Zinc, manganese, and copper amino acid complexes improve performance and bone characteristics of layer-type chicks under thermoneutral and cold stress conditions

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ABSTRACT Two experiments were designed to evaluate the effect of mineral-amino acid complexes (**AACM**) as a partial replacement of inorganic mineral (**IM**) in layer-type chicks' diets. Both studies had the same dietary treatments, where in experiment 1 (**Exp. 1**) was conducted under thermoneutral conditions from 0 to 35 D and chicks in experiment 2 (**Exp. 2**) were exposed to cold stress conditions at nighttime during the first 15 D and to thermoneutral condition from 16 to 35 D. For each trial, 1,200 one-day-old Lohmann Brown chicks were used, with 20 cage replicates with 30 chicks per cage. Treatments consisted of the control diet (IM; with 70, 70, and 8 mg/kg of zinc [**Zn**], manganese [**Mn**], and copper [**Cu**], respectively) and the treatment diet (AACM, with 40, 40, and 2.75 mg/kg of Zn, Mn, and Cu, respectively, from IM sources, along with 30, 30, and 5.25 mg/kg of Zn, Mn, and Cu, respectively). Data were submitted to analysis of variance, and means were compared using the t-test ($P < 0.05$). In Exp. 1, there were no significant differences between treatments on chick performance. However, AACM-fed chicks had higher thymus ($P = 0.03$) and cecum weight ($P < 0.01$), superior micromineral deposition in the tibias ($P < 0.01$), and reduced phosphorus excretion ($P = 0.03$). In Exp. 2, chicks fed with AACM had higher body weight gain ($P = 0.04$), better average daily feed intake ($P = 0.03$), lower phosphorus excretion ($P = 0.02$), and higher liver and pancreas weight ($P < 0.01$) in the last week of the study. In conclusion, chicks fed with AACM under thermoneutral conditions had higher bone mineralization and reduced excretion of phosphorus, and in adverse conditions, AACM improves performance and liver and pancreas weight, also reducing phosphorus excretion.

Key words: Lohmann layer-type chick, trace mineral, phosphorus, sustainability

INTRODUCTION

Normally, trace minerals such as copper (**Cu**), zinc (**Zn**), and manganese (**Mn**) are supplemented in diets as inorganic salts, such as sulfates, oxides, or carbonates. However, owing to the genetic enhancement of laying hens and the lack of mineral nutrition research, the industry uses them in higher concentrations in poultry diets (Pacheco et al., 2017). Therefore, the excess of microminerals above the animal requirements leads to an increase in costs and higher mineral excretion owing

to reduced absorption as the minerals compete for absorption sites from the gut lumen to the enterocytes.

The inorganic trace mineral sources (inorganic mineral [**IM**]) need to be solubilized into the ionic form before being absorbed, on reaching the gastrointestinal tract. However, such forms may interact with other dietary components, making them unavailable to the animal (Close, 2003). However, studies have shown that trace minerals linked to organic molecules in poultry diets generate better responses, even though they are included in smaller quantities than IM sources. Besides, the organic mineral source has peculiar characteristics of chemical stability, resulting in higher utilization by the animal (Maciel et al., 2010).

When minerals are complexed to an organic molecule such as amino acid (mineral-amino acid complexes [**AACM**]), they have higher absorption rate because their uptake will be mediated by the amino acid transporter and through the IM pathway, decreasing the competition

for absorption binding sites (Gao et al., 2014). This improvement in bioavailability has been proven in poultry research, with improvement in performance variables, such as body weight gain and feed conversion ratio (**FCR**) (Bao et al., 2007; Abdallah et al., 2009).

In the majority of studies in the literature, the efficacy of AACM in poultry nutrition has been demonstrated under thermoneutral environments (Mabe et al., 2003; Gheisari et al., 2010; Khatun et al., 2019) or under heat stress conditions (Laganá et al., 2007; Ribeiro et al., 2008). Nonetheless, information about the efficacy of AACM in layer birds reared under cold stress conditions is still limited. Under cold stress conditions, some metabolic functions are modified to increase body heat production, and it may affect animal performance. This effect is magnified especially in the first 15 D of life of chicks, a critical period in which birds do not have the fully developed thermoregulatory system, with possible negative effects reflected in their future zootechnical performance.

Beyond the lack of studies about cold stress, there is a shortage in studies related to layer-type chicks in the initial phase of life supplemented with AACM in the diets, and this research becomes a pioneer in this topic. Thus, the importance of studying the effect of AACM supplementation on performance and mineral concentration in the tibias, liver, and excreta as well as evaluating the bone characteristics of chicks in the initial phase raised under conditions of thermal comfort or cold stress becomes clear.

Therefore, we hypothesized that minerals, when complexed with organic molecules, can improve performance and bone quality when raised under conditions of thermal comfort or cold stress, in addition to reduction of mineral excretion.

MATERIALS AND METHODS

Experimental Design and Treatments

All management practices, as well as the slaughter and sampling procedures, carried out in these studies were previously approved by the Ethics Commission for Animal Research of the Federal Rural University of Pernambuco (CEUA no. 064/2016).

Two performance experiments (thermoneutral and cold stress) were conducted on 1,200 Lohmann Brown-Lite layer-type one-day-old chicks in each study from 0 to 35 D of age. Birds were raised in 40 cages per study, with dimensions of $60 \times 80 \times 30$ cm, equipped with a feeder plate and nipple drinker cup. Water supply to the birds was *ad libitum*, whereas the ADFI was adjusted weekly as per the breeder strain guideline recommendations.

