INTRODUCTION

The micronutrient Mn participates in many physiological processes in animals and humans. Manganese can be a cofactor of biological enzymes in the process of growth, carbohydrate and lipid metabolism, and clotting (Cao, 1987; Greger, 1999). Insufficient dietary Mn leads to the malfunction of ovulation, the regression of testis, and the early death of offspring (Cooper et al., 1963; Offiong and Abed, 1980). In chickens, Mn needs to be supplemented in the diet to meet the nutrition requirements because of low Mn content in the diet ingredients and the low absorption in the gut (Cao, 1987). The requirement of Mn has been extensively studied to prevent bone diseases and ensure the normal development of fast-growing broiler chickens. It has been shown by those studies that the organic forms of Mn supplementation had higher absorptivity and bioavailability than the inorganic form (Li et al., 2004; Ji et al., 2006; Bai et al., 2012). The bioavailability of organic Mn in broiler chicks fed with corn-soybean-based diets is closely associated with the chelation strength between Mn and the organic complex (Li et al., 2004, 2011). To date, few studies have been conducted on the dietary effect of different forms of Mn supplementation in broiler breeder hens with a special focus on reproduction.

Dietary Mn may function to regulate reproductive performance in several ways. As the cofactor of mevalonate kinase and farnesyl pyrophosphate synthase, Mn is speculated to regulate the synthesis of sexual hormone precursor cholesterol and consequently to affect the reproduction of animals. Furthermore, previous studies in layer hens have demonstrated the adverse effect of Mn deficiency on egg production while decreasing circulat-
Manganese is known to be capable to pass through the blood-brain barrier and deposit in the central nervous system, such as the cerebellum, hypothalamus, and pituitary (Rehnberg et al., 1981). Although there is no evidence showing direct effects of Mn on LH and FSH secretion, a few studies have demonstrated that Mn can act as the antagonist of Ca to regulate the secretion of prolactin (PRL; Merritt and Brown, 1984). In the brain, Mn also can act on the soluble guanylyl cyclase (GC) to activate the protein kinase G pathway to control the release of gonadotropin-releasing hormone-I (GnRH-I; Lee et al., 2007). The central toxic effect of exposures to high amount of Mn relates to the regenerative degradation of dopaminergic neurons and the resulting decrease of dopamine synthesis and secretion (Guilarte, 2011; Rivera-Mancia et al., 2011). As known, dopamine is a potent regulator for pituitary PRL (Fitzgerald and Dinan, 2008), thereby affecting FSH and LH secretion (Gregory et al., 2004; Henderson et al., 2008). It remains unknown whether the function of dopaminergic neurons is also modified by a nontoxic dose of Mn administered orally.

Broilers are genetically selected for fast growth, and broiler breeders have lower reproduction and more abnormalities in reproductive behaviors compared with layers. To maintain and promote the production of broiler breeder hens, the effects of dietary nutrition need to be better understood. The current study is aimed at determining the effect of dietary supplementation of Mn in both organic and inorganic forms on the reproductive performance of broiler breeder hens. Attempts were also made to reveal the possible central mechanism via which Mn may function to affect reproductive performance.

**MATERIALS AND METHODS**

The procedure for current animal experiment was approved by the Animal Welfare Committee in the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences.

**Bird Management and Sample Collection**

A total of 120 female Arbor Acres broiler breeders at 16 wk of age were purchased from a commercial farm (Huadu Broiler Company, Beijing, China), randomly allocated to 5 treatments with 6 replicates per treatment (n = 6), and kept in 3-story battery cages. The temperature and humidity were maintained at 21°C and 40%, respectively. All birds were feed restricted with the same amount of commercial diet to maintain the growth curve according to manufacturer’s instructions before the onset of the dietary treatments. The photoperiodic lighting was programmed with light on at 0800 h and off at 1600 h for the first 3 wk and gradually extended to 2300 h to promote sexual maturity. At the age of 30 wk, all birds were fed with a basal diet without Mn supplementation for 2 wk to deplete the body’s deposits of Mn. Then the birds were subjected to the 13-wk dietary treatments. Eggs were collected on a daily basis and laying rate was recorded every week. On the last day of wk 4 and 13 of the trial, serial blood samples were withdrawn every 4 h from 1000 h to 0600 h of the following day and serum was separated and stored at −20°C. All birds were killed by an overdose of anesthesia at the end of the trial, and heads were removed to collect samples of pituitary and hypothalamus. Tissue samples were snap-frozen in liquid nitrogen and then transferred to −80°C for storage.

