

Effect of dietary manganese on antioxidant status and expression levels of heat-shock proteins and factors in tissues of laying broiler breeders under normal and high environmental temperatures

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Abstract

To investigate the effect of Mn on antioxidant status and expression levels of heat-shock proteins/factors in tissues of laying broiler breeders subjected to heat challenge, we used a completely randomised design (n 6) with a factorial arrangement of 2 environmental temperatures (normal, 21 (SEM 1)°C and high, 32 (SEM 1)°C) × 3 dietary Mn treatments (an Mn-unsupplemented basal diet (CON), or a basal diet supplemented with 120 mg Mn/kg diet as inorganic Mn sulphate (iMn) or organic Mn proteinate (oMn)). There were no interactions ($P > 0.10$) between environmental temperature and dietary Mn in all of the measured indices. High temperature decreased ($P < 0.003$) Mn content, and also tended ($P = 0.07$) to decrease copper zinc superoxide dismutase (CuZnSOD) activity in the liver and heart. However, an increased manganese superoxide dismutase (MnSOD) activity ($P < 0.05$) and a slight increase of malondialdehyde level ($P = 0.06$) were detected in breast muscle. Up-regulated ($P < 0.05$) expression levels of heat-shock factor 1 (*HSF1*) and *HSF3* mRNA and heat-shock protein 70 (*HSP70*) mRNA and protein were found in all three tissues. Broiler breeders fed either iMn or oMn had higher tissue Mn content ($P < 0.0001$), heart MnSOD and CuZnSOD activities ($P < 0.01$) and breast muscle MnSOD protein levels ($P < 0.05$), and lower ($P < 0.05$) breast muscle *HSP70* mRNA and protein levels than those fed CON. Broiler breeders fed oMn had higher ($P < 0.03$) bone Mn content than those fed iMn. These results indicate that high temperature decreases Mn retention and increases *HSP70* and *HSF1*, *HSF3* expression levels in tissues of laying broiler breeders. Furthermore, dietary supplementation with Mn in either source may enhance heart antioxidant ability and inhibit the expression of *HSP70* in breast muscle. Finally, the organic Mn appears to be more available than inorganic Mn for bone in laying broiler breeders regardless of environmental temperatures.

Key words: Broiler breeders: Manganese: Heat stress: Antioxidant status: Heat-shock proteins/factors

The effect of heat stress on productive performance has been extensively studied in poultry, especially in high-producing hens^(1–4). High environmental temperature negatively influences the performance of laying commercial hens^(1,2) and broiler breeders^(3,4) by reducing feed intake, egg production and eggshell quality. In addition to altered productive performance, heat stress can also disturb the redox balance and induce oxidative stress, with the production of reactive oxygen species (ROS), in broiler breeders⁽⁵⁾ and commercial hens⁽⁶⁾. Although substantial attention has been paid to the roles of

antioxidant trace minerals (Se, Zn) in minimising the harmful effect of heat stress in broilers⁽⁷⁾ and commercial laying hens⁽⁸⁾, the role of Mn in stress reduction has not been well studied. Mn is a crucial component of the metalloenzyme manganese superoxide dismutase (MnSOD)⁽⁹⁾, which has a key role in the detoxification of superoxide free radicals. A series of studies in our laboratory have demonstrated that dietary Mn may increase heart MnSOD activity and reduce lipid peroxidation in broilers^(10,11) and commercial laying hens⁽¹²⁾, as well as up-regulate heart *MnSOD* expression^(13–16) in broilers under

Abbreviations: CON, Mn-unsupplemented basal diet; CuZnSOD, copper zinc superoxide dismutase; *HSF1* and *HSF3*, heat-shock factors 1 and 3; *HSP70* and *HSP90*, heat-shock proteins 70 and 90; HT, high temperature; iMn, basal diet supplemented with 120 mg Mn/kg as inorganic Mn; MDA, malondialdehyde; MnSOD, manganese superoxide dismutase; NT, normal temperature; oMn, basal diet supplemented with 120 mg Mn/kg as organic Mn; ROS, reactive oxygen species; SOD, superoxide dismutase.

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thermoneutral conditions. In addition, moderately chelated organic Mn is the most effective in augmenting these activities. Our recent results have shown that high environmental temperature significantly impaired egg production performance and eggshell quality and induced lipid peroxidation and tissue damage, whereas dietary supplementation of either organic or inorganic Mn improved eggshell strength and thermotolerance and reduced protein oxidation, and that the organic Mn with the moderate chelation strength could alleviate the negative effect of high temperature on egg production performance of broiler breeders at the period of 32–45 weeks of age⁽¹⁷⁾. However, it is not clear whether dietary supplementation with Mn, especially organic Mn with moderate chelation strength, can reduce oxidative damage and increase heat stress resistance with the enhancements of antioxidant ability in broiler breeders. Hyperthermia increases the synthesis of heat-shock proteins (HSP) in broilers to aid in thermotolerance^(18,19). However, hyperthermia induces *HSP70* expression via oxidative stress or the increased production of ROS^(20,21). In this scenario, *HSP70* accumulation might be used as a biomarker for potential heat stress damage⁽²²⁾. Increased activities of superoxide dismutase (SOD) and catalase enzymes in cells blocked for induction of HSP expression result in a less severe heat-stressed response⁽²³⁾. It is assumed that dietary Mn supplementation might inhibit the expression of *HSP/heat-shock factor (HSF)* by enhancing the antioxidant response of broiler breeders under high environmental temperature. Therefore, the objective of this study was to investigate the effect of dietary supplementation with Mn, especially organic Mn with moderate chelation strength, on tissue Mn content, antioxidant status and expression of *HSP/HSF* in tissues of laying broiler breeders reared at normal and high environmental temperatures.

Methods

Experimental design and treatments

A completely randomised design involving 2 environmental temperatures × 3 dietary Mn treatments was used in this experiment. The two environmental temperatures were a normal temperature of 21 (SEM 1)°C (NT) and a high temperature of 32 (SEM 1)°C (HT). The three dietary Mn treatments were a maize–soyabean meal basal diet without Mn supplementation (CON), the basal diet supplemented with 120 mg of Mn/kg of diet on an as-fed basis as either Mn sulphate (MnSO₄·H₂O, iMn) or an Mn proteinate (oMn) with a moderate chelation strength. Thus, there were a total of six different treatments (NT-CON, NT-iMn, NT-oMn, HT-CON, HT-iMn and HT-oMn).