The birds were vaccinated against colibacillosis, Newcastle disease, bronchitis, pneumovirus, coryza, and salmonella and beak trimmed at day 8. During the first week of life, 24 h of light (12-h natural + 12-h artificial) was offered; then, the light length was reduced by 1 h daily until reaching natural daylight length. The artificial light used was a fluorescent type, with an average intensity of 5 lux.

In the 2 studies, the birds were distributed in a completely randomized design, with 2 treatments and 20 replicates of 30 birds per cage. The treatments consisted of 2 diets, wherein the control diet (IM) was supplemented with Zn, Mn, and Cu in the concentration of 70, 70, and 8 mg/kg, respectively, and the treatment diet (AACM) was supplemented with an IM source: 40, 40, and 2.75 mg/kg of Zn, Mn, and Cu, respectively, associated with an AACM source 30, 30, and 5.25 mg/kg of Zn, Mn, and Cu, respectively. Zinc oxide, manganese oxide, and copper sulfate were used as IM, and AvailaZMC (Zinpro Corp., Eden Prairie, MN) was used in the treatment diet associated with IM. Table 1 presents the results of water and feed analysis of Ca, P, Zn, Mn, Cu, and Fe, analyzed by inductively coupled plasma optical emission spectrophotometry (**ICP-OES**, model Optima 7000 DV; PerkinElmer, Waltham, MA).

The diets used were isonutritional, differing only in the sources of minerals used, as described in Table 2. Diets were formulated as per Lohmann Brown-Lite guideline recommendations. In experiment 1 (**Exp. 1**), under thermal comfort, it was possible to use prestarter and starter diets. However, in experiment 2 (**Exp. 2**), owing to the low body weight gain resulting from stress conditions, it was not possible to offer the starter diet for birds

Table 1. Trace mineral content in water and feed.

Variable	Treatments	Ca	P	Zn	Mn	Cu
		(g/kg)			(mg/kg)	
Water (mg/L)	IM|AACM	7.47	<0.01	<0.01	0.01	<0.01
Prestarter feed, Exp. 1[1]	IM	11.41	6.18	97.50	99.25	9.31
	AACM	11.67	6.11	98.50	98.75	9.06
Starter feed, Exp. 1[2]	IM	10.55	5.29	106.37	103.00	9.56
	AACM	10.92	5.46	102.00	109.50	9.93
Prestarter feed, Exp. 2[3]	IM	11.38	6.25	98.37	105.50	9.25
	AACM	11.56	6.12	99.87	101.31	9.75

Analyzed values obtained by ICP-OES.

Abbreviations: AACM, amino acids-mineral complexed; Ca, calcium; Cu, copper; Exp. 1, experiment 1; Exp. 2, experiment 2; ICP-OES, inductively coupled plasma optical emission spectrophotometry; IM, inorganic mineral; Mn, manganese; P, phosphorus; Zn, zinc.

[1]Prestarter diet (1–4 wk) offered to experiment 1 chicks (thermal comfort).
[2]Starter feed (4–5 wk) offered to experiment 1 chicks (thermal comfort).
[3]Prestarter feed (1–5 wk) offered to experiment 2 chicks (cold stress).

Table 2. Ingredients and nutrient composition of the experimental diets.

	Experiment 1				Experiment 2	
	Prestarter feed[1]		Starter feed[2]		Prestarter feed[3]	
Ingredients, %	IM	AACM	IM	AACM	IM	AACM
Corn	58.35	58.35	63.46	63.46	63.26	63.26
Soybean meal, 46% CP	35.90	35.90	30.30	30.30	33.00	33.00
Soybean oil	1.80	1.80	0.19	0.19	0.14	0.14
Calcium carbonate	1.19	1.19	1.26	1.26	1.27	1.27
Dicalcium phosphate	1.55	1.55	1.43	1.43	1.19	1.19
Sodium bicarbonate	0.15	0.15	0.15	0.15	0.15	0.15
Salt	0.29	0.29	0.29	0.29	0.29	0.29
DL-methionine, 99%	0.20	0.20	0.15	0.15	0.29	0.29
Probiotic[4]	0.04	0.04	0.04	0.04	0.04	0.04
Absorbent[5]	0.20	0.20	0.20	0.20	0.20	0.20
Phytase[6]	0.01	0.01	0.01	0.01	0.01	0.01
Vitamin premix[7]	0.12	0.12	0.12	0.12	0.12	0.12
Inorganic mineral premix	0.10	0.00	0.10	0.00	0.10	0.00
Reduced inorganic mineral premix	0.00	0.10	0.00	0.10	0.00	0.10
Amino acids-minerals complexed[8]	0.00	0.075	0.00	0.075	0.00	0.075
Inert[9]	0.10	0.025	2.40	2.23	0.10	0.025
Total	100.00	100.00	100.00	100.00	100.00	100.00
Chemical composition						
AME, kcal/kg	2,950	2,950	2,850	2,850	2,900	2,900
CP, %	21.00	21.00	18.80	18.80	20.00	20.00
CP,[10] %	21.07	21.08	18.58	18.41	19.64	19.21
DM,[10] %	87.92	87.96	88.56	88.04	89.65	89.52
MM,[10] %	6.031	6.060	6.470	6.480	5.679	5.679
EE,[10] %	4.910	4.580	2.290	2.276	2.946	2.946
Digestible Met, %	0.491	0.491	0.412	0.412	0.411	0.411
Digestible Met + Cys, %	0.770	0.770	0.666	0.666	0.680	0.680
Digestible Lys, %	1.047	1.047	0.912	0.912	0.982	0.982
Ca, %	1.050	1.050	1.020	1.020	1.050	1.050
Available P, %	0.490	0.490	0.460	0.460	0.480	0.480
Na, %	0.180	0.180	0.180	0.180	0.180	0.180
CF, %	3.162	3.162	2.917	2.917	3.075	3.075