Artificial insemination was carried out twice every week after the first egg. Each hen was inseminated by 50 μL of semen collected from the same group of roosters. Reproductive traits, such as laying rate, hatchability, egg weight, eggshell quality, were determined at wk 4 and wk 8 to 9 of the trial. Eggs were collected for 7 or 14 d, stored at 13°C, and incubated at 37.8°C and 40 to 60% humidity for 18 d before transferred to the hatchery. Eggs were candled at embryonic d 6 and 18 to determine fertility. Each hatchling was weighed and hatching weight was recorded.

**Experimental Diets**

Birds from each treatment were fed accordingly with basal diets supplemented with 0 mg of Mn/kg (control), 120 mg of Mn/kg as both MnSO (120 mg/kg of iMn) and Mn proteinate (120 mg/kg of oMn), and 240 mg of Mn/kg as both MnSO (240 mg of iMn/kg) and Mn proteinate (240 mg of oMn/kg), respectively. The Mn proteinate was provided by a commercial company (Hebei Amino Acid Company, Hebei, China) and had a content of 10.22% Mn and a medium chelation strength of 61.91 (Q6). The ingredient and nutritional components of the basal diet are listed in Table 1. Contents of lysine and methionine were kept the same over different experimental diets by adding lysine and methionine to the basal diet and diets supplemented with MnSO. All nutrients except Mn in each diet met the requirement of broiler breeder hens according to the Arbor Acres manufacturer’s instructions.

**Eggshell Quality Assessment**

A total of 6 to 8 eggs from each replicate were used to assess the quality of eggshells. The eggshell strength measurement was determined by Egg Shell Force Gauge (F0241, Robotmation Co. Ltd., Tokyo, Japan), and the thickness of the eggshell was measured by TI-PVX Egg Shell thickness Gauge of Orka Food Technology Corp. (Herzliya, Israel).
### Table 1. Composition of ingredients and nutrient in corn-soybean basal diet fed to broiler breeder hens from 30 to 44 wk of age

<table>
<thead>
<tr>
<th>Ingredient (%)</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>65.15</td>
</tr>
<tr>
<td>Soybean</td>
<td>23.44</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>1.65</td>
</tr>
<tr>
<td>CaHPO4</td>
<td>1.45</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>7.3</td>
</tr>
<tr>
<td>Salt</td>
<td>0.3</td>
</tr>
<tr>
<td>L-lysine HCl</td>
<td>0.06</td>
</tr>
<tr>
<td>Mineral and vitamin premix¹</td>
<td>0.4</td>
</tr>
<tr>
<td>Cornstarch + Mn²</td>
<td>0.25</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME (kcal/kg)</td>
<td>2,800</td>
</tr>
<tr>
<td>CP (%)</td>
<td>15.15</td>
</tr>
<tr>
<td>Calcium (%)</td>
<td>3.31</td>
</tr>
<tr>
<td>Nonphytate phosphorus (%)</td>
<td>0.33</td>
</tr>
<tr>
<td>Lysine (%)</td>
<td>0.8</td>
</tr>
<tr>
<td>Methionine (%)</td>
<td>0.32</td>
</tr>
<tr>
<td>Manganese³ (mg/kg)</td>
<td>13.7</td>
</tr>
</tbody>
</table>

¹Provided per kilogram of diet: vitamin A, 15,000 IU; vitamin D₃, 4,500 IU; vitamin E, 36 IU; vitamin K₃, 3.9 mg; vitamin B₁, 4.5 mg; vitamin B₂, 10.5 mg; vitamin B₆, 4.5 mg; vitamin B₁₂, 0.024 mg; pantothenic acid calcium, 18 mg; niacin, 39 mg; folic acid, 1.5 mg; biotin, 0.18 mg; choline, 1,000 mg; Cu(CuSO₄·5H₂O), 8 mg; Fe(FeSO₄·7H₂O), 50 mg; Zn(ZnSO₄·7H₂O), 100 mg; I(CaI₂), 2 mg; Se(CaSe), 0.3 mg.

²Manganese supplement added in place of equivalent weights of corn starch.

³Analyzed composition.

