *a*, 96.6 ± 1.32 | <0.0001 | 0.97 |
|  |  |  | *b*, -0.30 ± 0.02 | <0.0001 |  |
|  | GLDA-Na4 | y = a + bx | *a*, 89.8 ± 2.37 | <0.0001 | 0.91 |
|  |  |  | *b*, -0.32 ± 0.04 | 0.0002 |  |
| Plasma APA | Control | y = a + bx | *a*, 102 ± 31.1 | 0 02 | 0.80 |
|  |  |  | *b*, 2.61 ± 0.53 | 0.003 |  |
|  | GLDA-Na4 | y = a + bx | *a*, 173 ± 22.2 | 0.0002 | 0.80 |
|  |  |  | *b*, 1.87 ± 0.38 | 0.003 |  |
| Bone Zn | Control | y = a + bx | *a*, 106 ± 6.30 | <0.0001 | 0.97 |
|  |  |  | *b*, 1.40 ± 0.11 | <0.0001 |  |
|  | GLDA-Na4 | y = a + bx | *a*, 128 ± 7.12 | <0.0001 | 0.95 |
|  |  |  | *b*, 1.29 ± 0.12 | <0.0001 |  |

1Relative zinc binding capacity describes the percentage amount of free zinc binding sites in blood plasma; 2Bone samples included the left femoral head of every animal; 2Treatment diets received 200 mg GLDA- Na4/kg and control diets received 200 mg hydrated silica/kg diet as a filler, respectively, each in the presence of varying dosages of dietary Zn from ZnSO4 \* H2O (+0, +5, +10, +15, +20, +25, +45 and +75 mg/kg) yielding average total dietary Zn concentrations of 30.9, 35.7, 40.5, 45.3, 50.8, 55.6, 74.2 and 103 mg/kg. 3Linear regression models were estimated on the basis of independent arithmetic group means relative to dietary zinc concentration (n = 8). Parameter estimates are presented as means ± SEs to indicate the precision of estimation. *P ≤ 0.05* was considered to be significant. a, Y-intercept of the respective linear regression curve; APA, alkaline phosphatase activity; b, slope of the respective linear regression curve; GLDA-Na4,L-glutamic acid N,N- diacetic acid, tetrasodium salt; ZBC, relative zinc binding capacity; Zn, zinc.

Supplemental Table 6. Broken-line regression analyses of the response of liver zinc (mg/kg DM) and relative hepatic *metallothionein 1A* gene expression 1(xfold) in weaned piglets fed 2control and 2treatment diets for 8d.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | 3Regression models | Parameter estimates | *P* values | *R2* |
| Liver Zn | Control | *y* = a1 + *b1x* for *x* ≤ *XB* | *XB*, 70.4 ± 8.30 | <0.0001 | 0.96 |
|  |  | *y* = a2 + *b2X* for *x* > *XB* | *YB*, 88.3 ± 7.04 | <0.0001 |  |
|  |  |  | *a1*, 59.55 ± 5.59 | 0.0001 |  |
|  |  |  | *a2*, 14.6 ± 0.00 | <0.0001 |  |
|  |  |  | *b1*, 0.41 ± 0.14 | 0.02 |  |
|  |  |  | *b2*, 1.05 ± 0.14 | <0.0001 |  |
|  | GLDA-Na4 | *y* = a1 + *b1x* for *x* ≤ *XB* | *XB*, 55.6 ± 14.2 | 0.004 | 0.96 |
|  |  | *y* = a2 + *b2X* for *x* > *XB* | *YB*, 92.8 ± 9.62 | <0.0001 |  |
|  |  |  | *a1,* 61.1± 14.0 | 0.01 |  |
|  |  |  | *a2,* 28.8 ± 8.21 | 0.28 |  |
|  |  |  | *b1*, 0.57 ± 0.23 | 0.04 |  |
|  |  |  | *b2*, 1.15 ± 0.22 | 0.0008 |  |
| Liver *MT1A* | Control | *y* = a1 + *b1x* for *x* ≤ *XB* | *XB*, 61.3 ± 1.96 | <0.0001 | 0.99 |
|  |  | *y* = a2 + *b2X* for *x* > *XB* | *YB*, 0.12 ± 0.02 | 0.001 |  |
|  |  |  | *a1,* -0.10 ± 0.04 | 0.05 |  |
|  |  |  | *a2,* -1.17 ± 0.00006 | <0.0001 |  |
|  |  |  | *b1*, 0.004 ± 0.0009 | 0.004 |  |
|  |  |  | *b2*, 0.02 ± 0.0009 | <0.0001 |  |
|  | GLDA-Na4 | *y* = a1 + *b1x* for *x* ≤ *XB* | *XB*, 45.3 ± 0.05 | <0.0001 | 0.98 |
|  |  | *y* = a2 + *b2X* for *x* > *XB* | *YB*, 0.37 ± 0.10 | 0.008 |  |
|  |  |  | *a1,* -0.83 ± 0.42 | 0.28 |  |
|  |  |  | *a2,* -2.38 ± 0.58 | 0.06 |  |
|  |  |  | *b1*, 0.03 ± 0.01 | 0.11 |  |
|  |  |  | *b2*, 0.06 ± 0.004 | <0.0001 |  |

1xfold differences in relative hepatic *MT1A* gene expression response in treatment and control groups were calculated relative to a gene expression response of 1.0 (not regulated) in the highest Zn supplied control group (103 mg/kg diet without GLDA-Na4 addition) using the relative gene expression value according to Livak and Schmittgen [37];2Treatment diets received 200 mg GLDA- Na4/kg and control diets received 200 mg hydrated silica/kg diet as a filler, respectively, each in the presence of varying dosages of dietary Zn from ZnSO4 \* H2O (+0, +5, +10, +15, +20, +25, +45 and +75 mg/kg) yielding average total dietary Zn concentrations of 30.9, 35.7, 40.5, 45.3, 50.8, 55.6, 74.2 and 103 mg/kg. 3Broken-line regression models were estimated on the basis of independent arithmetic group means relative to dietary zinc concentration (n = 8). Parameter estimates are presented as means ± SEs to indicate the precision of estimation. *P ≤ 0.05* was considered to be significant. *b1*, slope of the broken-line regression curves over dietary zinc doses ≤ *XB*; *b2*, slope of the broken-line regression curves over dietary zinc doses >*XB*; DM, dry matter; GLDA-Na4,L-glutamic acid N,N- diacetic acid, tetrasodium salt; *Mt1A*, *metallothionein 1A*; *XB*, *X* intercept of the breakpoint in the parameter response; *YB*, *Y* intercept of the breakpoint in the parameter response; Zn, zinc.