Abbreviations: AACM, amino acids-mineral complexed; CF, crude fiber; EE, ether extract; IM, inorganic mineral; MM, mineral matter.

[1]Prestarter diet (1–4 wk) offered to experiment 1 chicks (thermal comfort).

[2]Starter feed (4–5 wk) offered to experiment 1 chicks (thermal comfort).

[3]Prestarter feed offered to experiment 2 chicks (cold stress).

[4]Guarantee levels: *Bacillus licheniformis* (min) $> 16 \times 10^{10}$ UFC/g.

[5]Guarantee levels: bentonite, 666 g/kg; beta-glucans, 54 g/kg; manan oligosaccharides, 59.4 g/kg; bioactive phytogenic, 16.5 g/kg.

[6]Guarantee levels: phytase (min), 10,000 FTU/g.

[7]Guarantee levels (kg/kg of product): vitamin A, 8,000 IU; vitamin D3, 2,000 IU; vitamin E, 10,000 IU; vitamin K3, 2,000 mg; vitamin B1, 1,000 mg; vitamin B2, 4,000 mg; vitamin B6, 2,500 mg; vitamin B12, 11,000 mg; niacin, 25 g; calcium pantothenate, 10 g; folic acid, 550 mg; biotin, 50 mg;.

[8]AvailaZMC: zinc, 4%; manganese, 4%; copper, 0.7%; total amino acid, 19%.

[9]Kaolin.

[10]Analyzed levels.

as planned; then, over the first 5 wk, the prestarter diet was offered to the animals.

Temperature and Air Humidity Management

Temperature and relative air humidity were recorded daily using a data logger (model Hobo U12-012; Onset Computer Corporation, Bourne, MA), installed in the center of the shed, where temperatures were recorded every 10 min and 4 digital thermohygrometers (model 7663.02.0.00; Incoterm, Porto Alegre, RS, Brazil) were fixed at different places. Figure 1 shows the average temperatures of Exp. 1 (AT E1) and Exp. 2 (AT E2) and the relative humidity from both studies (UH E1 and UH E2).

Experiment 1 House temperature was controlled during the first 15 D of age to keep the birds under thermal comfort conditions. The average day and night temperatures were 31.57 ± 1.51°C and 31.81 ± 1.82°C, respectively (Figure 1).

Experiment 2 The birds were raised under thermal stress conditions with temperatures below their comfort zone during the night period (6:00 pm to 6:00 am) in the first 15 D of age. In the daytime period, the temperature was controlled to keep the animals in the thermoneutral zone. The average daytime temperature was 30.33 ± 1.08°C, whereas the nocturnal temperature was 27.01 ± 1.71°C (Figure 2).

Performance Measurements and Organ Collection

The birds were individually weighed at day 1 to have similar bulk weight among cages. Performance variables, such as live weight (g), average daily gain (**ADG**, g), ADFI (g), and uniformity (%), were measured weekly,
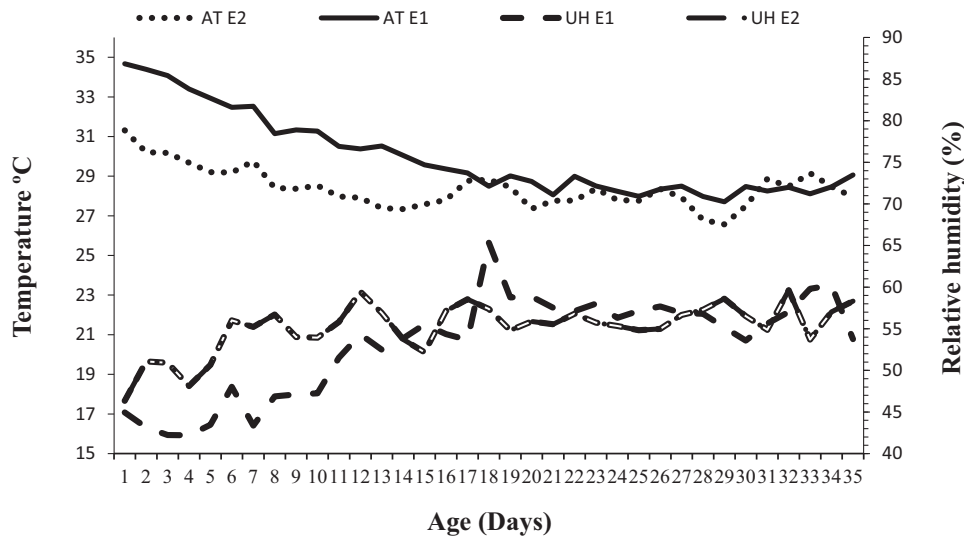
Figure 1. Average daily temperatures of experiment (E) 1 and 2 throughout the experimental period. Abbreviation: AT, average temperature.

and the FCR was calculated. Mortality was recorded as it occurred; any bird that died was weighed, and the weight was used to adjust ADFI. At day 35, three birds per cage were chosen as per the average live weight to be euthanized by cervical dislocation, so the tibias, thymus, bursa of Fabricius, spleen, liver, pancreas, and intestine were collected. The organs were weighed in a semianalytical balance precision of 0.01 g (model L3102iH; Bel Engineering, Milan, Italy), and the whole intestine and cecum were precisely measured using a tape measure. Then, the weight and relative length of the tibia were calculated as follows: (tibia weight/body weight) * 100.