### Measurement of Serum Microelements

A volume of 1 mL of serum samples was digested in 3 mL of grade-pure nitric acid using a microwave-assisted digestion method. After the digestion, samples were left to cool and then diluted with ultrapure water to the final volume of 25 mL. Blank digestion was carried out with the same procedure by using 1 mL of ultrapure water instead of serum sample. Manganese content in each digested sample was then analyzed by graphite furnace atomic absorption spectrometry (Hitachi Polarized Zeeman atomic absorption spectrophotometer Z-2000 series, Tokyo, Japan) with a procedure as follows: dried at 140°C for 40 s, ashed at 1,000°C for 20 s, atomized at 2,300°C for 4 s, and cleaned at 2,500°C for 4 s. Ammonium dihydrogen phosphate (2%) was used as the chemical modifier and coinjected with each digested sample placed into the furnace. The certified MnSO₄ stock standard solution (obtained from National Research Center for Certified Reference Materials, Beijing, China) was serially diluted with 2% nitric acid to a final concentration of 0, 2, 4, or 10 μg of Mn/L to generate the standard curve, and the concentration of Mn in each digested sample was calculated accordingly.

### Determination of Serum Cholesterol and Estradiol

Total cholesterol in the serum was measured with a commercial kit based upon the enzymatic method (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Serum was first extracted by ether as described by (Xie et al., 2010) and the content of estradiol (E₂) was determined using the RIA of the Beijing North Institute of Biological Technology (Beijing, China).

### Isolation of RNA, Reverse Transcription, and Q-PCR

Total RNA was isolated and purified using the column-based method (74104, RNeasy Mini Kit from Qiagen, Hilden, Germany) and the manufacturer’s instructions were followed. Because of the large volume of hypothalamus, the whole tissue sample was first homogenized with 5 volumes of TRizol (15596018, Life Technologies, Foster City, CA). For each sample, 0.5 mL of homogenate was then used for the following procedure of RNA extraction. The concentration of each isolated RNA sample was determined by NanoDrop Spectrophotometer (ND-1000, Gene Company Ltd., Wilmington, DE) and the integrity of RNA was checked by denatured RNA electrophoresis.

A total of 1 μg of RNA was used to obtain cDNA by reverse transcription using QuantiTech Reverse Transcription Kit (205311, Qiagen). To eliminate the contamination of genomic DNA, RNA samples were first incubated with gDNA Wipeout Buffer at 42°C for 2 min. A mixture of reverse transcriptase, RT buffer, and Oligo dT primer mix was then added to each sample, followed by incubation at 42°C for 30 min. Reactions were inactivated by a 3-min incubation at 95°C and cDNA was stored at −20°C until use.

Expression of genes coding for GnRH-I, tyrosine hydroxylase (TH), inducible nitric oxide synthase (iNOS) in the hypothalamus and genes coding for FSH, LH, PRL, GnRH-I receptor (GnRH-R), dopamine receptor D1 (DRD1), iNOS in the pituitary were quantified by real-time PCR using SYBR green dye. Primers (Invitrogen, Beijing, China) used in the PCR reactions were designed with the Primer-BLAST in National Center for Biotechnology Information services. To determine the amplification efficiency for each pair of primers, 5-fold serial dilutions of a cDNA template were analyzed by real-time PCR. The efficiency was calculated as the slope of the linear regression of plotting Ct versus log2-transformed template dilution. The target genes, primers, and amplification efficiency of each gene are given in Table 2. All samples were arranged in each plate to ensure the same reaction condition. The PCR reactions (20 μL) contained 1 μL of diluted cDNA, 250 nM each primer, and PCR buffer master mix from the Power SYBR Green Master Mix (4367659, Life Technologies) and were carried out in the ABI 7500 Real-Time PCR Detection System (Applied Biosystems, Foster City, CA). Each PCR reaction was conducted in triplicate, and the Ct value used in subsequent calculations was the mean of the triplicate reactions. The protocol of PCR was as follows: denaturation at 95°C for 2 min followed by 40 cycles of 95°C...
Table 2. Information of target genes and primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>ID</th>
<th>Primer set (5'-3')</th>
<th>Product size (bp)</th>
<th>Temperature (°C)</th>
<th>Amplification efficiency (%)</th>
</tr>
</thead>
</table>
| β-Actin | NM205518.1 | F: 5'-ACCTGAGGGCAAGTACTCTGCTCT-3'  
R: 5'-CATCCTGTATCTCTGATCTG-3' | 95                | 60              | 96              |
| GAPDH | NM204305.1 | F: 5'-CTTTGGGAATGGTGTTGAGCT-3'  
R: 5'-ACGCGCAGTTACCACATACAG-3' | 128               | 99              | 60              |
| FSH-β | NM204257.1 | F: 5'-ACGAGTGGGAGAAGCAAATGTA-3'  
R: 5'-TGTTTCATACACAACCTCCTGGAAG-3' | 151               | 60              | 99              |
| LH-β | HQ872606 | F: 5'-GGTGTAAATTGCTGTCGCCAC-3'  
R: 5'-ATCATTTCAGGAAACCCACAG-3' | 147               | 60              | 90              |
| PRL   | AB011438 | F: 5'-GGGTGTCGACCAAAACCTAATG-3'  
R: 5'-GAGGAGGACACACCAATGCA-3' | 120               | 60              | 94              |
| DRD1  | NM001144848 | F: 5'-TGGAACGACACACTATGGA-3'  
R: 5'-CCAGAGGAGGAAATCAATAGCA-3' | 153               | 60              | 60              |
| iNOS  | NM204961.1 | F: 5'-GGCATGGGTTGGATACAGG-3'  
R: 5'-GGCATGGGTTGGATACAGG-3' | 145               | 60              | 105             |
| GnRH-R | NM204653 | F: 5'-CGTGAATTTTGGAAGTACG-3'  
R: 5'-TGGAACGACACACTATGGA-3' | 123               | 60              | 97              |
| GnRH-I | NM001080877.1 | F: 5'-ACACTGGTCTTATGGCTGCCTCA-3'  
R: 5'-CATCGTACTCCTGCTTGCTGAT-3' | 116               | 60              | 105             |
| TH    | AJ251387.1 | F: 5'-GGCACTGGGTTGGAAATCAATAGCA-3'  
R: 5'-GGCACTGGGTTGGAAATCAATAGCA-3' | 142               | 60              | 101             |