Birds and diets

This study was approved by Animal Welfare Committee of Institutes of Animal Sciences, Chinese Academy of Agricultural Sciences. The managements of broiler breeders and dietary treatments for this experiment were the same as described previously⁽¹⁷⁾. Briefly, 144 18-week-old female broiler breeders (Arbor Acres; Huadu Broiler Company) were randomly allotted to one of six treatments with six replicates (four birds per

replicate) for each treatment based upon body weight. Four birds in each replicate were kept in two neighbouring galvanised steel cages (length 50 cm × width 50 cm × height 50 cm) with two birds per cage. All broiler breeders were handled in accordance with the Arbor Acres breeder management guidelines for lighting and feeding and allowed *ad libitum* access to tap water containing no detectable Mn during the adaptation period from 18 to 29 weeks of age. After the adaptation, all broiler breeders were fed with the same maize–soyabean meal basal diet with no Mn addition (Table 1, containing 32.5 g Ca/kg and 14.3 mg Mn/kg by analysis) to deplete Mn stores from 30 to 31 weeks of age. After Mn depletion, the room temperature for the groups of NT-CON, NT-iMn and NT-oMn was maintained at 21 (SEM 1)°C, whereas the room temperature for groups of HT-CON, HT-iMn and HT-oMn was increased step-wise from 21 to 32°C over 2 d for these birds to acclimatise to the experimental chronic heat challenge, and then maintained at 32 (SEM 1)°C for the rest of the experiment. Relative moisture in the two rooms was kept at 40 (SEM 5)% during the experimental period of 14 weeks (32–45 weeks of age). Our previous results⁽¹⁷⁾ on rectal temperature indicated that all of the laying broiler breeders under HT were in a heat stress status throughout the experimental period. The daily feed intake of birds in each replicate was recorded and reported previously⁽¹⁷⁾. All broiler breeders from both NT and HT were feed-restricted with the same amount of the treatment diets each day from 32 to 35 weeks of age. However, the feed intake of broiler breeders was still significantly lower from HT (125.5 g/bird per d) than NT (136.1 g/bird per d) during this period.

Table 1. Composition and nutrient levels of the basal diet (as-fed basis)

Items	Laying period (30–45 weeks of age)
Ingredient (g/kg)	
Ground yellow maize	651.5
Soyabean meal	234.4
Soyabean oil	16.5
CaCO ₃ *	73.0
CaHPO ₄ *	14.5
NaCl*	3.0
DL-Met (99%)	0.6
Premix†	4.0
Maize starch + Mn additive, etc.‡	2.5
Nutrient composition	
Metabolisable energy (MJ/kg)	11.7
Crude protein§	155
Lys§	8.8
Met§	3.4
Met + Cys§	6.1
Thr§	5.9
Ca§	32.5
Non-phytate P	3.2
Mn (mg/kg)§	14.3

* Reagent grade.

† The premix contained the following vitamins and minerals (per kg of diet): retinol, 4.5 mg; cholecalciferol, 0.10 mg; α -tocopherol acetate, 36.0 mg; menadione, 3.9 mg; thiamin, 4.5 mg; riboflavin, 10.5 mg; pyridoxine, 4.5 mg; cyanocobalamin, 0.024 mg; pantothenate, 18 mg; niacin, 39 mg; folic acid, 1.5 mg; biotin, 0.18 mg; choline, 1000 mg; Cu, 10 mg; Fe, 50 mg; Zn, 100 mg; I (KI), 2.0 mg; I, 2.0 mg; Se, 0.30 mg.

‡ Mn additive, etc., lysine-HCl or DL-methionine were added to diets by replacing an equal weight of maize starch.

§ Analysed values based on triplicate determinations.

From 36 to 45 weeks of age, in order to eliminate the potential effect of reduced feed intake under HT, the birds in NT were pair-fed the same amount of feed consumed by birds in HT on the previous day, and the feed intake of breeders in HT and NT were 130.8 and 130.9 g/bird per d, respectively.

The Mn-unsupplemented maize–soyabean meal basal diet for Mn-depleting and the experimental stages (Table 1) was formulated to meet or exceed the National Research Council⁽²⁴⁾ requirements for laying broiler breeders, except for Mn, which was added to the basal diet according to the experimental design. The MnSO₄·H₂O was reagent grade (purity > 99%; Beijing Chemical Company), whereas Mn proteinate (purity > 99%) was provided by Hebei Amino Acid Company. A single batch of basal diet was mixed and then divided into three aliquots according to the experimental treatments. Lysine and methionine levels in the control diet or diet supplemented with inorganic Mn were balanced by adding synthetic lysine-HCl and DL-methionine based upon supplemental amounts of lysine and methionine from Mn proteinate source. The analysed Mn contents in diets, which were very close to calculated values, are presented in Table 2.

Sample collections and preparations

Samples of the Mn sources, diets and tap water were collected for analyses of Mn, Ca and dietary crude protein (CP) contents. At the end of the experiment, two birds from each replicate were selected based on body weight and slaughtered humanely by carbon dioxide asphyxiation⁽⁸⁾. Heart, liver and breast muscle samples were collected immediately. A set of tissue sub-samples were snap-frozen in liquid N₂ and then stored at –80°C for the mRNA and protein analyses, whereas another set of sub-samples were kept on ice and stored at –20°C for subsequent measurements of Mn contents, malondialdehyde (MDA) levels and SOD activities. Tibiotarsal bones were boiled for approximately 10 min in deionised water, all soft tissues were removed, dried for 12 h at 105°C and then ashed in a muffle furnace at 550°C for 16 h to obtain bone ash samples. For each tissue, all samples from the two birds in each replicate were pooled into one sample of equal weight before analyses.

Measurements of manganese, calcium, crude protein and amino acid contents and Q_f value of manganese proteinate

The Mn and Ca contents in diets and Mn contents in Mn sources, tap water, tissues and bone ash were measured using an

inductively coupled plasma emission spectroscope (model IRIS Intrepid II; Thermo Jarrell Ash) after wet digestions with HNO₃ and HClO₄, as described by Luo *et al.*⁽⁹⁾. Validation of the mineral analysis was conducted using bovine liver powder (GBW (E) 080193; National Institute of Standards and Technology) as a standard reference material. Contents of CP and Ca in feed ingredients and diets were determined using Association of Official Analytical Chemists methods⁽²⁵⁾. Amino acids in Mn proteinate were analysed using an amino acid analyzer (model L-8500A; Hitachi Ltd), and the quotient of formation (Q_f) value of Mn proteinate was determined using polarography, as described by Li *et al.*^(13,15). The control basal diet was also sampled for analyses of amino acid contents.