Bone Strength Analysis and Seedor Index

All the tissues surrounding the tibia were collected without causing injury to the bone structure; then, the bones were weighed on a semianalytical scale (±0.01 g), and their length was measured using a digital caliper (precision of 0.01 mm, model Absolute Digital

AOS; Mitutoyo, SP, Brazil); then, the Seedor index (Seedor et al., 1991) was calculated by dividing the bone weight (mg) by its length (mm). This index is used as an indicator of bone density, wherein a higher index indicates a superior density. Bone strength analysis was performed using the universal tester (model TA-XT Plus; Stable Micro Systems, Surrey, UK), with a 50-kg load cell at a speed of 30 mm/min, at the Animal Products Evaluation Laboratory of the Federal University of Paraíba (LAPOA, UFPB).

Mineral Concentrations in the Tibia

For mineral composition analysis, the tibia previously submitted to bone strength analysis was used. The bones were dried in an oven at 105°C (model SL100; Solab, SP, Brazil) for 24 h and then calcined in a muffle furnace (model 2000F; Zezimaq, Minas Gerais, Brazil) for 4 h at 600°C (Yan et al., 2005). Then, approximately 0.5 g of the sample was weighed to be digested with 6 mL of
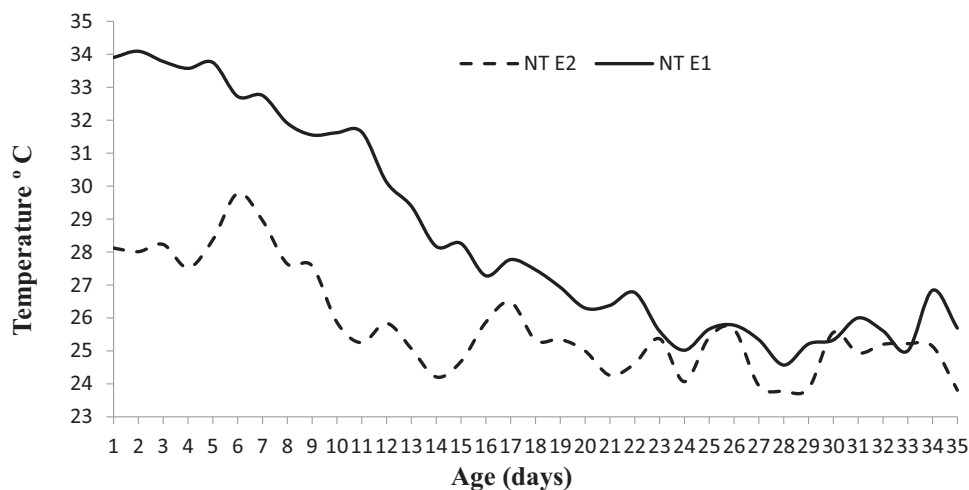
Figure 2. Average night temperatures of experiment (E) 1 and 2 throughout the experimental period. Abbreviation: NT, night temperature.

Table 3. Effect of amino acids-mineral complexed on body weight (BW), average daily gain (ADG), average daily feed intake (ADFI), feed conversion ratio (FCR), and uniformity (UNI) of layer chicks at 35 D, reared under thermoneutral conditions.

Treatments	BW (g)	ADG (g)	ADFI[1] (g)	FCR (g:g)	UNI (%)
7 D					
IM[2]	68.89	5.21	8.50	1.64	66.17
AACM[3]	69.42	5.28	8.50	1.61	70.83
P-value	0.541	0.555	0.970	0.162	0.235
SEM	0.431	0.435	0.061	0.014	1.965
14 D					
IM	123.26	7.69	13.00	1.69	69.66
AACM	123.35	7.61	13.22	1.74	75.56
P-value	0.943	0.521	0.080	0.142	0.394
SEM	0.629	0.062	0.665	0.010	3.066
21 D					
IM	202.74	11.35	21.54	1.90	72.68
AACM	204.26	11.56	21.68	1.88	78.25
P-value	0.425	0.212	0.461	0.255	0.087
SEM	0.932	0.567	0.096	0.013	1.629
28 D					
IM	290.36	12.52	28.05	2.25	62.22
AACM	289.69	12.20	28.34	2.33	69.64
P-value	0.573	0.102	0.091	0.066	0.062
SEM	0.591	0.688	0.084	0.025	4.747
35 D					
IM	346.08	7.96	31.38	3.97	69.97
AACM	346.50	8.12	31.14	3.88	69.64
P-value	0.858	0.599	0.581	0.402	0.323
SEM	1.163	1.313	0.301	0.074	2.243
Total period (1–35 D)					
IM	346.08	312.95	718.79	2.28	69.97
AACM	346.50	314.73	718.46	2.29	69.64
P-value	0.857	0.912	0.941	0.650	0.323
SEM	1.165	1.589	3.316	0.015	2.243

[1]Feed intake, g/D.
[2]IM: inorganic mineral.
[3]AACM: amino acids-mineral complexed.

nitric acid (65% of analytical purity) in an open system for 30 min, and finally, deionized water was added, producing a final volume of 50 mL. The quantification of minerals in the sample was carried out by ICP-OES.