1 GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FSH, follicle-stimulating hormone; LH, luteinizing hormone; PRL, prolactin; DRD1, dopamine receptor D1; iNOS, inducible nitric oxide synthase; GnRH-R, gonadotropin-releasing hormone-I receptor; TH, tyrosine hydroxylase.
2 F, forward; R, reverse.

for 60 s, 60°C for 30 s, and 72°C for 30 s. Dissociation curve analyses were run to ensure a single product of each reaction. For each reaction plate of the same target gene, the cDNA pool of all samples was used as the reference control sample. The geometric mean of internal references, β-actin and glyceraldehyde 3-phosphate dehydrogenase, was used to normalize the expression of the targeted genes (Vandesompele et al., 2002). The 2−ΔΔCt was used to calculate mRNA level of each target gene, where the average mean of Ct from the group fed with the basal diet was used as the calibrator.

**Statistical Analyses**

All data were analyzed using JMP 10.2 software (SAS Institute Inc., Cary, NC). Data were first subjected to homogeneity of variance analyses. One-way ANOVA was used to test the main effect on relative gene expression with log-transformed data. Data collected at wk 4 and 13 were considered as repeated measurements. For each reaction plate of the same target gene, the cDNA pool of all samples was used as the reference control sample. The geometric mean of internal references, β-actin and glyceraldehyde 3-phosphate dehydrogenase, was used to normalize the expression of the targeted genes (Vandesompele et al., 2002). The 2−ΔΔCt was used to calculate mRNA level of each target gene, where the average mean of Ct from the group fed with the basal diet was used as the calibrator.

Specific orthogonal contrasts were carried out to stress the effects of Mn supplementation (control vs. 120 mg of iMn/kg, 120 mg of oMn/kg, 240 mg of iMn/kg, 240 mg of oMn/kg), levels of dietary Mn (120 mg of iMn/kg, 120 mg of oMn/kg vs. 240 mg of iMn/kg, 240 mg of oMn/kg), and forms of Mn supplementation (120 mg of iMn/kg, 240 mg of iMn/kg and 120 mg of oMn/kg, 240 mg of oMn/kg). The significance of correlations between expression of mRNA and levels of serum hormone was determined using Pearson correlation analyses. All data were presented as mean ± SE with significance considered at the level of P < 0.05.

**RESULTS**

**Egg Production, Egg Quality, and Hatchability**

At the age of 35 wk, the average laying rate of broiler breeder hens reached 78.9 ± 1.5% and dropped remarkably to 66.4 ± 2.0% at the end of the trial (F1,25 = 71.733, P < 0.01), whereas average egg weights increased from 54.0 ± 0.5 g to 66.4 ± 0.4 g (F1,25 = 810.047, P < 0.01). There were no significant differences in average laying rate (Figure 1A, F4,36 = 0.521, P = 0.721) and average egg weight (Figure 1B, F4,37 = 1.563, P = 0.204) among dietary treatment groups. Manganese supplementation had a significant effect on eggshell strength (Figure 1C, F4,44 = 3.556, P = 0.0027); however, eggshell thickness of sampled eggs did not differ among treatments (Figure 1D, F4,38 = 0.913, P = 0.466).
The fertility of eggs after artificial insemination remained at around 98%. There was no indication that the fertility of eggs was affected by dietary treatments (Table 3, $F_{4,49} = 0.504$, $P = 0.733$). Mean hatchability of fertilized eggs varied among treatment groups from 91.7 ± 1.4% to 88.5 ± 1.6%, but these differences were not statistically significant (Table 3, $F_{4,48} = 0.411$, $P = 0.800$). Hatchling weight correlated with egg weight ($P < 0.001$, $r^2 = 0.40$), but did change after Mn supplementation (Table 3, $F_{4,36} = 0.5821$, $P = 0.678$).

