Sulfate and hydroxychloride trace minerals in poultry diets – comparative effects on egg production and quality in laying hens, and growth performance and oxidative stress response in broilers

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ABSTRACT Two experiments investigated the effect of sulfate and hydroxychloride trace minerals (TM), Zn, Cu, and Mn, in laying hens and broiler chickens. In Expt. 1, Lohmann Brown pullets (total of 1,344) at 21 wk of age were used for a 24-wk experiment. Each of the two treatments had 32 replicates with 21 hens per replicate. At 45 wk of age, three eggs per cage were randomly selected and used for internal quality assessment. In Expt. 2, Ross 308 broilers (total of 1,080) were allocated to two treatments. Each treatment had 30 replicates with 15 chicks per replicate. On day 28, after weighing, three birds were randomly selected from 15 randomly selected pens per treatment. The birds were euthanized and blood was collected for analysis for uric acid, C-reactive protein and methylmalonic acid. Samples were also taken from *pectoralis* muscle of each chicken and analyzed for mRNA expression of protein synthesis or hydrolysis genes. On day 35, 7 birds per pen were used for carcass evaluation. In Expt. 1, egg weight was greater ($P < 0.01$) in birds receiving sulfate TM from week 16 (of experiment) onwards whereas the percentage of cracked eggs was lower ($P < 0.01$) in hens receiving hydroxychloride TM. Percentage hen-day production tended to be greater ($P < 0.10$) in hens receiving hydroxychloride TM in weeks 4 to 8 only. In Expt. 2, birds receiving hydroxychloride TM had greater ($P < 0.05$) weight gain and tended to have greater ($P < 0.10$) feed intake on day 35. Expression of the gene, PSMA1, was lower ($P < 0.05$) whereas plasma level of uric acid and methyl malonic acid tended to be lower ($P < 0.10$) in birds receiving hydroxychloride TM. It was concluded that hydroxychloride TM reduced egg loss in hens at peak production and that improved growth performance response in broilers can be partly explained by reduction in proteolytic activities in the *pectoralis* muscle and greater resilience to oxidative stress.

Key words: hydroxychloride, trace mineral, laying hen, broiler, oxidative stress

2019 Poultry Science 98:4961–4971
http://dx.doi.org/10.3382/ps/pez261

INTRODUCTION

Trace minerals (**TM**) play crucial roles in metabolic processes contributing to growth and production of poultry birds. For example, Zn is a component of metalloenzymes (Prassad, 1984), whereas Cu and Mn are cofactors of many enzymes (Brandt and Schramm, 1986; Davis and Mertz, 1987). Common sources of the TM in poultry diets include inorganic (oxide or sulfates), organic and hydroxychloride all of which have different bioavailabilities (Miles et al., 1998; Batal et al., 2001; Luo et al., 2005). In addition, the inorganic TM sources may cause irritation of the intestinal mucosa, complex with other minerals and ultimately be excreted into the environment (Cao et al., 2002; Mwangi et al., 2017).

On the other hand, hydroxychloride TM sources do not have the limitations of the inorganic TM possibly due to their crystalline structure (Hawthorne and Sokolova, 2002) which ensures slow release during digestion, more optimal absorption and consequently less excretion. Previous research has shown that broilers receiving hydroxychloride Zn and Cu had superior performance compared to those receiving the inorganic TM (Arias and Koutsos, 2006; Liu et al., 2015; M'Sadeq et al., 2018). Nonetheless, positive growth performance response to supplemental TM from various sources is not consistently observed (Kidd et al., 1993).

In a previous study, we reported that hydroxychloride Zn and Cu enhanced growth performance and breast yield in broilers (Olukosi et al., 2018). Various mechanisms for this positive effect on growth has been proposed including greater bioavailability of

Received December 22, 2018.
Accepted April 18, 2019.

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hydroxychloride TM, enhancement of nutrient utilization or alleviation of oxidative stress (Batal et al., 2001, Rochell et al., 2017) but with the possible exception of bioavailability tests, many of these proposed mechanisms are speculative at best. In addition, there is a dearth of information on the use and effect of hydroxychloride Zn, Cu and Mn on egg production and egg quality in laying hens.

Therefore, the objectives of the experiments reported here were to study the influence of hydroxychloride or sulfate Zn, Cu and Mn on laying hens production efficiency and egg quality as well as the influence of hydroxychloride or sulfate Zn and Cu on growth performance and meat yield of broiler chickens. Two possible modes of action by which hydroxychloride TM accrue beneficial effects on growth performance of broilers were also investigated.

MATERIALS AND METHODS

All the animal experiment procedures in the current studies were approved by Scotland's Rural College's Animal Experiment Committee.

Experiment 1

A total of 1,344 Lohmann Brown (Classic) pullets at 21-wk-old were used for the 24-wk experiment. The birds were obtained at 16 wk old from a commercial grower and were adjusted to the research station for 5 wk prior to initiation of the experiment. The birds were allocated to 64 enriched colony cages with 21 birds per cage. Each cage was furnished with a nest, perch, and scratch pad. No additional furniture was used in the cages. The cages were allocated to experimental treatments in a randomized complete block design to ensure equal body weight of the birds at the beginning of the experiment.