Mineral Concentrations in the Liver

After weighing, the livers were individually stored in a freezer for further processing. These samples were defrosted at room temperature and dried in an oven at 105°C for 24 h (Gajula et al., 2011). Then, a fragment weighing 0.5 g was removed and digested in 6 mL of microwave-concentrated HNO_3 (model Mars Xpress: Technology Inside, CEM Corporation, Charlotte, NC) for 30 min, and in sequence, deionized water was added, producing a volume of 25 mL. The quantification of minerals in the sample was carried out by ICP-OES.

Mineral Concentrations in Excreta, Feed, and Water

For excreta collection, kraft paper sheets were lined under the experimental cages and spaced between them to prevent contamination. Fresh excreta located only in the center of the paper was collected and immediately stored in collection tubes. The excreta samples were collected twice a day for 2 consecutive days, on days 33 and 34, and then frozen in a freezer. Subsequently, the excreta from the 4 partial collections were defrosted, homogenized, and predried in a forced ventilation stove (model TE394/2; Tecnal, Sao Paulo, Brazil) at 55°C for 48 h and then ground in a stainless steel ball mill. The excreta were dried in an oven at 105°C for 24 h, and then, the samples weighing 0.5 g were digested in 6 mL of nitric acid; then, the volume was adjusted to 25 mL with deionized water. The samples were sent for wet digestion through the microwave for 30 min. Mineral quantification was carried out by ICP-OES.

Feed samples were collected, stored in plastic bags, and kept in in a freezer. Subsequently, they were ground in a ball mill and dried in an oven at 105°C, following the same digestion procedure as for excreta.

During the experimental period, water samples were collected in plastic containers and frozen in a freezer. The quantification of minerals in water was carried out by ICP-OES.

Statistical Analysis

Data were tested for homogeneity of variances and normality of errors and then submitted for analysis of variance with the GLM procedure of SAS software (SAS Institute, Cary, NC). In Exp. 1, the variables of bursa weight, intestine weight, and cecum length were transformed by the Box-Cox transformation, whereas in Exp. 2, the transformation occurred in the variables of

Table 4. Effect of amino acids-mineral complexed on the organ weight and intestine length of layer chicks at 35 D, reared under thermoneutral conditions.

Treatments	Liver	Thymus	Spleen	Bursa	Pancreas	Intestine	Cecum	Intestine
				(g)				(cm)
IM[1]	11.51	1.93[b]	1.60	1.34	1.19	24.53	11.30[a]	113.78
AACM[2]	11.72	2.10[a]	1.61	1.27	1.2	24.76	12.20[b]	113.15
P-value	0.393	0.031	0.930	0.412	0.667	0.713	<0.01	0.462
SEM	0.117	0.172	0.053	0.040	0.067	0.021	0.442	0.125

[a,b]Means lacking a common superscript letter differ, $P < 0.05$.
[1]IM: inorganic mineral.
[2]AACM: amino acids-mineral complexed.

Table 5. Effect of amino acids-mineral complexed on the bone variables of layer-type chicks at 35 D, reared under thermoneutral conditions.

Treatments	Tibia weight[3] (g)	Tibia length[4] (mm)	Seedor index (mg mm^{-1})	Bone strength (Kgf)
IM[1]	1.00	19.21	52.31	8.95
AACM[2]	0.98	19.28	51.16	8.66
P-value	0.159	0.661	0.079	0.236
SEM	0.007	0.082	0.112	0.446

[1]IM: inorganic mineral.
[2]AACM: amino acids-mineral complexed.
[3]Relative weight.
[4]Relative length.

bursa and spleen weight. The means were compared using the Student t-test ($P < 0.05$).

RESULTS

Experiment 1

Under thermoneutral environment conditions, the experimental diets did not influence ($P > 0.05$) BW, ADG, ADFI, FCR, and uniformity (Table 3). Table 4 presents the data from the organ weights and the length of the birds' intestines. There was no significant difference ($P > 0.05$) between treatments for the weight of the liver, spleen, bursa of Fabricius, pancreas, and intestine; however, the AACM diet affected ($P = 0.03$) thymus weight and the length of the cecum ($P < 0.01$).

From the bone variables presented at Table 5, it was found that the diets did not influence ($P > 0.05$) the characteristics evaluated in laying chicks raised in a thermoneutral environment. The AACM supplementation increased ($P < 0.01$) the amount of Zn, Cu, and Mn, without affecting calcium and phosphorus (**P**)

Table 6. Concentration of zinc (Zn), manganese (Mn), copper (Cu), calcium (Ca), and phosphorus (P) in the liver, tibia, and excreta of layer-type chicks at 35 D, reared under thermoneutral conditions.