**Serum Mn, Cholesterol, and E2**

There was a significant dietary treatment effect on the serum Mn content (Figure 2A, $F_{4,28} = 3.081$, $P = 0.035$). It was revealed by contrast analyses that the dietary supplementation of Mn had a tendency to elevate the serum Mn compared with the control basal diet ($P = 0.057$). Birds fed with 240 mg of Mn/kg had significantly higher serum Mn than those fed with 0 or 120 mg of Mn/kg (Figure 2B, $P < 0.05$). There was no difference in serum Mn between inorganic and organic Mn supplementation groups (Figure 2C, $P = 0.137$).

Dietary treatments with different Mn supplements did not change serum cholesterol content (Figure 3, $F_{4,48} = 0.204$, $P = 0.935$). Serum E2 displayed diurnal changes with the bottom level after 1800 h and the peak level at 1000 h (Figure 4B), and the circulating level dropped remarkably as the birds aged ($F_{1,288} = 70.398$, $P < 0.001$). The average content of E2 of all 6 bleeds did not differ among treatment groups (Figure 4A, $F_{4,199} = 0.926$, $P = 0.450$).

Figure 1. Laying performance and egg quality of broiler breeder hens after dietary supplementation of Mn as MnSO₄ (iMn) or Mn proteinate (oS Mn) for 4 and 13 wk. A. Laying rate on a weekly basis; B. average weight of eggs collected at wk 4 and 13 of the trial; C. eggshell strength, determined by 6 to 8 eggs per replicate; D. eggshell thickness, determined by 6 to 8 eggs from each replicate. All data were presented as mean ± SE. *An asterisk indicates significant difference between the Mn-supplemented group and the control (CON) basal diet, which was determined by post hoc contrast analyses. Significant effects were considered when $P < 0.05$.  

EFFECTS OF MANGANESE ON REPRODUCTION
Table 3. Fertility, hatchability, and hatchling weight of eggs collected from wk 4 and wk 8 to 9 of the trial\textsuperscript{1}

<table>
<thead>
<tr>
<th>Item</th>
<th>Wk 4 Fertility (%)</th>
<th>Wk 4 Hatchability (%)</th>
<th>Wk 4 Hatchling weight (g)</th>
<th>Wk 8 to 9 Fertility (%)</th>
<th>Wk 8 to 9 Hatchability (%)</th>
<th>Wk 8 to 9 Hatchling weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>98.3 ± 1.1</td>
<td>94.3 ± 2.0</td>
<td>43.0 ± 1.0</td>
<td>98.6 ± 0.7</td>
<td>87.1 ± 4.2</td>
<td>46.5 ± 0.7</td>
</tr>
<tr>
<td>120 mg of iMn/kg</td>
<td>98.0 ± 1.2</td>
<td>92.0 ± 3.4</td>
<td>44.4 ± 1.5</td>
<td>97.7 ± 1.0</td>
<td>92.5 ± 3.9</td>
<td>46.3 ± 1.3</td>
</tr>
<tr>
<td>120 mg of oMn/kg</td>
<td>98.4 ± 1.0</td>
<td>90.4 ± 2.6</td>
<td>42.3 ± 0.8</td>
<td>97.0 ± 0.8</td>
<td>86.6 ± 3.5</td>
<td>46.0 ± 1.7</td>
</tr>
<tr>
<td>240 mg of iMn/kg</td>
<td>97.4 ± 2.6</td>
<td>93.6 ± 2.2</td>
<td>43.3 ± 0.8</td>
<td>99.3 ± 0.5</td>
<td>90.0 ± 4.2</td>
<td>45.3 ± 0.9</td>
</tr>
<tr>
<td>240 mg of oMn/kg</td>
<td>95.7 ± 3.5</td>
<td>88.8 ± 5.3</td>
<td>44.1 ± 0.7</td>
<td>97.7 ± 0.8</td>
<td>87.6 ± 2.8</td>
<td>47.4 ± 1.1</td>
</tr>
</tbody>
</table>

\textsuperscript{1}iMn, Mn supplement as inorganic form, MnSO\textsubscript{4}; oMn, Mn supplement as Mn proteinate.