Determinations of malondialdehyde levels and superoxide dismutase activities in tissues

Heart, liver and breast muscle samples were homogenised in 10% (w/v) physiological saline on ice for 60 s and then sonicated with an ultrasonic wave cell grinder (JY92-11; Ningbo) for 1 min (on 1 s, interval 2 s). The homogenates were centrifuged at 1000 g for 15 min at 4°C and supernatants were collected to determine total protein contents, and MDA levels and SOD activities. Total protein concentration was determined using a BCA Protein Assay Kit (Cat no. 23225; Pierce). The levels of MDA in the supernatant were determined using a commercial assay kit (Cat no. A003-1; Nanjing Jiancheng Bioengineering Institute) and expressed as MDA content per mg protein. The total superoxide dismutase (TSOD) and MnSOD activities were measured according to the nitrite method described by Li *et al.*⁽¹³⁾, and copper zinc superoxide dismutase (CuZnSOD) activities were calculated by subtracting MnSOD activity from TSOD activity. The MnSOD and CuZnSOD activities in the tissues were expressed as nitrite units per mg protein.

RNA extractions, reverse transcriptions and real-time PCR

Total RNA was isolated from heart, liver and breast muscle samples using Trizol reagent (Cat no. 15596018; Life Technologies) according to the manufacturer's instructions. The concentration of each isolated RNA sample was determined using a NanoDrop Spectrophotometer (ND-2000; Gene Company Ltd),

Table 2. Analysed manganese content in experimental diets* (Mean values and standard deviations)

Items	Dietary Mn treatments		
	CON	iMn	oMn
Added Mn (mg/kg)	0	120	120
Analysed dietary Mn (mg/kg)†			
Mean	14.3	133	132
SD	0.04	0.43	0.74

CON, Mn-unsupplemented basal diet; iMn, basal diet + 120 mg Mn/kg as MnSO₄·H₂O; oMn, basal diet + 120 mg Mn/kg as the Mn proteinate with a moderate chelation strength of 61.9 quotient of formation (Q_f) (10.2% Mn).

* The Mn concentrations are on an as-fed basis.

† Mean values are based on triplicate determinations.

and the integrity of the RNA was checked using denatured RNA electrophoresis.

A total of 1 µg of RNA was used to obtain complementary DNA (cDNA) by reverse transcription using the QuantiTect Reverse Transcription Kit (Cat no. 205311; Qiagen). The samples were treated with RNase-Free DNase and reverse-transcribed according to the manufacturer's instructions. Expression levels of genes coding for *HSP70*, *HSP90*, *HSF1*, *HSF3* and *MnSOD* in the heart, liver and breast muscle were quantified by real-time PCR using Power SYBR Green Master Mix (Cat no. 4367659; Life Technologies) with an ABI 7500 Real-Time PCR Detection System (Life Technologies). The target genes and primers (Invitrogen) of each target gene are given in Table 3. All samples were arranged in the same plate to ensure that the analysis was run under the same reaction conditions. The protocol of PCR was as follows: denaturation at 95°C for 2 min followed by forty cycles at 95°C for 60 s, 60°C for 30 s and 72°C for 30 s. 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 (*GAPDH*), was used to normalise the expression of the targeted genes⁽⁵⁾. The $2^{-\Delta\Delta C_t}$ was used to calculate the mRNA level of each target gene, where the average mean of C_t from the NT-CON group was used as the calibrator.

Tissue preparations and Western blotting

Frozen tissue samples (40 mg) were minced and homogenised in 0.7 ml of ice-cold RIPA lysis buffer (Cat no. P0013B; Beyotime Institute of Biotechnology) supplemented with protease inhibitor (Cat no. 4693159001; Roche). The homogenates were centrifuged at 12 000 *g* for 5 min at 4°C, and then the cytosolic fractions were collected. Total protein concentration was determined using a BCA Protein Assay kit. Protein extract (30 µg) from each sample was then loaded onto 10 % SDS-PAGE gels, and the separated proteins were transferred onto nitrocellulose membranes (Cat no. IPVH00010; Merck-Millipore). After the transfer, membranes were blocked for 1 h at room temperature in blocking buffer with 5 % skimmed milk and then

incubated overnight at 4°C with the following primary antibodies purchased from Abcam: HSP70 (1:2000, ab69412), HSP90 (1:2000, ab64182), MnSOD (1:3000, ab13533) and GAPDH (1:5000, ab22555). After four washes for 10 min each with Tris-buffered saline containing Tween, membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:5000, Cat no. CW0103A; ComWin Biotech) for 1 h at room temperature. After four washes for 10 min each, bands were visualised by enhanced chemiluminescence using a SuperSignal West Pico Trial Kit (Cat no. 34077; Pierce). The signals were recorded with an ImageQuant LAS 4000 scanner (GE Healthcare Life Sciences) and analysed with the TotalLab Quant software (TotalLab). GAPDH protein was used to normalise the expression levels of the targeted proteins, and the average expression of proteins in the NT-CON was used as a calibrator.

Statistical analyses

All data were analysed by two-way ANOVA using the general linear model procedure of the SAS 9.2 (SAS Institute Inc.), and the model included the main effects of temperature, dietary Mn and their interactions. Each replicate served as the experimental unit. Differences among means were tested by the least significant difference method, and statistical significance was set at $P \leq 0.05$ with a trend at $0.05 < P \leq 0.10$.