The birds received the experimental diets (mash form) that were formulated to meet the nutrient requirement of Lohmann Brown (Lohmann, 2016) hens in Phase 1 of production (Table 1). The wheat-based diets were supplemented with vitamin-TM premix that was devoid of supplemental Zn, Cu, and Mn. Diet 1 was then supplemented with analytical grade Zn (as zinc sulfate monohydrate, Acro Organics, Belgium), Cu (as copper sulfate pentahydrate, Sigma Aldrich, United Kingdom) and Mn (as manganese sulfate monohydrate, Sigma Aldrich, United Kingdom). Diet 2 was supplemented with hydroxychloride Zn (IntelliBond® Z, Trouw Nutrition, Netherlands), hydroxychloride Cu (IntelliBond® C, Trouw Nutrition, Netherlands), and hydroxychloride Mn (IntelliBond® Mn, Trouw Nutrition, Netherlands).

The intended supplemental levels were 15, 80, and 80 ppm for Cu, Zn, and Mn, respectively for both sulfate and hydroxychloride minerals. The supplemental levels of each of the minerals and sources are shown in Table 2. The hens were provided the diets in ad libitum basis. New feed batch was manufactured every 8 wk and feed weigh back was done bi-weekly throughout the experiment. Hens were weighed at the beginning and end of the experiment.

Egg Production Total eggs laid were collected and weighed daily to determine egg mass and for calculation of hen-day production. After weighing, eggs with physical defects, denoted faulty (small, shell-less, thin shelled, double yolk, deformed, etc.) were noted and recorded.

Egg Internal Quality Assessment All the eggs produced on day 3 of the last week of the experiment were collected (hens were 45-wk-old). Three eggs per cage (n = 96 per treatment) were randomly selected and used for internal quality assessment of the eggs. All the egg quality assessments were done using egg quality equipment QCM+^{TM} and QCC^{TM} (Technical Services and Supplies Ltd., York, United Kingdom). The egg qualities assessed were albumen height, Haugh unit, yolk weight, Roche yolk color, albumen weight, and shell weight. Shell thickness was measured using a micrometer screw gauge (part of the equipment). The weights of the albumen, yolk and shell were also expressed relative to the egg weight.

Yolk Trace Minerals Analysis The yolk from the three eggs per cage used for internal egg quality assessment were pooled per cage, frozen, and lyophilized. The dried yolks were then used for analysis of Cu, Mn, and Zn, as described below.

Experiment 2

A total of 1,080 Ross 308 male broilers at 0-day-old were used for the 35-D experiment. The broiler chicks were allocated to 2 treatments in a randomized complete block design. Each treatment had 30 replicates with 15 chicks per replicate pen. The diets were wheat–soybean meal based and were supplemented with vitamin-trace mineral premix that was Zn- and Cu-free (basal Zn and Cu levels were 34.3 and 7.6 ppm, respectively). Diet 1 was subsequently supplemented with sulfate Zn and Cu, whereas diet 2 was supplemented with hydroxychloride Zn and Cu at levels of 80 ppm and 15 ppm, respectively, as previously described (Olukosi et al., 2018). The diets were fed in three phases, namely the starter (day 0 to 9), grower (day 9 to 21), and finisher (day 21 to 35). The ingredient and expected chemical composition of the experimental diets are shown in Table 1. The birds and feed were weighed on days 0, 9, 21, and 35. Feed was supplied as crumbles for the first 9 D, and as pellets from day 9 until end of the experiment. Paracox 8 (MSD Animal Health, Milton Keynes, United Kingdom) was administered in feed to birds on day 5 of age according to manufacturer's guide (0.1 mL per bird, diluted 5 × with distilled water).

Blood Collection and Analysis On day 28, after weighing, three birds were randomly selected from

Table 1. Ingredient composition of the experimental diets.

Items	Expt. 1	Expt. 2		
		Day 0 to 9	Day 9 to 21	Day 21 to 35
Wheat	553.2	475.9	506.4	545.92
Barley	150.0	150.0	150.0	150
Soybean meal 48	182.0	330.0	295.0	240
Soybean oil	18.0	8.0	15.0	35
DL Met	1.60	1.60	1.60	1.4
L-Lysine	1.60	1.60	1.60	1.8
L Threonine	0.15	0.15	0.15	0.18
Calcium carbonate	73.0	9.50	9.50	8.0
DCP	11.0	14.0	11.5	8.5
Salt NaCl	2.50	2.50	2.50	2.4
Sodium bicarbonate	2.80	2.80	2.80	2.8
VTE Premix[1]	4.00	4.00	4.00	4
$CuSO_4.5H_2O$[2]	+/−	+/−	+/−	+/−
$ZnSO_4.H_2O$[2]	+/−	+/−	+/−	+/−
$MnSO_4.H_2O$[2]	+/−	–	–	–
Yellow carophylle	0.003	–	–	–
Lucantin Red	0.002	–	–	–
Xylanase[3]	0.075	+	+	+
Phytase[4]	0.060	+	+	+
Total	1000.0	1000.0	1000.0	1000
Calculated Nutrients & Energy				
Protein, g/kg	166.8	229.6	216.0	193.9
ME, kcal/kg	2,744	3,122	3,148	3,239
Ca, g/kg	32.1	8.53	7.80	6.30
Non-phytate P, g/kg	2.70	3.49	2.99	2.37
Digestible amino acids, g/kg				
Arg	10.1	15.0	13.9	12.2
His	4.1	5.8	5.5	4.8
Ile	6.5	9.5	8.8	7.8
Leu	11.8	16.7	15.6	13.9
Lys	8.9	13.1	12.1	10.8
Met	4.0	4.9	4.7	4.2
Cys	3.1	3.9	3.8	3.5
Phe	4.5	8.1	7.2	5.9
Tyr	3.2	5.9	5.3	4.3
Thr	5.8	8.4	7.8	6.9
Trp	2.1	2.9	2.8	2.5
Val	7.5	10.5	9.8	8.8
TSAA	7.1	8.8	8.5	7.7
Phe+Tyr	13.0	18.7	17.5	15.4