\textbf{Relative Abundance of Gene mRNA in the Pituitary and Hypothalamus}

In the pituitary, only in the FSH mRNA abundance was the significant main effect of dietary treatment observed (Figure 5A, $F_{4,24} = 3.2651$, $P = 0.033$). Contrast analyses showed that birds fed with diets supplemented with 240 mg of Mn/kg had higher FSH gene expression than birds fed with basal diets and experimental diets with 120 mg of Mn/kg supplement (insert of Figure 5A, $P < 0.001$). No difference was observed between inorganic and organic Mn supplements ($P = 0.791$). Other genes in the pituitary, including PRL (Figure 5B, $F_{1,24} = 1.0529$, $P = 0.404$), LH (Figure 5C, $F_{4,24} = 1.275$, $P = 0.313$), DRD1 (Figure 5D, $F_{4,24} = 0.485$, $P = 0.746$), GnRH-R (Figure 5E, $F_{4,24} = 0.567$, $P = 0.689$), and iNOS (Figure 5F, $F_{4,24} = 1.081$, $P = 0.390$) did not express differently among treatment groups. Correlation analyses showed that mRNA abundance of PRL positively correlated with GnRH-R ($P = 0.022$, $r^2 = 0.227$), whereas FSH negatively correlated to DRD1 gene expression ($P = 0.012$, $r^2 = 0.235$).

In the hypothalamus, relative expression of 3 genes coding for GnRH-I, TH, and iNOS was quantified. There was a significant effect of dietary treatment on GnRH-I mRNA abundance (Figure 6A, $F_{4,25} = 4.684$, $P = 0.007$). Manganese supplements significantly increased GnRH-I gene expression in the brain. Contrast analyses also showed that 240 mg of Mn/kg significantly induced GnRH-I mRNA expression in the brain (Figure 6B, $P < 0.05$). Different forms of Mn exhibited very different effects on GnRH-I mRNA expression with a 2-fold GnRH-I mRNA increase with the inorganic Mn supplement over the organic form ($P = 0.016$). Abundance of TH mRNA (Figure 6C, $F_{4,28} = 0.696$, $P = 0.602$) or iNOS (Figure 6D, $F_{4,28} = 0.587$, $P = 0.675$) was not affected by dietary treatments.

\textbf{DISCUSSION}

Over the 13 wk of trial, there were no significant differences observed in egg production, hatchability, hatchling weight, or serum cholesterol despite the increased serum Mn after dietary supplementation of Mn. Dietary Mn supplementation, however, did affect gene expression of GnRH-I in the brain and FSH in the pituitary and as well as eggshell quality. These results suggest that there might be a central mechanism for dietary Mn in affecting the reproduction of broiler breeder hens, and it may take a longer time to show these effects in reproductive behavior.

In the current study, the reproductive performance and behavior of broiler breeders, such as egg production and hatchability, were not altered by dietary Mn deficiency, although previous studies confirmed that low dietary Mn negatively affected egg productions in birds. Attia et al. (2010) found that supplementation of 10 to 40 mg of Mn/kg to the corn-soybean meal significantly increased the laying rate and egg weight in In-cha hens (Siena X Plymouth Rock) after 16 wk. Layer hens fed with a Mn-deficient diet from 1 d old had reduced laying rate, hatchability, eggshell thickness, and force, and increased broken egg rate and soft egg rate at peak egg production (Zhang et al., 1992). A study using Lohmann layers at the peak production did not find an effect of Mn supplementation on laying rate and egg production for 14-wk dietary treatments (Chen and Wu, 2002), whereas another trial using the same layers showed a decrease in egg production after exposure to Mn-unsupplemented diets for 32 wk (Feng and Feng, 1998). The inconsistency in the behavioral effects of Mn among those studies may be due to the differences in chicken breeds, the duration of dietary treatments, as well as Mn contents in feed ingredients. In present study, there was no difference in the serum Mn content between birds receiving diets supplemented with 0 and 120 mg of Mn/kg. The eggshell strength, however, was lowered by the Mn-deficient diet treatment. Previous studies found that the ultrastructure of eggshell could be altered by a Mn-deficient diet, where large irregular mammillary knobs were observed (Leach and Gross, 1983; Luo et al., 2003). Hexosamine and hexuronic acid contents in the eggshell were also decreased by low Mn intake (Leach and Gross, 1983). It is likely that the homeostasis of serum Mn was maintained by redirecting Mn deposition in the body, such as eggs.