Results

Manganese contents of manganese sources, and amino acid contents and Q_f value of manganese proteinate

Mn contents of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ and Mn proteinate were 32.2 and 10.2 % on an as-fed basis, respectively. The Mn proteinate contained amino acids (percentage of the product) with aspartic acid (6.77), serine (2.05), glutamic acid (4.49), threonine (0.57), glycine (12.36), arginine (1.56), alanine (3.76), proline (7.80), valine (1.04), phenylalanine (1.28), isoleucine (0.46), leucine (1.23), lysine (6.75) and methionine (0.34) on an as-fed basis. The chelation strength (Q_f value) of the Mn proteinate was

Table 3. Primer sequences for real-time PCR amplifications

Target genes	Gene bank ID	PCR products (bp)	Primer sequences (5'–3')
<i>β-Actin</i>	NM205518.1	95	F: 5'-ACCTGAGCGCAAGTACTCTGTCT-3' R: 5'-CATCGTACTCCTGCTTGCTGAT-3'
<i>GAPDH</i>	NM204305.1	128	F: 5'-CTTTGGCATTGTGGAGGGTC-3' R: 5'-ACGCTGGGATGATGTTCTGG-3'
<i>HSP70</i>	JX827854.1	144	F: 5'-CGTCAGTGCTGTGGACAAGAGTA-3' R: 5'-CCTATCTCTGTTGGCTTCATCCT-3'
<i>HSP90</i>	NM001109785.1	108	F: 5'-GAGTTTACTGACCCGAGCA-3' R: 5'-TCCCTATGCCGGTATCCACA-3'
<i>HSF1</i>	L06098.1	191	F: 5'-CAGGGAAGCAGTTGGTTCACACACG-3' R: 5'-CCTTGGGTTTGGGTTGCTCAGTC-3'
<i>HSF3</i>	L06126.1	71	F: 5'-CAACAGGACTGAGGAGCAGG-3' R: 5'-TCCACCTCTCCTCTCGGAAG-3'
<i>MnSOD</i>	NM204211.1	137	F: 5'-TTCTGACCTGCCTTACGACTAT-3' R: 5'-CCAGCGCCTCTTTGTATTCT-3'

F, forward; R, reverse; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *HSP70* and *HSP90*, heat-shock proteins 70 and 90; *HSF1* and *HSF3*, heat-shock factors 1 and 3; *MnSOD*, manganese superoxide dismutase.

analysed to be 61.9, which was categorised as a moderate chelation strength based on the classification of Li *et al.*⁽¹³⁾.

Manganese contents in tissues

No interactions ($P > 0.10$) between temperature and dietary Mn were observed in Mn content in the liver, heart, breast muscle and bone (Table 4). Temperature affected ($P < 0.003$) Mn content in the liver and heart, but not ($P > 0.19$) in the breast muscle and bone (Table 4). The Mn content in measured tissues was affected ($P < 0.0001$) by dietary Mn. Compared with NT, HT decreased ($P < 0.003$) the Mn content in the liver and heart. Broiler breeders fed either an iMn or an oMn diet had 1.5–2.0-fold higher ($P < 0.0001$) Mn content in the liver, heart, breast muscle and bone than those fed the CON diet, with no differences ($P > 0.48$) between the two Mn sources except for bone Mn content. Broiler breeders in the oMn group had higher ($P < 0.03$) bone Mn content than those in the iMn group.

Tissue malondialdehyde levels and superoxide dismutase activities

MDA levels and SOD activities in the liver, heart and breast muscle of broiler breeders are listed in Table 5. No interactions ($P > 0.20$) between temperature and dietary Mn were observed in all of the above-mentioned indices. Both temperature and dietary Mn had no effect ($P > 0.14$) on MDA levels in the liver and heart. The breast muscle MDA level tended ($P = 0.06$) to be influenced by temperature, but not by dietary Mn ($P > 0.28$). Dietary Mn influenced the activities of MnSOD ($P < 0.0001$) and CuZnSOD ($P < 0.01$) in the heart, but not ($P > 0.29$) in the liver and breast muscle. Temperature affected ($P = 0.02$) MnSOD activity in breast muscle and tended ($P = 0.07$) to influence the CuZnSOD activity in the liver and heart. The temperature did not affect ($P > 0.26$) the activities of MnSOD in the liver and heart, as well as CuZnSOD in breast muscle. Compared with NT, HT increased ($P < 0.05$) MnSOD activity and tended ($P = 0.06$) to increase the MDA level in breast muscle, but it tended ($P = 0.07$) to decrease the CuZnSOD activities in the liver and heart. Broiler breeders fed diets supplemented with Mn of either source had 9.4 and 12.0% higher ($P < 0.001$) activities of MnSOD and CuZnSOD in the heart, respectively,

than those in CON, with no differences ($P > 0.78$) between the two Mn sources.

mRNA expression levels of heat-shock proteins, heat-shock factors and manganese superoxide dismutase in tissues

The mRNA expression levels of HSP70, HSP90, HSF1, HSF3 and MnSOD genes in the liver, heart and breast muscle of broiler breeders are listed in Tables 6–8. No interactions ($P > 0.15$) between temperature and dietary Mn were observed in all of the above indices. Neither temperature nor dietary Mn influenced ($P > 0.57$) HSP90 mRNA expression levels in the liver and heart (Tables 6 and 7). The mRNA expression levels of HSP90 ($P < 0.007$) in breast muscle, HSP70 ($P < 0.001$) in liver and heart and HSF1 ($P < 0.006$), HSF3 ($P < 0.04$) and MnSOD ($P < 0.04$) in liver, heart and breast muscle were affected by temperature, but not ($P > 0.16$) by dietary Mn (Tables 6–8). Both temperature and dietary Mn influenced ($P < 0.05$) HSP70 mRNA expression levels in breast muscle (Table 8). Compared with NT, HT increased the mRNA expression levels of HSP70, HSF1, HSF3 and MnSOD in the liver, heart and breast muscle ($P < 0.05$) and HSP90 only in breast muscle ($P < 0.005$). Broiler breeders fed either iMn ($P < 0.05$) or oMn ($P < 0.01$) had lower breast muscle HSP70 mRNA expression levels than those in CON, with no difference ($P > 0.62$) between the two Mn sources.