[1]VTE premix was free of Zn, Cu, and Mn in Expt. 1 and free of Zn and Cu for Expt. 2.
[2]Intellibond Zn, Cu, and Mn were used in Expt. 1; whereas Intellibond Zn and Cu were used in Expt. 2.
[3]In Expt. 1, Econase XT, 12,000 BXU/kg was used. For Expt. 2, Danisco AXTRA XB was used.
[4]In Expt. 1, AB Vista Quantum blue was added at 300 FTU/kg. For Expt. 2, Danisco Phyzyme XP was added at the rate of 500 FTU/kg.

15 randomly selected pens per treatment. The birds were euthanized and blood was collected into specialized trace mineral-free tubes (S-Monovette, Sarstedt, Germany). The blood from the 3 birds per pen was pooled and subsequently centrifuged at 2000 × *g* for 15 min in order to separate the plasma. The plasma was frozen at −20°C prior to analysis for uric acid, C-reactive protein, and methylmalonic acid as indicators of stress response in the birds.

Gene Expression Analysis Two randomly selected birds (among the 3) from each of 15 pens as described above were used for analysis of mRNA expression levels of selected protein synthesis and degradation genes. After euthanasia, 2 approximately 3- to 4-g samples were taken from left *pectoralis major* muscle of each chicken and fixed in RNA-later (Qiagen, Manchester, United Kingdom) prior to analysis. The protein synthesis genes analyzed for were mechanistic target of rapamycin serine/threonine kinase (mTOR), ribosomal protein S6 kinase 1 (S6K1), and eukaryotic initiation factor, 4E-binding protein 1 (4E-BP1). The proteolytic genes analyzed for were cathepsin B (CB), 20S proteasome (PSMA1), muscle atrophy F Box (MAFbx), and muscle ring finger 1 (MurF1). The housekeeping

Table 2. Supplementation levels (g/ton) of sulfate and hydroxychloride trace minerals in Expt. 1 and 2.

Diet	Sulfate			Hydroxychloride		
	Zn	Cu	Mn	Zn	Cu	Mn
			Experiment 1			
1	220	59	246			
2				145.5	27.8	181.8
			Experiment 2			
1	220	59	–			
2				145.5	27.8	–

Table 3. GenBank accession number, sequences of forward and reverse primers and fragments sizes used for real-time PCR.

Target	Accession number	Primer sequence	Size (bp)
MTOR	XM_417,614.5	F: 5′-GCAGGCAATGATGGTAAGTGAAG-3′; R: 5′-AGACGAGATGCTTCTTCCAACC-3′	90
RPS6KB1	NM_001,03072	F: 5′-CGCTGCCTCACGTCTAGGA-3′; R: 5′-CCAGTTAATGTGTCTGAAGAACGG-3′	79
EIF4EBP1	XM_424,384.5	F: 5′-GGGCGGAACCAGGATTATTTATG-3′; R: 5′-CGGAAGGTCAGAAGGTGGTG-3′	88
CTSB	NM_205,371.2	F: 5′-CTGCCAACTCCTGGAACACTG-3′; R: 5′-AGCCACGATCTCGGACTCG-3′	95
PSMA1	NM_205,020.1	F: 5′-CGTTGACAACCATATCGGTATCTC-3′; R: 5′-CGAGATACTGGAAGAGGTCTATCAA-3′	126
FBXO32	NM_001,03095	F: 5′-CCAGTGGAGCTCATGGCTG-3′; R: 5′-GCATCTTCAGAGCCTTTAACTAGG-3′	112
TRIM63	XM_0,152,9775	F: 5′-ATCTACAAGCAGGAGTTCTCCAG-3′; R: 5′-TCGCAGGTGACGCAGTAGA-3′	104

MTOR—mechanistic target of rapamycin (serine/threonine kinase).
RPS6KB1–ribosomal protein S6 kinase B1.
EIF4EBP1–eukaryotic translation initiation factor 4E binding protein 1.
CTSB—cathepsin B.
PSMA1–proteasome subunit alpha 1.
FBXO32–F-box protein 32.
TRIM63–tripartite mortif containing 63, transcript variant X1.

gene used was glyceraldehyde 3-phosphate dehydrogenase. The *Gallus gallus* gene-specific primers for all the genes of interest (Table 3) were designed by PrimerDesign (Southampton, United Kingdom). The procedures for RNA extraction and quantitative real-time PCR were as described previously (Walk et al., 2018).