Manganese deficiency was found to lead to changes in circulating ovarian steroids in layer hens (Cao and Chen, 1987; Feng and Feng, 1998). Steroidogenesis begins with cholesterol. Because Mn is one of the cofactors of enzymes in the biosynthesis of cholesterol (Klimis-Tavantzis et al., 1983b), it was speculated to also regulate the steroid synthesis. Although an increased serum Mn was found in the birds supplemented with
240 mg of Mn/kg, there were no differences in serum cholesterol and E2 among all treatments of present study. This finding was consistent with previous studies, which showed that Mn did not affect the hepatic cholesterol and fatty acid synthesis and circulating cholesterol.

**Figure 2.** Serum Mn of broiler breeder hens after dietary supplementation of Mn as MnSO4 (iMn) or Mn proteinate (oMn) for 13 wk. A. Average content of Mn in serum from birds fed with diets supplemented with 0, 120, or 240 mg of Mn/kg as MnSO4 or Mn proteinate; B. Average serum Mn of birds fed with diets supplemented with 0, 100, or 200 mg of Mn/kg; C. Serum Mn of birds fed with diets with inorganic or organic Mn supplements. All data were presented as mean ± SE. Different letters (A,B) indicate significant differences among each level of Mn supplementation, which was determined by contrast analyses. Significant effects were considered when $P < 0.05$. CON = control.

**Figure 3.** Serum cholesterol in broiler breeder hens fed with diets supplemented with Mn as MnSO4 (iMn) or Mn proteinate (oMn) for 4 and 13 wk. All data were presented as mean ± SE. CON = control.

**Figure 4.** Effects of dietary Mn supplements as MnSO4 (iMn) or Mn proteinate (oMn) on serum estradiol (E2) in broiler breeder hens. A. Average serum E2 in birds fed with 0, 120, and 240 mg of Mn/kg; B. Diurnal pattern of serum E2 in broiler breeder hens. All data were presented as mean ± SE. Different letters indicate significant differences among time points (uppercase letters for wk 4, lowercase for wk 13). Significant effects were considered when $P < 0.05$. CON = control.
lesterol in rats (Klimis-Tavantzis et al., 1983b), hens (Klimis-Tavantzis et al., 1983a), and roosters (Cao et al., 1990) in vivo. In avian species, the steroidogenesis in follicles is mainly regulated by LH, whereas FSH stimulates the maturation of granulosa cells and attained ability of cells to respond to LH, FSH, and other signals (Scanes, 1999). Due to a lack of antibodies, circulating LH was not directly determined in the current study. Analysis of LH mRNA expression did not show Mn to influence LH after a 13-wk dietary treatment. The comparable levels of LH among each treatment, to a certain extent, supported the unaltered serum E2.

We found that dietary supplementation of 240 mg of Mn/kg significantly increased the serum Mn content as well as pituitary FSH mRNA abundance, but not LH mRNA. The unchanged LH mRNA expression after 13-wk dietary treatments suggested that it might take a longer time for dietary Mn to have a significant impact on ovulation and egg production in broiler breeders. The upregulated FSH mRNA expression in

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**Figure 5.** Gene expression of follicle-stimulating hormone (FSH; A), prolactin (PRL, B), luteinizing hormone (LH; C), dopamine receptor D1 (DRD1, D), GnRH-R (GnRH receptor, E), and iNOS (inducible nitric oxide synthase, F) in the pituitary of broiler breeder hens after dietary Mn treatments for 13 wk. The gene expression was quantified by real-time PCR using geometric mean of β-actin and glyceraldehyde 3-phosphate dehydrogenase as the internal standard. Insert in the FSH (A) showed the effect of different levels of Mn supplementation on the abundance of FSH mRNA. All data are presented as mean ± SE. Different uppercase letters (A,B) indicate significant differences among each level of Mn supplementation. Significant effects were considered when \( P < 0.05 \). iMn = dietary supplementation of Mn as MnSO4; oMn = dietary supplementation with Mn proteinate; CON = control.
the high-Mn dietary treatment could be induced by several mechanisms. First, Mn could directly target the FSH-producing cells. Although there was no direct evidence, it has been shown that Mn can regulate the release of PRL (Merritt and Brown, 1984) and also can activate MnSOD expression at the transcriptional level (Li et al., 2011). Second, Mn could regulate FSH synthesis and secretion by affecting dopamine system. It is known that excess Mn exposure causes degenerative changes in dopaminergic neurons (Guilarte, 2011). In the current study, the nontoxic high doses of Mn had few effect on mRNA expression of tyrosine hydroxylase (rate-limited enzyme in dopamine synthesis and commonly used as the marker for dopaminergic neurons) in the hypothalamus of broiler breeder hens. In the pituitary, FSH was shown to be negatively correlated to DRD1 gene expression, indicating that 240 mg of Mn/kg interacted with the dopamine system most likely at the pituitary level. Third, the regulatory effect of Mn could depend on the GnRH-I, the primary regulator of the hypothalamic-pituitary-gonadal axis. Manganese is capable of passing through the blood-brain barrier, which makes it possible for Mn to regulate the synthesis and secretion of GnRH-I in the brain. Previous work in both male and female rats showed that injecting MnCl2 into the brain could induced LH, FSH, and E2 secretion (Pine et al., 2005; Lee et al., 2006; Prestifilippo et al., 2008). The central regulatory effect of Mn depends on the release of GnRH-I (Pine et al., 2005). In addition to FSH, GnRH-I gene expression in the brain also exhibited a dose-independent increment from 0 to 240 mg of Mn/kg in our study. The transcriptionally increased GnRH-I expression suggested that the central mechanism was involved in the regulation of Mn on reproductive performance of broiler breeder hens.