Protein expression levels of heat-shock protein 70, heat-shock protein 90 and manganese superoxide dismutase in tissues

The results and representative immunoblots of HSP70, HSP90 and MnSOD protein expression in liver, heart and breast muscle are shown in Table 9 and Fig. 1(a–c), respectively. No interactions ($P > 0.21$) between temperature and dietary Mn were observed in all of the above indices. The protein expression levels of HSP70, HSP90 and MnSOD in the liver and heart and HSP90 in breast muscle were not affected ($P > 0.36$) by dietary Mn. The temperature affected ($P < 0.004$) the protein expression levels of HSP70 in the liver and heart, as well as HSP90 in breast muscle but not ($P > 0.12$) in the liver and heart. The breast muscle HSP70 and MnSOD protein expression levels were influenced ($P < 0.02$) by both temperature and

Table 4. Effects of environmental temperature (TEMP) and dietary manganese on tissue manganese content (µg/g) of broiler breeders* (Mean values with their standard errors)

Tissues	NT†			HT†			SEM	TEMP†			Dietary Mn†				P		
	CON	iMn	oMn	CON	iMn	oMn		NT	HT	SEM	CON	iMn	oMn	SEM	TEMP	Mn	TEMP × Mn
Liver	1.18	2.21	2.12	1.11	1.70	1.75	0.10	1.84 ^a	1.52 ^b	0.06	1.15 ^b	1.96 ^a	1.93 ^a	0.07	0.0008	<0.0001	0.11
Heart	0.37	0.56	0.59	0.30	0.54	0.50	0.02	0.51 ^a	0.45 ^b	0.01	0.34 ^b	0.55 ^a	0.55 ^a	0.02	0.0021	<0.0001	0.21
Breast muscle	0.32	0.59	0.54	0.42	0.56	0.57	0.03	0.48	0.52	0.02	0.37 ^b	0.58 ^a	0.56 ^a	0.02	0.20	<0.0001	0.22
Bone	2.02	3.23	4.04	2.12	3.46	4.11	0.30	3.10	3.23	0.17	2.07 ^c	3.35 ^b	4.08 ^a	0.21	0.57	<0.0001	0.96

NT, normal temperature; HT, high temperature; CON, Mn-unsupplemented basal diet; iMn, basal diet + 120 mg Mn/kg as MnSO₄·H₂O; oMn, basal diet + 120 mg Mn/kg as the Mn proteinate with a moderate chelation strength of 61.9 quotient of formation (Q_f) (10.2% Mn).

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

* The Mn contents in the liver, heart and breast muscle were on a fresh basis, and the bone Mn content was on an ash basis.

† The values represented the means of 6, 18 and 12 replicate cages (n 6, 18 and 12), respectively.

Table 5. Effects of environmental temperature (TEMP) and dietary Mn on tissue superoxide dismutase (SOD) activities and malondialdehyde (MDA) content of broiler breeders (Mean values with their standard errors)

Tissues	Indices	NT*			HT*				TEMP*			Dietary Mn*				P		
		CON	iMn	oMn	CON	iMn	oMn	SEM	NT	HT	SEM	CON	iMn	oMn	SEM	TEMP	Mn	TEMP × Mn
Liver	MnSOD (NU/mg of protein)†	33.9	36.1	34.7	34.6	35.6	34.6	1.75	34.9	34.9	1.01	34.2	35.8	34.6	1.24	0.99	0.65	0.94
	CuZnSOD (NU/mg of protein)†	41.9	42.4	40.4	37.1	40.8	40.5	1.34	41.6 ^A	39.5 ^B	0.77	39.5	41.6	40.4	0.95	0.07	0.30	0.21
	MDA (nmol/mg of protein)	0.66	0.64	0.64	0.73	0.64	0.56	0.07	0.65	0.64	0.04	0.69	0.64	0.60	0.05	0.64	0.93	0.38
Heart	MnSOD (NU/mg of protein)	54.6	59.0	58.9	53.1	58.8	58.6	0.70	57.5	56.8	0.41	53.8 ^b	58.9 ^a	58.8 ^a	0.50	0.27	<0.0001	0.58
	CuZnSOD (NU/mg of protein)	20.0	21.9	22.2	18.5	20.9	21.0	0.78	21.4 ^A	20.2 ^B	0.45	19.2 ^b	21.4 ^a	21.6 ^a	0.55	0.07	0.009	0.95
	MDA (nmol/mg of protein)	0.91	0.85	1.00	0.92	0.79	0.68	0.10	0.92	0.80	0.08	0.91	0.82	0.84	0.05	0.86	0.15	0.63
Breast muscle	MnSOD (NU/mg of protein)	24.5	25.3	26.6	28.9	29.1	30.2	1.47	25.5 ^b	28.4 ^a	0.85	26.7	27.2	26.9	1.03	0.02	0.94	0.38
	CuZnSOD (NU/mg of protein)	27.7	26.0	27.4	30.4	27.4	27.3	2.29	27.0	28.3	1.33	29.0	26.7	27.3	1.63	0.49	0.58	0.83
	MDA (nmol/mg of protein)	0.25	0.25	0.25	0.33	0.27	0.38	0.05	0.25 ^B	0.32 ^A	0.03	0.29	0.26	0.31	0.03	0.06	0.29	0.55

NT, normal temperature; HT, high temperature; CON, Mn-unsupplemented basal diet; iMn, basal diet + 120 mg Mn/kg as MnSO₄·H₂O; oMn, basal diet + 120 mg Mn/kg as the Mn proteinate with a moderate chelation strength of 61.9 quotient of formation (Q) (10.2% Mn); NU, nitrite units.
^{A,B} Mean values within a row with unlike superscript uppercase letters were significantly different for a trend (0.05 < P < 0.10).
^{a,b} Mean values within a row with unlike superscript lowercase letters were significantly different (P < 0.05).
 * The values represented the means of 6, 18 and 12 replicate cages (n 6, 18 and 12), respectively.
 † One NU was defined as the amount of enzyme needed to obtain 50% inhibition of nitrite formation.

Table 6. Effects of environmental temperature (TEMP) and dietary manganese on mRNA expression of heat-shock proteins (HSP), heat-shock factors (HSF) and manganese superoxide dismutase (MnSOD) in the liver of broiler breeders* (Mean values with their standard errors)

Indices	NT†			HT†				TEMP†			Dietary Mn†				P		
	CON	iMn	oMn	CON	iMn	oMn	SEM	NT	HT	SEM	CON	iMn	oMn	SEM	TEMP	Mn	TEMP × Mn
HSP70	1.00	0.78	0.89	2.88	2.61	2.83	0.29	0.89 ^b	2.77 ^a	0.17	1.94	1.69	1.86	0.21	0.0004	0.92	0.99
HSP90	1.00	1.08	1.18	1.49	0.90	0.72	0.23	1.08	1.04	0.13	1.25	0.99	0.95	0.16	0.74	0.58	0.91
HSF1	1.00	0.85	1.11	1.57	1.47	1.45	0.16	0.99 ^b	1.50 ^a	0.09	1.29	1.16	1.28	0.11	0.005	0.81	0.78
HSF3	1.00	0.70	1.09	1.53	1.35	1.26	0.15	0.93 ^b	1.38 ^a	0.09	1.27	1.03	1.18	0.10	0.003	0.38	0.36
MnSOD	1.00	0.95	1.09	1.75	1.61	1.77	0.39	1.02 ^b	1.71 ^a	0.22	1.38	1.28	1.42	0.27	0.03	0.93	0.99

NT, normal temperature; HT, high temperature; CON, Mn-unsupplemented basal diet; iMn, basal diet + 120 mg Mn/kg as MnSO₄·H₂O; oMn, basal diet + 120 mg Mn/kg as the Mn proteinate with a moderate chelation strength of 61.9 quotient of formation (Q) (10.2% Mn).
^{a,b} Mean values within a row with unlike superscript letters were significantly different (P < 0.05).
 * The geometric mean of internal references, β-actin and glyceraldehyde-3-phosphate dehydrogenase was used to normalise the expression of targets genes, and the average expression of genes mRNA in the NT-CON was used as a calibrator.
 † The values represented the means of 6, 18 and 12 replicate cages (n 6, 18 and 12), respectively.