Treatments	Zn (mg/kg)	Mn (mg/kg)	Cu (mg/kg)	Ca (mg/kg)	P (mg/g)
Tibia composition					
IM[1]	326.89[b]	7.61[b]	6.91[b]	425.56	221.61
AACM[2]	374.43[a]	8.66[a]	7.56[a]	445.10	231.98
P-value	<0.01	<0.01	0.012	0.068	0.076
SEM	7.530	0.155	0.131	5.313	2.908
Liver composition					
IM[1]	142.68	6.81	21.35[a]	561.98	11.43
AACM[2]	142.16	6.40	18.14[b]	555.69	11.27
P-value	0.932	0.171	<0.01	0.781	0.443
SEM	3.051	0.142	0.574	11.117	0.108
Excreta composition					
IM[1]	302.28[b]	303.99	34.96	29.74	11.40[a]
AACM[2]	336.79[a]	319.98	36.11	26.94	10.61[b]
P-value	<0.01	0.192	0.503	0.081	0.035
SEM	4.716	6.061	0.864	0.814	0.190

[a,b]Means lacking a common superscript letter differ, $P < 0.05$.
Abbreviations: Ca, calcium; Cu, copper; Mn, manganese; P, phosphorus; Zn, zinc.
[1]IM: inorganic mineral.
[2]AACM: amino acids-mineral complexed.

contents in the tibia (Table 6). When analyzing the minerals in the liver, it was observed that birds fed with IM had a higher concentration of Cu ($P < 0.01$). When assessing the content of minerals in the excreta, it was observed that birds fed with AACM showed higher Zn excretion ($P < 0.01$), whereas birds fed with IM showed increase in the excretion of P ($P = 0.03$), as presented in Table 6.

Experiment 2

Table 7 shows the effect of treatments on performance variables of layer-type chicks reared under cold stress conditions. Diets supplemented with AACM promoted higher ADG ($P = 0.04$) and better FCR ($P = 0.03$) in the evaluation period from 29 to 35 D and also improved FCR ($P = 0.03$) in the overall study period.

The AACM treatment positively affected liver and pancreas weights ($P < 0.01$); however, they did not affect ($P > 0.05$) the other organs' weight (Table 8). There were differences neither ($P > 0.05$) in tibia weight, length, and Seedor index nor in bone strength (Table 9).

When the birds were raised under cold stress conditions, mineral concentrations in the tibia and liver were not influenced ($P > 0.05$). However, when analyzing the minerals contents in the excreta, birds fed with IM showed higher ($P = 0.02$) P excretion (Table 10).

DISCUSSION

In these studies, it was possible to observe that AACM can reduce P excretion, increase the deposition of microminerals in the tibias, and improve performance and liver and pancreas weight.

Mechanisms of thermogenic responses that play a role in maintaining homeothermy during early stages of neonate chicks exposed to cold stress are poorly studied as chicks are believed to be poikilothermic. In the geographic region where this study was carried out, the thermal amplitude is generally wide, with high temperatures during the day, but night temperatures are below the comfort zone for the chicks. Therefore, maintaining a controlled environment without large amplitudes is essential for birds' development in the first weeks of life.

Cold stress conditions can lead to considerable losses in poultry production, affecting its growth and performance.

Table 7. Effect of amino acids-mineral complexed on the body weight (BW), average daily gain (ADG), average daily feed intake (ADFI), feed conversion ratio (FCR), and uniformity (UNI) of layer chicks at 35 D, reared under cold stress conditions.

Treatments	BW (g)	ADG (g)	ADFI[1] (g)	FCR (g:g)	UNI (%)
7 D					
IM[2]	59.90	3.98	7.45	1.87	57.30
AACM[3]	61.00	4.13	7.58	1.84	58.80
P-value	0.086	0.101	0.235	0.277	0.642
SEM	0.301	0.295	0.057	0.011	1.648
14 D					
IM	110.95	6.85	13.93	2.03	72.78
AACM	110.37	6.80	13.88	2.04	72.22
P-value	0.424	0.526	0.787	0.598	0.913
SEM	0.364	0.260	0.086	0.012	2.323
21 D					
IM	182.52	10.22	21.32	2.09	72.10
AACM	179.95	9.94	20.79	2.10	71.65
P-value	0.125	0.157	0.121	0.797	0.888
SEM	0.842	0.687	0.171	0.011	1.590
28 D					
IM	245.45	8.99	25.36	2.83	66.11
AACM	245.42	9.32	25.54	2.75	60.01
P-value	0.981	0.066	0.405	0.101	0.318
SEM	1.026	0.618	0.102	0.021	3.463
35 D					
IM	293.45	6.79[b]	29.85	3.81[a]	62.99
AACM	298.52	7.52[a]	30.03	3.49[b]	63.65
P-value	0.177	0.041	0.765	0.036	0.808
SEM	1.841	1.265	0.308	0.077	1.302
Total period (1–35 D)					
IM	293.45	261.56	656. 46	2.51[a]	62.99
AACM	298.52	266.35	653.94	2.46[b]	63.65
P-value	0.171	0.197	0.686	0.035	0.801
SEM	1.845	1.829	0.902	0.025	1.301

[a,b]Means lacking a common superscript letter differ, $P < 0.05$.
[1]Feed intake, g/D.
[2]IM: inorganic mineral.
[3]AACM: amino acids-mineral complexed.

