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Effect of dietary supplementation of organic minerals on laying performance of hens

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Abstract
This study evaluated the effects of different dietary levels and sources of zinc (Zn) and manganese (Mn) on egg production and characteristics in laying hens. A total of 144 layers aged 22 weeks were fed a basal diet as per BIS (1992) specifications for 16 weeks, and were randomly allocated to 6 groups with 24 layers each. First group was kept as negative control (T_1) containing mineral mixture without zinc and manganese and T_2 (positive control containing mineral mixture with inorganic salts of zinc and manganese) while experimental groups T_3, T_4, T_5 and T_6 were supplemented with organic zinc and organic manganese @ 50 and 100% in substitution of inorganic sources. Each group had 4 replicates of 6 hens. Results showed that egg weight and egg mass production during 22-32 weeks of age in different treatments was found similar ($P>0.05$) and not influenced by any supplementation level of organic minerals. Shell thickness was greater in the organic Zn-added groups ($P<0.05$), and its relationship with the supplemental level of Zn showed linear effect. Laying hens that received 100% organic minerals (T_6) resulted into significantly ($P<0.05$) higher egg shell weight (5.21) with respect to negative control group (4.74) during 22-24 weeks of age. Egg shell thickness (mm) in all groups differed widely, ranging from 0.30 for T_1 to 0.39 for T_6 during 22-24 weeks of age. Results obtained in last two weeks of experiment (36-38) showed significantly ($P<0.05$) increased egg shell thickness in all organic minerals fed groups (0.40, 0.40, 0.40 and 0.41 respectively) in comparison to negative control (0.33). while albumin index of all the birds reached the maximum value of 11.21 at the end of 30th week and albumin index of the control groups and birds treated with organic minerals was similar (not differed significantly ($P<0.05$) during the whole experimental trial.

Keywords: Eggshell quality, laying hen, manganese, zinc

Introduction

India is the third- largest egg producer in the world after China and the USA and the fourth-largest chicken producer in the world after China, Brazil and the USA (Statista, a statistic portal, 2017) [14]. In poultry diet the micro minerals deserve attention as they exert essential functions in the organism, but in modern poultry nutrition effective use of micro minerals is overlooked. Micro minerals, especially, zinc and manganese are important component of many enzymes or catalysts or constituent of hundreds of protein, of vital relevance to the animal metabolism, hormone secretion pathways.

Zinc has been found in every tissue of the bird but the element tends to accumulate in the bones rather than the liver, which is the main storage organ of the other trace elements. Most of the zinc in blood is present in the erythrocytes. Zinc is present as a co-factor in over 300 metalloenzymes, representing all six classes of enzymes and plays an essential role in many metabolic processes, including protein synthesis (O'Dell, 1992; Salim *et al.*, 2008) [9, 11]. Important zinc metalloenzymes in the hen include carboxypeptidases and DNA polymerases. These enzymes play important role in the hen's immune response, in skin and wound healing and for hormone production (testosterone and corticosteroids). Zinc and manganese, as cofactors of various metalloenzymes responsible for carbonate and mucopolysaccharide synthesis, play an important role in eggshell formation and affect mechanical properties of eggshell by modifying crystallographic structure of eggshell (Mabe *et al.*, 2003) [8]. Manganese also participate in various structural and physiological functions, including, reproductive functions, formation of skeletal and cartilage tissue.

Poultry derives the minerals required for normal growth and metabolism from the diet and in commercial poultry diets, the majority of these trace minerals are supplemented as inorganic forms (sulphate or oxide salts) which suffer from high rates of loss due to dietary antagonism. Use of organically complexed trace minerals can help to prevent these losses, due to increased stability in the upper gastrointestinal tract. While measuring mineral requirements, source solubility and previous nutritional status of the birds are extremely important factors because mineral absorption requires previous solubilization of the original mineral source in the intestinal lumen. Also competition for the same or similar carriers is a major interference when metals are transferred from the lumen into the enterocyte (Hill & Matrone, 1970) [7]. Use of organically complexed or chelated minerals in premixes has been suggested as a solution to this problem.

Material and Methods

One hundred and fourty four laying hens were randomly divided into six treatment groups with 4 replicates of 6 birds in each following completely randomized design (CRD).
Feed ingredients used in the diet formulations were analyzed for the proximate nutrients (AOAC, 2013) [1]. The chemical composition of different feed ingredients is presented in Table 1.

Table 1: Chemical composition (%DM basis) and metabolizable energy (Kcal/Kg) of feed ingredients used in formulating the experimental diets of laying hens

Ingredients	CP	CF	EE	Ash	ME*	Cost/100kg
Maize	9.10	3.44	2.44	2.25	3300	1545
Soyabean meal	45.2	3.16	3.93	8.47	2250	2722
Fish meal	47.4	1.76	5.15	26.6	2210	3847
Groundnut cake	40	5.6	1.5	7.68	2400	2222

* calculated value (BIS, 1992)

The basal diet of laying hens was formulated as per BIS (1992) standards. The ingredient composition and chemical composition of the layers' control ration (T_1), has been given in Table 2.