Table 7. Effects of environmental temperature (TEMP) and dietary manganese on mRNA expression of heat-shock proteins (HSP), heat-shock factors (HSF) and manganese superoxide dismutase (MnSOD) in the heart of broiler breeders* (Mean values with their standard errors)

Indices	NT†			HT†				TEMP†			Dietary Mn†				P		
	CON	iMn	oMn	CON	iMn	oMn	SEM	NT	HT	SEM	CON	iMn	oMn	SEM	TEMP	Mn	TEMP × Mn
HSP70	1.00	0.97	0.83	1.86	2.05	1.48	0.27	0.93 ^b	1.80 ^a	0.16	1.44	1.51	1.15	0.19	0.0008	0.43	0.76
HSP90	1.00	1.28	1.17	1.16	1.28	1.18	0.22	1.16	1.21	0.12	1.08	1.28	1.18	0.16	0.73	0.60	0.91
HSF1	1.00	1.08	1.13	1.35	1.37	1.60	0.14	1.07 ^b	1.44 ^a	0.08	1.18	1.23	1.36	0.09	0.002	0.38	0.77
HSF3	1.00	0.82	0.88	1.46	1.06	1.11	0.23	0.90 ^b	1.21 ^a	0.13	1.24	0.94	1.00	0.13	0.03	0.17	0.73
MnSOD	1.00	0.97	0.83	1.86	2.05	1.48	0.09	0.93 ^b	1.80 ^a	0.05	1.44	1.51	1.15	0.10	0.0008	0.43	0.76

NT, normal temperature; HT, high temperature; CON, Mn-unsupplemented basal diet; iMn, basal diet + 120 mg Mn/kg as MnSO₄·H₂O; oMn, basal diet + 120 mg Mn/kg as the Mn proteinate with a moderate chelation strength of 61.9 quotient of formation (Q) (10.2% Mn).

^{a,b} Mean values within a row with unlike superscript letters were significantly different (P < 0.05).

* The geometric mean of internal references, β-actin and glyceraldehyde-3-phosphate dehydrogenase was used to normalise the expression of target genes, and the average expression of each gene's mRNA in the NT-CON was used as a calibrator.

† The values represented the means of 6, 18 and 12 replicate cages (n 6, 18 and 12), respectively.

Table 8. Effects of environmental temperature (TEMP) and dietary manganese on mRNA expression of heat-shock proteins (HSP), heat-shock factors (HSF) and manganese superoxide dismutase (MnSOD) in breast muscle of broiler breeders* (Mean values with their standard errors)

Indices	NT†			HT†				TEMP†			Dietary Mn†				P		
	CON	iMn	oMn	CON	iMn	oMn	SEM	NT	HT	SEM	CON	iMn	oMn	SEM	TEMP	Mn	TEMP × Mn
HSP70	1.00	0.56	0.57	1.16	0.93	0.77	0.17	0.71 ^b	0.95 ^a	0.10	1.08 ^a	0.75 ^b	0.67 ^b	0.11	0.04	0.01	0.76
HSP90	1.00	0.68	0.92	1.32	1.90	1.28	0.50	0.87 ^b	1.50 ^a	0.29	1.16	1.29	1.10	0.35	0.006	0.82	0.16
HSF1	1.00	0.68	0.85	1.55	1.60	1.22	0.23	0.84 ^b	1.45 ^a	0.13	1.28	1.14	1.03	0.11	0.0002	0.39	0.30
HSF3	1.00	0.96	0.95	1.83	1.64	1.30	0.35	0.97 ^b	1.59 ^a	0.21	1.41	1.30	1.12	0.25	0.03	0.68	0.75
MnSOD	1.00	1.13	1.14	1.66	1.56	1.40	0.12	1.09 ^b	1.54 ^a	0.07	1.33	1.34	1.27	0.08	0.0001	0.82	0.27

NT, normal temperature; HT, high temperature; CON, Mn-unsupplemented basal diet; iMn, basal diet + 120 mg Mn/kg as MnSO₄·H₂O; oMn, basal diet + 120 mg Mn/kg as the Mn proteinate with a moderate chelation strength of 61.9 quotient of formation (Q) (10.2% Mn).

^{a,b} Mean values within a row with unlike superscript letters were significantly different (P < 0.05).

* The geometric mean of internal references, β-actin and glyceraldehyde-3-phosphate dehydrogenase was used to normalise the expression of targets genes, and the average expression of gene's mRNA in the NT-CON was used as a calibrator.

† The values represented the means of 6, 18 and 12 replicate cages (n 6, 18 and 12), respectively.