Early research in poultry raised under acute cold stress conditions has shown a clear suppression in body development and egg production (Nguyen et al., 2016). At the molecular level, an acute hypothermic condition significantly increases the gene expression of hepatic leptin in chickens at 5 wk of age (Dridi et al., 2008). Depending on the magnitude and period of the stress, the birds show lower ADG and ADFI and worsening of FCR, which was observed in Exp. 2.

The initial phase is the period of greater bird's development in which intense cellular and enzymatic metabolism occur, followed by greater nutritional requirements, and consequently, more nutrients that act as enzymatic cofactors, such as minerals and vitamins, are needed. This is true for birds in general, especially for layer-type chicks, whose bone structure is going to change to have greater egg output persistence and retain high eggshell quality. By offering AACM diets, the damage caused by stress was reduced owing to the greater availability of minerals. Thus, even under stress conditions, the animals showed improvement in performance, with greater ADG and better FCR at the end of the experimental period. This fact may be related to the action of Zn in improving the intestinal epithelium quality, enhancing the absorption of nutrients, and boosting improvement in animal performance (Tse et al., 2010). Although Exp. 1 did not show any effect among the evaluated treatments for zootechnical performance, it was possible to observe that in all experimental periods, except the last week, birds fed with the AACM diet had a trend for greater uniformity.

The AACM supplementation showed an increase in the thymus weight of birds raised in thermoneutral environments. The improvement in immune organ weight by mineral supplementation is well documented in the literature (Moghaddam and Jahanian, 2009; Feng et al., 2010; Gheisari et al., 2010), mainly in studies on Zn, which has a close relationship with immune response in animals. Although, concentrates on which IM was utilized in studies, the required amounts are higher than AACM to obtain improvement in the immune system. Salabi et al. (2011) studied the relative weight of the bursa of Fabricius, liver, and spleen of broilers slaughtered at 43 D and reported a significant increase in bursa and spleen weight of birds supplemented with higher doses of IM (135 mg/kg of Zn) when compared with those supplemented with 90 and 45 mg/kg of Zn bound to organic molecules. Kidd et al. (1994) analyzed the effects of diets supplemented with Zn on turkeys by feeding them with adequate levels of this mineral and found that the addition of 30 or 45 mg/kg of Zn methionine significantly increased the cellular immune response of young turkeys.

A variable commonly used to estimate bird immunity is the lymphoid organ's weight that can be easily measured and reflect the body's ability to produce lymphoid cells during the immune response (Pope,

Table 8. Effect amino acids-mineral complexed on the organ weight and intestine length of layer-type chicks at 35 D, reared under cold stress conditions.

Treatments	Liver	Thymus	Spleen	Bursa	Pancreas	Intestine	Cecum	Intestine
				(g)				(cm)
IM[1]	10.05[b]	1.42	1.03	1.56	1.19[b]	27.40	12.36	125.19
AACM[2]	10.30[a]	1.56	1.05	1.50	1.24[a]	27.99	12.36	122.33
P-value	0.017	0.260	0.675	0.316	<0.01	0.216	0.821	0.086
SEM	0.108	0.048	0.013	0.038	0.345	0.013	0.821	0.094

[a,b]Means lacking a common superscript letter differ, $P < 0.05$.
[1]IM: inorganic mineral.
[2]AACM: amino acids-mineral complexed.

Table 9. Effect of amino acids-mineral complexed on the bone variables of layer-type chicks at 35 D, reared under cold stress conditions.

Treatments	Tibia weight[3] (g)	Tibia length[4] (mm)	Seedor index (mg mm^{-1})	Bone strength (Kgf)
IM[1]	0.86	20.82	41.48	7.63
AACM[2]	0.86	20.41	41.95	7.73
P-value	0.608	0.060	0.440	0.739
SEM	0.005	0.108	0.121	0.307

[1]IM: inorganic mineral.
[2]AACM: amino acids-mineral complexed.
[3]Relative weight.
[4]Relative length.

1991). Zinc has an impact on major mediators of immunity such as enzymes, thymus peptides, and cytokines, which explains the importance of their levels in the regulation of lymphoid cell proliferation, activation, and apoptosis (Dardenne, 2002).

In addition to the thymus, the cecum is also involved in the immune response process of the birds through the cecal tonsils. Cecal tonsils are the more important lymphoid tissue in the cecum, comprising the major concentration of lymphocytes associated with the bird's intestine, containing about 50% of B lymphocytes and 35% of T lymphocytes (Befus et al., 1980). Rezaian and Hamedi (2007) reported that there is an association between the thymus and cecal tonsils as the thymus involution process is synchronized with the higher growth rate and a greater increase in lymphatic nodular distribution in the cecal tonsils. However, unlike the thymus, the tonsils are not present soon after birth as their appearance depends on antigenic stimuli produced in the intestinal mucosa, which will occur with the normal microbiota establishment or with the intake of feed. Therefore, supplementation with AACM may have

Table 10. Concentration of zinc (Zn), manganese (Mn), copper (Cu), calcium (Ca), and phosphorus (P) on the liver, tibia, and excreta of layer-type chicks at 35 D, reared under cold stress conditions.