Table 2: Ingredient Composition of Basal Ration for Laying Hens

Feed ingredients	Percentage
Maize	56
Groundnut cake	15
Soybean Meal	15
Fish Meal	7
Mineral Mixture	3
Salt	0.5
Shell Grit	3.5

Feed additives included Spectromix-10g, Spectrimix-BE-10g per 100 Kg of ration. For the experimental diets, mineral mixture was prepared using inorganic forms of all components except Zn and Mn. Organically complexed Zn and Mn were separately added in mineral mixture at proportion of their inorganic form in positive control group. The basal ration was formulated as per BIS (1992) specifications to meet energy and protein requirements of birds. First group was kept as negative control (T_1) containing mineral mixture without zinc and manganese and T_2 (positive control containing mineral mixture with inorganic salts of zinc and manganese) while experimental groups T_3, T_4, T_5 and T_6 were supplemented with organic zinc and organic manganese @ 50 and 100% in substitution of inorganic sources.The laying hens were reared in deep litter system at poultry farm and were offered feed and water *ad libitum* through linear feeder and waterer. Proper ventilation was provided and a photoperiod of sixteen hours per day was provided. Body weight of individual birds was measured at the start and end of the experiment. Egg production were recorded daily, separate record for individual bird were maintained for entire experimental period i.e. 22-38 weeks of age of laying hens. Per cent hen day egg production was calculated by using following formula:

$$\text{Per cent hen day egg production} = \frac{\text{Total no.of eggs produced during the period}}{\text{Total no.of hen days during the period}} \times 100$$

At the end of every two weeks the egg weights were recorded. Egg weights were measured by using egg analyzer and egg mass production was calculated using following formula:

$$\text{Egg mass production (g/day)} = \frac{\text{Per cent hen day egg production}}{100} \times \text{weight of egg}$$

After every two weeks 24 eggs were collected randomly, one from each replication of each treatment to estimate egg quality parameter. The egg shell was broken at the middle portion with the help of blunt end of knife. The egg contents, egg shell weight and shell thickness were measured using egg analyzer.
The width and length of each egg was taken using Vernier caliper to calculate egg shape index as per the formula:

$$\text{Shape index} = \frac{\text{Maximum width of egg}}{\text{Maximum length of egg}} \times 100$$

Maximum length and the maximum width of thick albumin were measured with the help of Vernier Caliper for average width of albumin. Albumin height was measured with the help egg analyzer. The albumin index was measured by using the following formula.

$$\text{Albumin index percent} = \frac{\text{Average height of albumin}}{\text{Average width of albumin}} \times 100$$

Haugh unit is the product of log of albumin height and egg weight and was calculated by following formula given by Raymond Haugh (1937) [6].

$$\text{Haugh Unit} = 100 \log (H + 7.57 - 1.7\, W^{0.37})$$

Where, H = Albumin Height, W= Egg Weight

Statistical Analysis

The resultant data were statistically analysed according to the procedure laid down by Snedecor and Chochran, (1994). Analysis of variance was used to study the differences among treatment means and they were compared by using Duncan's Multiple Range Test (DMRT) as modified by Kramer (1956).

Results and Discussion

Hen Day Egg Production

In layers feed, partial (50%) or complete substitution of inorganic Zn and Mn with amino acid complexes increased hen-day egg production, but only in later phase of the experiment (from 28 to 38 weeks of laying cycle). There were no significant differences between treatments (P>0.05) at 22-28 weeks of hen's age. The average hen day egg production was recorded significantly (P<0.05) higher in T_6 and T_5 groups during 30-32 weeks of age and in T_6 during 32-38 weeks of age with respect to negative control and inorganic minerals supplemented groups. Zn is an important cofactor of CA, which performs a vital role in eggshell formation and a diet for aged laying hens should contain 80 mg/kg Zn (Guo *et al.*, 2002) [5]. Proteins with zinc-binding domains are estimated to represent approximately 10% of proteome, and zinc plays important part in a wide array of processes including cell proliferation, immune development and response, reproduction, gene regulation, and defense against oxidative stress and damage (Song *et al*., 2009) [13].

Egg Weight

Results showed that egg weight in different treatments was found similar (P>0.05) and not influenced by any supplementation level of organic minerals as compared to the negative and inorganic source group. Organic Zn, Mn and Cu at a dosage lower than NRC recommendation is sufficient to maintain laying performance and can improve eggshell and albumen qualities of the egg in laying hens. (Gheisari *et al.*, 2011) [4].