Carcass Processing Carcass processing (at the Carcass Evaluation Unit of Scotland's Rural College) was done on day 35 on seven representative birds per pen. The birds were fasted overnight and the details of the procedures for carcass processing were as previously described (Olukosi et al., 2018). In addition to the criteria evaluated in Olukosi et al. (2018), the carcasses were scored for white stripping and woody breast adapting the scoring methods of Kuttappan et al. (2012) and Sihvo et al. (2017), respectively and were assigned scores of 0 (normal) to 2 (severe). In addition, drip loss was measured in the carcasses as described by Rémignon et al. (1996).

Foot Pad and Litter Scoring Foot pad scoring (**FPS**) was done on day 35 on 5 randomly selected birds per pen. Litter scoring (**LS**) was done, by visual assessment, at the end of the study after birds had been removed. The procedures for FPS and LS were as previously stated (Olukosi et al., 2018).

Chemical Analysis

The diets for each experiment were milled through 0.5 mm sieve prior to analysis. Diets were subsequently analyzed for dry matter, N, ether extract, acid, and neutral detergent fibers, ash, and minerals. Analysis for uric acid, C-reactive protein (not reported due to unexplained variability/non-detection) and methyl malonic acid were done at Synlab (Synlab Vet GmbH, Standort Augsburg, Germany).

Dry matter was determined by drying the samples in a drying oven at 100°C for 24 h (Method 934.01, AOAC, 2006). Nitrogen was determined by the combustion method (Method 968.06, AOAC, 2006). Total (acid hydrolyzed) fat analysis was done using AOAC method 925.32 (AOAC, 2006). Acid and neutral detergent fibers were analyzed using the Ankom nylon bag technique (Ankom 220 Analyzer). Ash content was determined in a muffle furnace by ashing the sample overnight at 600°C (Method 942.05, AOAC, 2006). Minerals content (diets and dried yolk) was determined using inductively coupled plasma—optical emission spectroscopy (AOAC, 2006) following digestion, in turn, in concentrated HNO_3 and HCl.

Statistical Analysis

All the data were analyzed by the Mixed Model procedure of SAS as appropriate for a randomized complete block design. The blocks were the random variables, with treatments as the fixed variable. Significance was declared when $P \leq 0.05$.

RESULTS

Analyzed composition of the diets are shown in Tables 4 and 5 and they show that the expected nutrient profile was met for both experiments.

Expt. 1–Layers

There were no effects of the TM sources on any of the egg production performance or external characteristics of the eggs (Table 6). In weeks 4 to 8, egg FCR was lower ($P < 0.01$) in hens receiving the sulfate TM. Average egg weight was greater ($P < 0.01$) in birds

Table 4. Analyzed composition (% as fed) of the experimental diets (Expt. 1).

Items	Sulfate	Hydroxychloride
Dry matter	89.4	89.3
Crude protein	16.7	16.5
Acid hydrolyzed fat	3.86	3.52
Crude fiber	2.50	2.60
Ca	4.63	4.10
Cu, ppm	26.0	29.0
Ash	13.7	12.7
Mn, ppm	115	121
P	0.46	0.45
Na	0.22	0.19
Zn, ppm	114	112

receiving sulfate TM from week 20 onwards whereas the percentage of cracked eggs was lower ($P < 0.01$) in hens receiving hydroxychloride TM.

In Expt. 1, Roche yolk color, albumen weight and analyzed yolk Mn level were greater ($P < 0.05$) in eggs from hens receiving diets with sulfate TM (Table 7). Yolk weight: egg weight tended to be greater ($P = 0.077$) whereas albumen weight: egg weight tended to be lower ($P = 0.051$) for hens receiving hydroxychloride TM. There were no treatment effects on the other characteristics.

Expt. 2–Broiler Chickens

The growth performance effect of the two TM sources in broiler diets (Expt. 2) are shown in Table 8. In the starter phase (day 0 to 9), gain: feed tended ($P = 0.078$) to be greater in the birds fed the diet with hydroxychloride Zn and Cu but there was no effect of the TM sources on weight gain or feed intake. In the overall starter and grower phases (days 0 to 28), finisher phase (days 21 to 35) and overall period (days 0 to 35), birds receiving hydroxychloride Zn and Cu had greater ($P < 0.05$) weight gain and tended to have greater ($P < 0.10$) feed intake. There was no significant treatment effect on gain: feed during these periods.

The mRNA expression profile of selected protein synthesis and degradation genes are shown in Table 9. There were no significant treatment effects on any of the genes examined except PSMA1 (20S proteasome), which was lower ($P < 0.05$) in birds receiving hydroxychloride Zn and Cu.

The levels of uric acid and methyl malonic acid, markers of stress response, tended to be lower ($P < 0.10$) in the plasma of broilers receiving hydroxychloride Zn and Cu (Table 10). There was no effect of the treatments on FPD or LS but the FPD and LS levels were within the normal range.

There were no significant treatment effects on carcass yield characteristics or quality scores in the broiler chickens receiving the experimental diets (Table 11).