Table 9. Effects of environmental temperature (TEMP) and dietary manganese on protein expression of heat-shock protein 70 (HSP70), heat-shock protein 90 (HSP90) and manganese superoxide dismutase (MnSOD) in tissues of broiler breeders* (Mean values with their standard errors)

Tissues	Indices	NT†			HT†				TEMP†			Dietary Mn†				P		
		CON	iMn	oMn	CON	iMn	oMn	SEM	NT	HT	SEM	CON	iMn	oMn	SEM	TEMP	Mn	TEMP × Mn
Liver	HSP70	1.00	1.29	1.47	1.98	2.36	1.90	0.17	1.25 ^b	2.07 ^a	0.10	1.49	1.81	1.68	0.12	0.002	0.54	0.51
	HSP90	1.00	1.06	1.19	1.57	1.37	1.11	0.15	1.10	1.25	0.09	1.29	1.21	1.15	0.11	0.13	0.80	0.22
	MnSOD	1.00	1.23	1.08	1.08	1.15	1.55	0.16	1.10	1.25	0.09	1.04	1.19	1.31	0.11	0.33	0.37	0.34
Heart	HSP70	1.00	0.83	0.75	1.15	1.06	1.26	0.11	0.86 ^b	1.15 ^a	0.07	1.08	0.94	1.01	0.08	0.003	0.51	0.29
	HSP90	1.00	0.95	0.86	0.92	1.03	1.12	0.12	0.94	1.02	0.07	0.96	0.99	0.99	0.08	0.37	0.97	0.38
	MnSOD	1.00	0.93	0.88	0.81	0.73	0.79	0.13	0.94	0.78	0.08	0.90	0.83	0.84	0.09	0.11	0.75	0.85
Breast muscle	HSP70	1.00	0.80	0.67	1.47	1.29	1.15	0.13	0.82 ^b	1.30 ^a	0.06	1.23 ^a	1.04 ^b	0.90 ^b	0.07	<0.0001	0.01	0.99
	HSP90	1.00	1.09	0.82	1.78	1.69	1.73	0.17	0.97 ^b	1.77 ^a	0.10	1.38	1.39	1.27	0.12	<0.0001	0.93	0.46
	MnSOD	1.00	1.79	2.35	2.80	3.55	3.23	0.12	1.71 ^b	3.20 ^a	0.08	1.90 ^b	2.66 ^a	2.78 ^a	0.08	0.0003	0.01	0.22

NT, normal temperature; HT, high temperature; CON, Mn-unsupplemented basal diet; iMn, basal diet + 120 mg Mn/kg as MnSO₄·H₂O; oMn, basal diet + 120 mg Mn/kg as the Mn proteinate with a moderate chelation strength of 61.9 quotient of formation (Q) (10.2% Mn).

^{a,b} Mean values within a row with unlike superscript letters were significantly different (P < 0.05).

* The glyceraldehyde-3-phosphate dehydrogenase protein was used to normalise the expression levels of the target proteins, and the average expression level of protein in the NT-CON was used as a calibrator.

† The values represented the means of 6, 18 and 12 replicate cages (n 6, 18 and 12), respectively.

dietary Mn. Compared with NT, HSP70 protein expression levels in the three tissues were increased (P < 0.0004) under HT, whereas the protein expression levels of HSP90 and MnSOD only in breast muscle were increased (P < 0.0004)

by 1-fold under HT. Broiler breeders fed either iMn (P < 0.05) or oMn (P < 0.01) had higher MnSOD but lower HSP70 protein expression levels in breast muscle than those in CON.

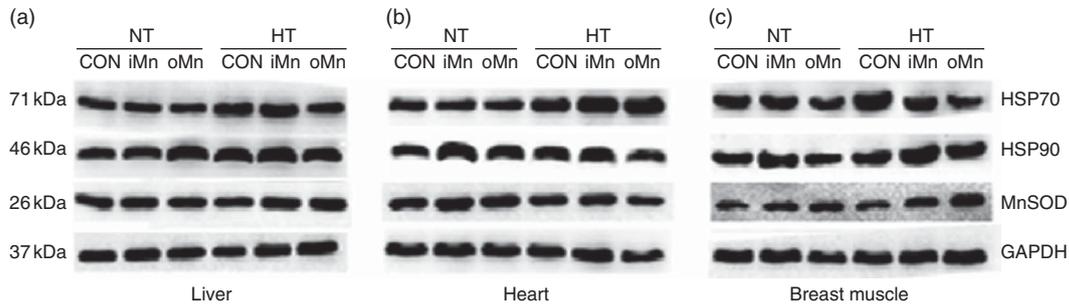


Fig. 1. Representative immunoblots demonstrating heat-shock protein 70 (HSP70), heat-shock protein 90 (HSP90) and manganese superoxide dismutase (MnSOD) protein expression in the liver, heart and breast muscle of laying broiler breeders subjected to varying temperature and Mn supplementation (a–c). NT, normal temperature; HT, high temperature; CON, Mn-unsupplemented basal diet; iMn, basal diet + 120 mg Mn/kg as $\text{MnSO}_4 \cdot \text{H}_2\text{O}$; oMn, basal diet + 120 mg Mn/kg as the Mn proteinate with a moderate chelation strength of 61.9 quotient of formation (Q) (10.2% Mn); GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Discussion

The negative effect of heat stress on mineral balance in broilers has been previously reported^(26,27). In the present study, significantly decreased Mn content in the liver and heart was observed in heat-stressed broiler breeders. The decreased Mn content may have been because of reduced Mn absorption coupled with increased Mn excretion under HT⁽²⁸⁾. Thus, a higher dietary Mn level may be required for broiler breeders exposed to HT compared with NT. Dietary supplementation of Mn of either Mn source increased tissue Mn content under both NT and HT in this study, which was consistent with previous studies in broilers^(12–15) and laying hens⁽¹¹⁾. Specifically, the organic Mn with moderate chelation strength demonstrated greater bioavailability than the inorganic Mn in bone. A series of studies also suggest that organic Mn has higher absorption and bioavailability than inorganic Mn for broilers^(13–16,28,29) and lambs⁽³⁰⁾, and organic Mn sources with moderate chelation strengths exhibit the greatest availability^(13–15).

Heat stress is believed to produce ROS and induce oxidative damage in broilers^(31,32) and laying hens⁽⁶⁾. In the current study, HT showed a clear trend towards elevating breast muscle MDA content and the increased plasma MDA level was also observed in our another study⁽¹⁷⁾, suggesting that lipid peroxidation occurs under the HT condition. However, at the same time, HT led to an increase in MnSOD activity in breast muscle. Both SOD (MnSOD and CuZnSOD) may allow superoxide radicals to be scavenged and protect cells against toxic oxygen radicals. Therefore, the damage to the breast muscle membrane resulting from free radicals under HT may have induced the corresponding increase in its MnSOD activity because of its function in self-protection during heat stress. The above results suggest that heat stress may disturb the redox balance and induce oxidative damage in the breast muscle of laying broiler breeders.