Egg Mass Production

The results showed that none of treatments had showed significant effect on egg mass production till 22-32 weeks of age (Table 5). During 32-34 weeks egg mass was significantly (P<0.05) increased in all organic minerals supplemented groups (T_3, T_4, T_5 and T_6) and inorganic minerals treated group (T_2) in comparison to negative control group (T_1). However, during 34-36 weeks also, no significant difference was observed among the dietary treatments.

Results of egg mass production during 36-38 weeks indicated significantly (P<0.05) higher values in organic minerals treated groups from the negative control group (T_1).

Eggs from hen fed the control diet or that supplemented organic trace minerals proved to be similar (P>0.05). These minerals (Zn and Mn) have role in egg shell synthesis and calcite crystal deposition during shell formation. There was no effect of organic trace minerals supplementation appeared on egg mass production (Darvishi *et al.*, 2020) [3].

Table 3: Mean values of percent hen day egg production during progressive age (weeks) under different dietary treatments

Weeks/ Treatment	T_1	T_2	T_3	T_4	T_5	T_6
22-24	70.18±0.44	69.72±0.57	70.40±0.49	70.85±0.60	69.43±0.39	70.33±0.40
24-26	69.95±0.57	70.94±0.60	70.18±0.78	70.40±0.49	71.20±0.77	70.11±0.59
26-28	71.46±0.42	70.99±0.77	70.15±0.59	71.86±0.44	71.06±0.58	71.52±0.57
28-30	71.26^{ab}±0.38	71.18^{ab}±0.60	71.12^{ab}±0.65	70.67^{b}±0.35	71.36^{ab}±0.27	72.25^{a}±0.34
30-32	70.60^{b}±0.31	70.75^{b}±0.73	71.91^{ab}±0.39	71.95^{ab} ±0.35	72.33^{a}±0.45	73.13^{a}±0.38
32-34	71.20^{c}±0.33	71.66^{bc}±0.64	72.02^{bc}±0.60	72.42^{abc}±0.66	73.23^{ab}±0.68	74.04^{a}±0.44
34-36	71.00^{b}±0.22	72.42^{ab}±0.51	72.87^{a}±0.23	72.31^{ab}±0.75	72.11^{ab}±0.52	73.07^{a}±0.58
36-38	71.08^{d}±0.49	71.75^{cd}±0.43	72.61^{bc}±0.35	73.04^{bc}±0.53	73.58^{ab}±0.43	74.62^{a}±0.32

Means bearing different superscripts in a row differ significantly (P<0.05)

Table 4: Egg weight (g) during progressive age (weeks) under different dietary treatments

Weeks/ Treatment	T_1	T_2	T_3	T_4	T_5	T_6
22-24	51.89±1.35	52.18±1.58	50.01±1.39	51.50±1.50	50.12±1.18	50.14±1.16
24-26	49.87±0.55	51.20±0.70	50.10±0.89	51.78±0.65	51.70±0.87	51.35±0.93
26-28	51.47±0.47	51.80±0.69	52.74±0.57	52.24±0.53	51.50±0.57	51.22±0.61
28-30	52.33±0.49	51.85±0.87	51.88±0.61	52.42±0.79	52.47±0.34	52.62±0.52
30-32	51.68±0.54	52.25±0.45	51.98±0.52	51.90±0.51	52.17±0.15	52.57±0.75
32-34	50.95±0.44	51.68±0.65	51.20±0.49	51.93±0.51	51.62±0.45	51.58±0.76
34-36	51.82±0.52	51.65±0.63	52.21±0.57	51.57±0.50	52.10±0.64	52.01±0.59
36-38	51.94±0.61	52.39±0.52	52.17±0.68	51.92±0.83	52.00±0.66	52.24±0.76

Table 5: Egg mass production (g/day/hen) during progressive age (weeks) under different dietary treatments

Weeks/ Treatment	T_1	T_2	T_3	T_4	T_5	T_6
22-24	36.43±1.09	36.36±0.94	35.20±0.96	36.51±1.27	34.80±0.94	36.66±0.63
24-26	34.88±0.42	35.92±0.28	35.16±0.68	36.45±0.62	36.81±0.70	36.01±0.79
26-28	36.77±0.16	36.76±0.63	36.99±0.19	37.55±0.48	36.60±0.56	37.35±0.63
28-30	37.29±0.53	36.92±0.92	36.90±0.74	37.86±0.40	37.04±0.26	37.55±0.35
30-32	36.49±0.47	36.97±0.49	37.38±0.35	37.39±0.37	37.73±0.33	37.71±0.65
32-34	36.27^{c}±0.22	37.02^{abc}±0.17	36.87^{bc}±0.43	37.60^{ab}±0.33	37.79^{ab}±0.30	38.19^{a}±0.68
34-36	36.80±0.29	37.41±0.65	38.05±0.51	37.28±0.44	37.56±0.19	38.01±0.69
36-38	36.91^{b}±0.31	37.59^{ab}±0.56	37.73^{ab}±0.60	37.91^{ab}±0.31	38.26^{ab}±0.47	38.79^{a}±0.61