DISCUSSION

The objective of the current studies was to study the impact of replacing sulfate TM (Zn, Cu, and Mn in Expt. 1, and Zn and Cu in Expt. 2) with hydroxychloride TM on the performance responses in poultry. Specifically, in Expt. 1, the impact of the TM source in laying hens and egg quality was investigated. The specific aim in Expt. 2 was to investigate possible growth and carcass yields responses as well as modes of action by which hydroxychloride TM produce improvement in growth performance response.

Trace minerals are essential in poultry diets to ensure optimal growth and production. This is in view of their many functions in biochemical processes that are necessary for growth, development, and egg production (Richards et al., 2010). However, TM from inorganic sources tend to dissociate and react with other minerals in upper digestive tract, and this reduces their availability and leads to greater excretion (Miles et al., 1998; Underwood and Suttle, 1999). On the other hand, the crystalline structure of hydroxychloride TM (Hawthorne and Sokolova, 2002) and their low solubility in water (Cao et al., 2002) invariably make them to dissolve

Table 5. Analyzed composition (% as fed) of the experimental diets (Expt. 2).

	Day 0 to 9		Day 9 to 21		Day 21 to 35	
Items	Sulfate	Hydroxychloride	Sulfate	Hydroxychloride	Sulfate	Hydroxychloride
Dry matter	87.0	87.1	87.5	87.5	87.4	87.3
Crude protein (N × 6.25)	23.8	25.0	21.7	21.3	18.5	19.2
Acid hydrolyzed fat	2.83	2.89	3.56	3.83	4.77	4.82
Acid detergent fiber	3.74	3.64	4.17	3.51	3.53	3.29
Neutral detergent fiber	9.1	9.9	7.7	8.0	8.3	9.1
Ash	4.8	4.8	5.4	5.2	5.3	4.6
Ca	0.71	0.71	1.06	0.95	0.99	0.81
Na	0.11	0.14	0.19	0.18	0.19	0.16
Mg	0.15	0.16	0.15	0.14	0.14	0.13
Cu, ppm	24	23	20	21	20	21
Fe, ppm	89	86	119	105	136	103
Mn, ppm	94	93	148	137	141	124
Zn, ppm	110	120	137	120	122	126
K	0.89	0.97	0.87	0.82	0.70	0.73
P	0.76	0.71	0.62	0.65	0.52	0.54

Table 6. Egg production response to experimental diets (Expt. 1).[1]

	Egg mass, g/pen daily	% Cracked	% Dirty	% Faulty[2]	Av. egg wt., g	% HDP[3]	Egg FCR
			Week 1 to 4				
Sulfate	1087.1	1.12	0.03	0.74	55.5	94.6	2.42
Hydroxychloride	1096.1	0.88	0.05	0.88	56.0	94.5	2.39
Pooled SEM	14.5	0.105	0.016	0.067	0.625	0.375	0.030
P-value	0.663	0.126	0.381	0.157	0.659	0.852	0.597
			Week 4 to 8				
Sulfate	1200.8	0.96	0.11	0.60	62.8	92.8	2.23
Hydroxychloride	1180.4	0.72	0.12	0.81	61.2	93.4	2.32
Pooled SEM	15.2	0.094	0.038	0.098	0.926	0.259	0.028
P-value	0.410	0.066	0.845	0.119	0.265	0.084	0.036
			Week 16 to 20				
Sulfate	1191.6	0.64	0.07	0.13	61.5	93.6	2.27
Hydroxychloride	1190.2	0.50	0.14	0.09	60.8	94.0	2.31
Pooled SEM	6.35	0.078	0.026	0.028	0.124	0.396	0.031
P-value	0.8804	0.210	0.113	0.276	0.001	0.400	0.456
			Week 20 to 24				
Sulfate	1171.4	0.99	0.31	0.31	61.7	92.3	2.16
Hydroxychloride	1176.6	0.55	0.25	0.33	61.1	92.9	2.18
Pooled SEM	6.26	0.070	0.052	0.061	0.140	0.395	0.011
P-value	0.563	<0.001	0.402	0.867	0.004	0.267	0.294
			Week 1 to 24				
Sulfate	1175.8	0.84	0.11	0.52	60.7	94.0	2.28
Hydroxychloride	1170.6	0.62	0.11	0.61	59.8	94.4	2.31
Pooled SEM	4.826	0.048	0.018	0.050	0.206	0.255	0.009
P-value	0.458	0.003	0.911	0.210	0.005	0.241	0.058

n = 32 replicate cages with 21 hens per replicate cage.

[1]Data not presented for weeks 8 to 16. There were no treatment effects on any of the responses except lower ($P < 0.05$) % cracked eggs in hens receiving hydroxychloride Zn, Cu, and Mn.

[2]Faulty eggs indicate eggs with any one, or more, of the following defects: small size, double yolk, thin shell, shell-less, or malformed.

[3]Hen-day production.