Treatments	Zn (mg/kg)	Mn	Cu	Ca	P (mg/g)
Tibia composition					
IM[1]	397.01	8.48	7.12	439.08	227.94
AACM[2]	385.04	8.91	7.18	440.09	229.76
P-value	0.215	0.098	0.821	0.892	0.667
SEM	7.530	0.154	0.131	5.315	2.908
Liver composition					
IM[1]	154.58	8.87	20.09	548.84	14.54
AACM[2]	146.14	8.56	19.93	532.62	14.44
P-value	0.272	0.267	0.809	0.461	0.778
SEM	3.802	0.131	0.317	10.902	0.160
Excreta composition					
IM[1]	350.44	488.48	59.19	44.99	20.55[a]
AACM[2]	364.13	470.69	57.38	42.38	18.79[b]
P-value	0.173	0.301	0.565	0.378	0.023
SEM	5.035	8.567	1.521	1.430	0.404

[a,b]Means lacking a common superscript letter differ, $P < 0.05$.
Abbreviations: Ca, calcium; Cu, copper; Mn, manganese; P, phosphorus; Zn, zinc.
[1]IM: inorganic mineral.
[2]AACM: amino acids-mineral complexed.

accelerated the process of nodular lymphatic distribution in the cecal tonsils, favoring the longer cecum length. The results found in this study have great importance because the initial stage of laying hens is a critical period of life, in which animals are subject to challenges caused by several types of environmental stressors when they are still under development.

The AACM supplementation in birds' diets raised under cold stress conditions increased liver and pancreas weights. The liver is a storage organ of minerals, mainly Cu. Baker et al. (1991) evaluated Cu bioavailability of chelated sources compared with nonchelated sources by the amount of Cu in this organ. The best use of Cu is in the complexed form, which possibly increases the synthesis of ceruloplasmin, providing higher liver weight. Zinc is also required by the liver to release retinol-binding protein responsible for vitamin A transport (Cozzolino, 1997). With this, the association of these factors may have contributed to the increase in hepatic synthesis, reflecting on greater liver weight.

The pancreas is an endocrine and exocrine organ, which requires a large amount of Zn for biological processes, including the production of digestive enzymes (Shannon et al., 2011). Huang et al. (2009) reported that the pancreas is the soft tissue most sensitive to Zn diet for chicks, and therefore, it is common for high concentrations of this element to be found in this organ. Thus, increased production of digestive enzymes and accumulation of Zn may explain the increase in pancreas weight of birds that are fed with minerals with higher bioavailability (AACM).

Although the average data of bone variables, in birds submitted to cold stress, had been systemically higher in the treatment with AACM, there were no statistically significant differences. These results corroborate those of the study by Brito et al. (2006), who evaluated the use of microminerals as an organic complexed source in pullets from 7 to 12 wk of age and did not find significant differences between treatments for the same variables evaluated.

In the present study, a reduction in mineral concentration in the liver was observed when providing the AACM diet, with Cu significantly affected. These results corroborate those found by other authors evaluating mineral supplementation in organic complexed form compared with IM for broiler chickens (Paik et al., 1999; Aksu et al., 2010; El-Husseiny et al., 2012). This probably

happened because after uptake of minerals in the gut, they are transported to the liver, where they are stored. However, when complexed to organic molecules, minerals can be redirected to other metabolic pathways and thus used in other organs for both protein synthesis and bone development (Gao et al., 2014). Another important finding is the fact that decreasing the levels of toxic minerals in the liver, especially Cu, would provide a reduction in toxicity from this mineral, with less amounts of the mineral excreted by the kidneys (Aksu et al., 2010).

On the other hand, unlike what happened in the liver, it was observed that the animals when raised in thermoneutral temperatures showed a significant increase in the concentration of Zn, Mn, and Cu in bone tissue, proving what was previously reported about the redirection of minerals to the target tissues, in this case, the bone. By considering bone as a multifunctional, complex, and heterogeneous tissue responsible for supporting the muscles, it is known that its growth and development are closely linked to the general growth of the animal's body. Thus, in the rearing phase, the birds must present an adequate bone structure and development, avoiding the occurrence of imbalance in mineral homeostasis and consequently problems in future phases, because the importance of this tissue in mineral support for eggshell synthesis is known.

Referring to the results of mineral excretion, it was observed that the partial replacement of IM by AACM negatively influenced Zn excretion in birds raised under thermoneutral conditions, and this may be related to the combination of both IM and AACM sources at the same total supplementation levels as the control. It is known that complexed minerals have higher bioavailability than conventional inorganic sources; thus, the 2 sources blend in a single diet cause an excessive supply of minerals, leading to higher excretion.

In contrast, in both studies, there was a reduction in P excretion. It is also observed that birds that were fed with AACM showed a tendency toward increase in P content in the tibia; the P may have been directed to bone deposition and development, not to undergo antagonism with other minerals, which would cause higher excretion.

Although the present studies were carried out with layer chicks until 5 wk of age, during when bone tissue is still in development and collagen fibers still lacked a defined organization, it is necessary to carry out further studies on chicks in several stages of rearing for better understanding of the functionality of the supply of microminerals bound to organic molecules in their body development.

Thus, the results observed in these studies demonstrated that in a thermal comfort environment, AACM supplementation supported an increase in thymus weight and length of the cecum, a reduction in P excretion, and an improvement in bone quality and the immune response of the bird. However, lower Cu deposition in the liver is the result, suggesting that more research must be carried out to understand the mechanisms involved in this response. In adverse conditions, such as cold stress, the supplementation of AACM can improve performance, increase liver and pancreas weight, and reduce P excretion.

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