**Figure 6.** Gene expression of gonadotropin-releasing hormone-I (GnRH-I; A), tyrosine hydroxylase (TH, D), and inducible nitric oxide (NO) synthase (E) in the hypothalamus of broiler breeder hens fed with 0, 120, or 240 mg of Mn/kg supplements as MnSO4 (iMn) or Mn proteinate (oMn) for 13 wk. The gene expression was quantified by real-time PCR using geometric mean of β-actin and glyceraldehyde 3-phosphate dehydrogenase as the internal standard. B. The effects of 0, 120, and 240 mg of Mn/kg supplementation on the abundance of GnRH-I mRNA. C. Abundance of GnRH-I mRNA in birds fed with diets with inorganic or organic Mn supplements. All data are presented as mean ± SE. Different uppercase letters (A, B) indicate significant differences among each level of Mn supplementation. *An asterisk indicates significant difference between inorganic and organic supplements. Significant effects were considered when *P* < 0.05. CON = control.
Lee et al. (2007) found that Mn regulated GnRH-I release depending on the GC/protein kinase G pathway. It functions at the soluble GC level but not at the production of nitric oxide (NO; Lee et al., 2007). Although we did not directly analyze the NO in the hypothalamus because of technical issues, the analysis of NO synthase mRNA expression revealed no difference in iNOS mRNA expression among dietary treatments with different levels of Mn. It suggested that the NO pathway may not be activated by Mn supplementation. Dietary Mn supplementation increased the expression of both GnRH-I and FSH genes; however, no correlations were found between the amounts of RNA encoding GnRH-I, GnRH-R, and FSH. In the pituitary, GnRH-R locates not only at gonadotropin-expression cells, but also at somatotropin-, somatolactin-, and PRL-expressing cells (Stefano et al., 1999). This makes the regulatory effect of GnRH-I in the pituitary more complicated.

Comparisons between inorganic and organic Mn supplementation showed that the supplementation of Mn as MnSO₄ had a more profound stimulatory effect on gene expression of GnRH-I than supplemental Mn proteinate. Extensive studies in broiler chickens found that the absorption of organic forms of Mn was higher than that of inorganic forms in the intestine (Ji et al., 2006) and the organic form of Mn with medium chelation strength (Q₂ of 10–100) had the highest bioavailability reflecting by higher expression of heart MnSOD mRNA and Mn intestinal transporter DMT1 mRNA (Li et al., 2004). In contrast to the different bioavailability between inorganic and organic Mn, serum Mn contents were found to be comparable both in previous studies and the current one. Because Mn needs to pass through the blood-brain barrier to target brain neurons, the organic source of Mn may not favor the permeability of Mn ions because of its complex structure. Therefore, regarding central stimulation of GnRH-I mRNA by Mn, the inorganic form performed better than the organic form with moderate chelation strength, although it had lower bioavailability.

In conclusion, dietary supplementation of Mn could improve the eggshell quality in the long-term. The central mechanism of nontoxic dose of Mn might employ the transcriptional activation of hypothalamic GnRH-I gene and pituitary FSH gene, which may not be mediated by dopamine or NO pathway. The inorganic form of Mn supplement showed a higher stimulatory effect of the GnRH-I mRNA than the Mn proteinate despite similar serum Mn contents.

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