Table 7. Egg quality characteristics and yolk trace mineral levels in response to the experimental diets (Expt. 1).[1]

Items	Sulfate	Hydroxychloride	Pooled SEM	*P*-value
Egg Weight, g	64.4	63.0	0.530	0.050
Albumen Height, mm	11.5	11.3	0.162	0.405
Haugh Unit	103.7	103.6	0.972	0.998
Yolk weight, g	16.3	16.3	0.153	0.720
Yolk weight: egg weight	0.253	0.258	0.002	0.077
Yolk color roche	9.42	6.90	0.165	<0.001
Shell Thickness, mm	0.338	0.333	0.003	0.240
Shell weight, g	8.61	8.53	0.128	0.693
Shell weight: egg weight	0.129	0.132	0.001	0.244
Shell density, mg/cm^2	111.5	112.7	1.04	0.404
Albumen weight, g	39.5	38.2	0.442	0.033
Albumen weight: egg weight	0.617	0.610	0.003	0.051
Shell, %	13.0	13.2	0.150	0.244
Yolk Cu, ppm	3.14	3.34	0.161	0.379
Yolk Mn, ppm	2.89	2.46	0.098	0.004
Yolk Zn, ppm	78.8	79.2	1.02	0.772

n = 32 replicate cages with 3 eggs per replicate cage.

[1]Trace minerals were analyzed in lyophilized yolks (yolk pooled per cage).

comparatively more slowly in the digestive tract. Hydroxychloride Zn and Cu have been noted to have greater bioavailability (Cao et al., 2002; Batal et al., 2001) and less reactivity with feed components (Miles et al., 1998; Pang and Applegate, 2006; Lu et al., 2010).

Egg Production and Quality

The influence of replacing sulfate TM (Zn, Cu and Mn) with hydroxychloride TM on egg production efficiency in the current study was marginal. The marginally higher egg FCR in hens receiving hydroxychloride TM was possibly driven by equally marginally greater FI (128.3 g vs. 128.9 g in hens receiving sulfate vs. hydroxychloride TM, respectively). Others have similarly observed no effect of TM supplementation in hens receiving organic or inorganic sources of Zn, Cu, and Mn (Fernandes et al., 2008; Stefanello et al., 2014; Xiao et al., 2015). It is possible that observation of no effect of the TM source in the current study was because

Table 8. Growth performance response of broilers to the experimental diets (Expt. 2).

Diet	Wt. gain, g	FI, g/bird	G:F	Wt. gain, g	FI, g/bird	G:F	Wt. gain, g	FI, g/bird	G:F
	Starter 1: day 0 to 9			Starter 2: day 9 to 21			Overall starter phase: day 0 to 21		
Sulfate	166.2	199.2	833.1	694.89	923.9	751.96	861.09	1123.10	766.40
Hydroxychloride	169.7	200.0	847.7	704.55	943.8	746.02	874.26	1143.82	763.96
Pooled SEM	2.24	1.85	5.67	8.20	9.40	3.41	9.70	10.8	2.92
P-value	0.275	0.776	0.078	0.412	0.144	0.228	0.345	0.185	0.559
	Day 0 to 28			Day 21 to 35			Overall: d 0 to 35		
Sulfate	1505.4	2100.6	716.6	1446	2292	631	2307	3415	676
Hydroxychloride	1553.6	2153.2	721.2	1500	2346	639	2374	3490	680
Pooled SEM	15.7	18.7	3.18	14.6	20.0	5.09	20.6	28.2	3.87
P-value	0.039	0.056	0.313	0.015	0.066	0.266	0.030	0.071	0.416

n = 30 pens with 18 broiler chickens per replicate pen (there were 15 chickens per pen between days 28 and 35).

Table 9. Expression profile for selected genes for protein synthesis and degradation in male broilers chickens receiving diets in which dietary Zn and Cu were provided in sulfate or hydroxychloride forms for 28 D (Expt. 2).

Genes	Sulfate	Hydroxychloride	Pooled SEM	*P*-value
MTOR	0.93	0.86	0.096	0.581
RPS6KB1	1.24	1.06	0.119	0.290
EIF4EBP1	1.16	1.14	0.045	0.773
CTSB	1.09	0.98	0.092	0.433
PSMA1	1.11	0.84	0.061	0.030
FBOXO32	1.11	0.89	0.103	0.152
TRIM63	1.06	0.97	0.081	0.529

n = 15 replicate pens with 2 birds per replicate pen.
All genes were expressed relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).
MTOR- mechanistic target of rapamycin.
RPS6KB1–ribosomal protein S6 kinase 1.
EIF4EBP1–eukaryotic initiation factor 4E-binding protein 1.
CTSB—cathepsin B.
PSMA1–20S proteasome.
FBOXO32–muscle atrophy F box.
TRIM63–muscle ring finger-1.

of the age of the hens, which were at peak production. For example, Toghyani et al. (2019) showed beneficial effects of hydroxychloride TM on % hen-day egg production, egg mass, and FCR in the post peak period.

There was consistently lower proportion of cracked eggs from hens receiving hydroxychloride TM in the current study. This may be an indication of greater breaking strength of the egg shells although this was not measured in the current study. Trace minerals (Zn, Cu, and Mn) play crucial roles as part of enzymes involved in the process of eggshell or membrane formation or by direct involvement in the formation of structural component of the eggshell. Such processes include the formation of mucopolysaccharides (Leach, 1976; Leach and Gross, 1983); desmosine, and isodesmosine (Chowdury, 1990) or as a component of carbonic anhydrase enzyme (Nys et al., 1999).