Evidence suggests that HSP, especially HSP70, are involved in the development of thermotolerance in broilers^(18,19) and in human and mouse cells^(33–35). In the present study, the up-regulations of both *HSP70* mRNA and protein in the heart, liver and breast muscle of broiler breeders exposed to HT might favour an anti-heat stress response. Some reports have confirmed that the expression levels of *HSP* are regulated mainly at the level of transcription by HSF^(36,37). *HSF1*- and *HSF3*-mediated mechanisms of cellular adaptation to heat shock

have been examined in detail in chicken embryo fibroblasts, and *HSF3* is considered an avian-specific factor⁽³⁸⁾. As expected, the synchronised changes in transcriptional activation between *HSF1*, *HSF3* and *HSP70* mRNA and protein were observed in the liver, heart and breast muscle under HT. Generally, *HSF1*, *HSF3* can bind the heat-shock element in the promoter of the *HSP70* to up-regulate *HSP70* in response to heat stress⁽³⁹⁾. The up-regulation of HSP70 under HT might be dependent upon the presence of ROS and/or oxidative stress induced by heat stress. Previous research indicates that ROS-mediated activation of p38 mitogen-activated protein kinase (p38MAPK) can increase *HSF1* DNA-binding activity to increase *HSP70* expression in cells⁽⁴⁰⁾. As previously reported, expression of *HSF* is mainly altered by certain stressors, and it exhibits a stress-specific pattern⁽⁴¹⁾. This could very well explain why dietary Mn supplementation did not affect the expression of *HSF* mRNA in the present study.

In addition, the increased expression levels of *HSP90* mRNA and protein were induced only in breast muscle of birds under chronic heat challenge, but not in the liver and heart, suggesting that the abundance and inducibility of *HSP90* might be tissue dependent⁽⁴²⁾. The muscle was the most sensitive and responsive tissue of heat-stressed broiler breeders based on the expression of *HSP90*. However, the expression levels of *HSF1* and *HSF3* mRNA in the liver and heart were also increased by HT. In fact, the *HSP90* expression levels were regulated not only by HSF⁽³⁶⁾ but also by other non-HSF transcription factors, such as signal transducer and activator of transcription (STAT) family⁽⁴³⁾ and nuclear factor IL-6⁽⁴⁴⁾. It is reported that the STAT-3 signalling pathway has inhibitory effects on HSF-mediated activation of *HSP90*⁽⁴⁵⁾. Therefore, it would be possible to have no response of *HSP90* expression in the liver and heart under HT. In addition to *HSP*, antioxidant enzymes are induced by stressors and provide an organism with multiple protective options^(40,46). The increases of *MnSOD* mRNA and protein expression levels, as well as MnSOD activity, were observed in the breast muscle of broiler breeders subjected to HT, suggesting that these up-regulated responses in the breast muscle might be essential for reducing lipid peroxidation damage and protecting mitochondria stability and integrity. The enhancement of MnSOD expression under heat exposure could be caused by the presence of elevated ROS levels via activation of p38MAPK and protein kinase B⁽⁴⁰⁾.

Mn is a crucial cofactor of MnSOD, which is the most dominant dismutase involved in scavenging free radicals.

Severe Mn deficiency (1 mg/kg) in purified diets decreased MnSOD activities in heart and other organs (liver, kidney and brain) of rats, mice and chickens⁽⁴⁷⁾. However, previous studies from our laboratory have shown that moderate Mn deficiency (about 16 mg/kg) in a practical maize–soyabean meal diet depressed MnSOD activity only in the heart, but not in the liver and pancreas, and then further led to Mn-responsive abnormalities in heart mitochondrial ultrastructure, indicating that the heart of broilers is more sensitive to Mn deficiency than other tissues^(9,10). In the present study, we also found that supplemental Mn significantly increased MnSOD activity, as well as CuZnSOD activity, in the heart regardless of Mn source, indicating that Mn-supplemented broiler breeders might be better prepared to deal with oxidative stress with enhanced antioxidant ability. Similar results have been observed in commercial laying hens⁽¹²⁾ in our previous study under thermoneutrality conditions. Our other studies further demonstrated that heart *MnSOD* mRNA level in broilers is more sensitive to dietary Mn level than is MnSOD activity^(13–15). However, in this study, no differences in expression of heart *MnSOD* mRNA or protein were observed in broiler breeders receiving dietary Mn treatments. This discrepancy between broiler chicks and laying broiler breeders might mainly be because of differences in metabolic and physiological statuses at different developmental stages. It appears that Mn accumulation in broiler breeders in the pre-period was sufficient to meet their Mn need for heart *MnSOD* mRNA and protein expression levels. Breast muscle MnSOD protein level was increased by dietary Mn supplementation regardless of Mn source, suggesting that the protection of breast muscle against ROS might depend, in part, upon an endogenous pool of antioxidant enzymes stored as proteins during heat stress. These findings also suggest that MnSOD expression in breast muscle and heart may be up-regulated by dietary Mn at different levels of translation and post-translational modification, respectively.

Heat shock, as a promoter of oxidative stress, creates a redox imbalance by increasing the generation of ROS⁽³¹⁾. Subsequent cellular damage caused by accumulation of ROS has been suggested as a key factor for activation of HSP genes^(7,8). When cells are subjected to heat shock with an increase in lipid peroxidation, HSP70 accumulates and might serve as a tissue biomarker for potential stress damage⁽²²⁾. Thus, constitutive and inducible HSP70 expression levels might be regarded as a response to damage resulting from a strong stress to the organism^(20,21). In addition, the increased activities of SOD and catalase likely scavenge free radicals that inhibit the expression of HSP proteins and improve cell survival⁽²³⁾. In the present study, compared with birds fed the control diet, broiler breeders fed diets supplemented with Mn from either source had lower expression levels of *HSP70* mRNA and protein in breast muscle regardless of environmental temperatures. It appears that dietary Mn supplementation may inhibit HSP70 expression by enhancing the activities of SOD and subsequent removal of ROS. Similarly, it has been reported that reduced induction of HSP70 protein occurs in Se-fed chickens because of the higher glutathione peroxidase activity in the liver⁽⁷⁾.

In conclusion, high environmental temperature decreases Mn retention and increases *HSP70* and *HSF1*, *HSF3* expression levels in tissues of laying broiler breeders. Breast muscle is

more susceptible to heat stress because of the induction of lipid peroxidation, as well as the up-regulation of *HSP90* expression. Dietary supplementation with Mn for either source may thus enhance heart antioxidant capacity and inhibit the expression of HSP70 in muscle. Organic Mn proteinate with a moderate chelation strength might be more available to bone than inorganic Mn sulphate in laying broiler breeders regardless of environmental temperatures.

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The authors declare that there are no conflicts of interest.

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