The potential for stronger shell was not reflected in the measured shell thickness and shell weight, or shell weight: egg weight ratio of the eggs from hydroxychloride TM-fed hens. Stefanello et al. (2014) similarly reported no difference in egg weight or mass from hens receiving organic or inorganic sources of Zn, Cu and Mn but a lower egg loss (i.e., eggs that were broken, cracked, porous, or thin shelled) in hens receiving the organic TM. This has also been previously reported by others (Zamani et al., 2005). However, factors such as age of the hens, basal TM level or the inclusion levels of the organic vs. inorganic TM, among others, may influence the observed effect (Mabe et al., 2003; Maciel et al., 2010). Nonetheless, the overall indication is that the use of the more bioavailable TM sources (organic or hydroxychloride) often results in improved shell quality (Xiao et al., 2015), which may, or may not, be reflected in shell thickness.

The yolk TM content is a sensitive response used in TM bioavailability assays (Kim et al., 2016). In the current experiment, yolk Mn content was lower in hens receiving hydroxychloride TM. Mabe et al. (2003) indicated that Mn is mainly distributed in the egg shell whereas Zn and Cu are largely distributed in the egg yolk. Consequently, it appears that unlike Zn and Cu, a more reliable indication of efficacy of Mn source will be its concentration in the eggshell and this should likely be the focus of a future investigation. The greater albumen weight in the hens receiving sulfate TM is clearly associated with greater egg weight of the hens, as indicated by the albumen: egg weight ratio. Hence, it does not seem that the larger albumen was due to the dietary sources of TM per se. On the other hand, the marginal increase in yolk: egg weight likely indicates greater yield of yolk in hens receiving hydroxychloride TM. Fernandes et al. (2008) similarly observed increased yolk percentage in hens receiving organic Zn, Mn, and Se, which are more bioavailable sources of the TM.

Broiler Growth Performance and Carcass Yield

In the current experiment, the main effect of TM source was apparent from day 28 when the weight gain for broiler chickens receiving hydroxychloride TM was superior. It stands to reason that the superior weight gain was partially driven by a marginally greater feed intake in the broilers receiving hydroxychloride TM, which will also translate to greater intake of the TM. But it does not seem that the marginally greater feed intake in the treatment is sufficient to fully explain the

Table 10. Plasma levels of markers of oxidative stress and foot pad and litter scores in male broilers chickens receiving diets in which dietary Zn and Cu were provided in sulfate or hydroxychloride forms for 28 D (Expt. 2).

Genes	Sulfate	Hydroxychloride	Pooled SEM	*P*-value
Plasma markers, μmol/L				
Uric acid	370.7	334.9	18.9	0.091
Methyl malonic acid	0.064	0.037	0.009	0.063
Foot and litter scores				
Foot pad score	2.68	3.04	0.166	0.135
Litter score	1.92	2.01	0.072	0.417

n = 15 replicate pens with 3 birds per replicate pen.

Table 11. Carcass yield and characteristics in male broilers in response to provision of Zn and Cu in sulfate or hydroxychloride forms for 35 D (Expt. 2).

Items		Sulfate	Hydroxychloride	Pooled SEM	*P*-value
	Abdominal fat	1.23	1.21	0.025	0.577
	Eviscerated	70.9	71.2	0.142	0.269
	Thigh	18	18	0.063	0.825
% yield	Drumstick	13.3	13.3	0.066	0.645
	Wings	10	10	0.039	0.859
	Back + ribs	28.1	28.2	0.123	0.820
	Breast	29.3	29.3	0.196	0.905
%	Drip loss	8.36	8.34	0.170	0.921
Scores	White striping	0.438	0.371	0.038	0.234
	Wooden breast	0.087	0.110	0.019	0.411

n = 30 replicates with 7 birds per replicate.

comparatively much greater weight gain observed in the treatment. We (Olukosi et al., 2018) and others (Huang et al., 2007; Liu et al., 2015) have reported improvement in weight gain of broilers following Zn and Cu supplementation. Growth performance response to Zn and Cu supplementation to diets devoid of these TM can be explained by alleviation of growth-suppression effect of deficiency of the TM. On the other hand, improvement in growth performance in diets with different sources of Zn and Cu, as in the current experiment, is more likely due to differences in availability of the TM from these sources (Miles et al., 1998; Batal et al., 2001).

There were no differences in the carcass yield, or scores, in response to hydroxychloride or sulfate TM in the current experiment. In a previous study (Olukosi et al., 2018), we reported greater % breast yield and lower % back + ribs in broilers receiving hydroxychloride Zn and Cu. There is limited information on the effect of hydroxychloride vs. sulfate Zn and Cu on carcass traits. Iqbal et al. (2011) and Varun et al. (2017) reported no difference in carcass quality of 6-wk-old broiler chickens receiving organic vs. sulfate Zn and Cu. Liu et al. (2011), on the other hand, reported increased intramuscular fat in breast meat of broilers receiving Zn supplementation. It appears that the difference in the observation of the effect on the TM in our previous (Olukosi et al., 2018) and current studies was the negative effect of high level of sulfate TM on breast yield in the earlier study. The low breast yield in that treatment led to overall greater depression in % breast yield in the broiler chickens receiving sulfate, compared with hydroxychloride, Zn and Cu. It may be that at lower supplemental levels, the margin of difference in effect of the TM sources on % breast yield is minimal.

The diet type might also influence the efficacy of different trace mineral sources. A diet high in non-starch polysaccharides (**NSP**), such as the wheat-based diet used in the current study, may result in higher digesta viscosity in the digestive tract (Choct, 2006). Addition of xylanases to degrade NSP will reduce the challenging effect of a wheat based diet. In contrast to the previous study (Olukosi et al., 2018), xylanase was added to the diets of the broiler chickens in the current study. As a result, it might be expected that the diet was less challenging, and the contrasts between the mineral sources may have been smaller.

Gene Markers of Protein Synthesis and Degradation

Because of the preponderance of evidence indicating positive effect of hydroxychloride Zn and Cu, relative to inorganic TM (Luo et al., 2005; Zhang and Guo, 2009; Olukosi et al., 2018), the possible explanations for this observation are of interest in the current experiment. One line of investigation was the expression profile of mRNA for proteogenic and proteolytic genes in the *pectoralis major* muscle of 28-day-old broiler chickens at a time that coincides with the period of accelerated growth. Protein accretion results from a positive balance of protein synthesis, or hypertrophy, and catabolic events (Rehfeldt et al., 1997). Comparison of the proteolytic systems in the muscle suggests that the more efficient broiler types have markedly reduced protein catabolism (Dransfield and Sosnick, 1999).

There was a significant reduction in 20S proteasome (PSMA1) mRNA expression in *pectoralis* muscle of

broiler receiving hydroxychloride Zn and Cu in the current experiment. Proteasome is a protease complex that is responsible for selective hydrolysis of intracellular proteins (Tanaka, 2009). Ekmay et al. (2013) observed age-related decrease in mRNA expression of PSMA1 in broiler breeders, and that this was closely associated with decrease in fractional breakdown rate of protein in *pectoralis* muscle. Therefore, the lower mRNA expression of PSMA1 as observed in the current experiment indicates that part of the positive effect of hydroxychloride TM on growth is by reducing protein catabolism in the chickens. Nonetheless, because only PSMA1 (among all the genes investigated) was affected by the treatment, and the sampling was done at only one point, more thorough investigation will be necessary to establish this point.

Effect of Zn and Cu Sources On Plasma Marker of Stress Response in Broilers

The second line of investigation regarding the explanation for positive effects of hydroxychloride Zn and Cu on growth was the influence of the TM sources on stress response. Rapid growth rate can reduce stress-tolerance and increase susceptibility to opportunistic pathogens (Huff et al., 2005) which may in turn reduce performance. Oxidative stress in broiler chickens has been associated with poor performance (Fellenberg and Speisky, 2006) and therefore it is reasonable to expect that alleviation, or reduction, of such stress will result in improved growth performance response.

Elevated plasma methylmalonic acid is used in diagnosis of specific vitamin deficiency but is also induced by any condition, including stress, that impairs folate utilization (Allen et al., 1990; Ho et al., 2003). Uric acid, on the other hand, is one of most important antioxidants and is elevated in plasma in response to oxidative stress (Glantzounis et al., 2005) mostly serving a protective function. The plasma levels of methylmalonic and uric acids were lower in broiler chickens receiving hydroxychloride Zn and Cu in the current experiment. The lower plasma levels of both of these metabolites in the broiler chickens receiving hydroxychloride TM could indicate that this TM source supports a more robust tolerance to oxidative stress. Clearly, more investigations along this line will help to verify this observation. But in support of that reasoning, Bartlett and Smith (2003) showed that broilers receiving diets with comparatively high Zn level had more competent immune response under heat stress.

In addition, Perez et al. (2017) showed an increased level of superoxide dismutase (**SOD**), an antioxidant enzyme, when feeding increasing levels of Zn and Mn in both laying hens and broiler chickens challenged with lipopolysaccharides. In laying hens, a similar SOD level was reached with lower levels of Zn (50 ppm) and Mn (45 ppm) from hydroxychloride TM compared to higher levels (100 ppm Zn and 90 ppm Mn) from sulfate sources. In the current study, reduction in oxidant markers were observed in broiler chickens fed hydroxychloride TM. Although SOD level was not measured in Expt. 2 of current study, Perez et al. (2017) observation supports the notion of similar mechanism of hydroxychloride TM augmenting the antioxidant response in order to lower oxidative stress in laying hens.

Conclusion

The observations from the current experiments indicate that hydroxychloride Mn, Zn, and Cu reduced egg loss by reducing the proportion of cracked eggs, even though its effect on egg production was minimal in hens at peak production. In addition, the positive effect of hydroxychloride Zn and Cu on weight gain in broiler chickens is partly explained by its effect on reducing proteolysis in the *pectoralis* muscle and alleviating oxidative stress during rapid growth.

ACKNOWLEDGMENTS

The authors acknowledge the assistance of Moira Webley, Jenna Morrison, and Andrew Scott (Scotland's Rural College, UK) in care of animals and carcass evaluation. SRUC receives support from the Scottish government (RESAS).

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