Means bearing different superscripts in a row differ significantly (P<0.05)

Table 6: Egg shape index during progressive age (weeks) under different dietary treatments

Weeks/ Treatment	T_1	T_2	T_3	T_4	T_5	T_6
22-24	72.04±0.97	71.89±0.53	72.78±0.70	73.10±0.53	72.60±0.77	73.22±0.49
24-26	71.96^{b}±0.51	72.87^{ab}±0.66	72.82^{ab}±0.51	73.35^{ab}±0.50	73.00^{ab}±0.39	73.86^{a}±0.53
26-28	73.32±0.33	72.90±0.43	73.15±0.17	72.45±0.46	73.23±0.81	73.45±0.55
28-30	72.38±0.40	72.67±0.30	72.35±0.44	71.55±0.31	72.93±0.49	72.73±0.67
30-32	73.14±0.42	72.44±0.30	73.23±0.58	72.52±0.35	73.03±0.17	73.05±0.41
32-34	70.85^{b}±0.79	71.31^{ab}±0.50	72.63^{ab}±0.42	71.91^{ab}±0.89	72.58^{ab}±0.35	73.00^{a}±0.47
34-36	72.47±1.00	73.08±0.72	72.10±0.58	72.55±0.65	73.08±0.63	73.18±0.56
36-38	72.27^{b}±0.42	72.07^{b}±0.37	72.98^{ab}±0.44	73.27^{ab}±0.41	73.90^{a}±0.40	73.18^{ab}±0.30

Means bearing different superscripts in a raw differ significantly (P<0.05)

Egg Shape Index

Egg shape index during 22-24 weeks is similar irrespective of treatments (Table 6) but during 24-26 weeks of age significantly (P<0.05) higher egg shape index was recorded in T_6 (73.86) as compared to T_1 (71.96). The results here showed that there was no effect of dietary supplementation of organic minerals on egg shape index in duration of 26-32 weeks. In the course of 32-34 weeks of age 100% supplementation of organic minerals (T_6) in place of inorganic minerals (T_1) resulted into significantly (P<0.05) higher egg shape index. Laying hens that received organic minerals did not show any significant difference from the negative control and inorganic minerals fed group during 34-36 weeks. However, during 36-38 weeks of the experiment laying hens supplemented with organic minerals (T_5) manifested significantly (P<0.05) higher egg shape index (73.90) in comparison to T_1 (72.27) and T_2 (72.09). Zn and Mn are cofactor for various metalloenzymes responsible for carbonate and mucopolysaccharide synthesis, which play role in maintaining egg shape and egg shell formation. Laying performance and egg quality parameters (egg weight, egg shape index) are usually not sensitive to supplementation level of Zn and Mn when hens are fed with a conventional corn-soybean meal diet containing higher Zn levels (Tabatabaie *et al.*, 2007) [15].

Egg Shell Weight and Egg Shell Thickness

Laying hens that received 100% organic minerals (T_6) resulted into significantly (P<0.05) higher egg shell weight (5.21) with respect to negative control group (4.74) during 22-24 weeks of age. Organic minerals supplemented @ 100% (T_6) showed significantly higher (P<0.05) egg shell weight in comparison to 50% organic minerals supplemented groups (T_3) during 26-28 weeks. Nevertheless, in the course of 28-32 weeks supplementation of organic minerals at different levels did not affect egg shell weight significantly. Layers fed with different levels of organic minerals (T_3, T_5 and T_6) showed significantly (P<0.05) higher egg shell weight (5.19, 5.04 and 5.09 respectively) as compared to negative control group (4.61) during 32-34 weeks. No significant differences were recorded on supplementation of organic minerals with respect to negative control and inorganic minerals fed group during period of 34-36 weeks. Dietary supplementation of inorganic (5.03) as well as organic minerals at different levels (5.02, 509, 5.07 and 5.13 respectively) showed significantly (P<0.05) higher egg shell weight as compared to negative control group (4.80) in course of 36-38 weeks of age.

Egg shell thickness (mm) in all groups differed widely, ranging from 0.30 for T_1 to 0.39 for T_6 during 22-24 weeks of age and marked significant difference in T_6 group from negative control group. Organic minerals supplemented groups (T_3, T_4, T_5 and T_6) have also shown significant variation from negative control group (T_1) at 24-26 weeks of age. With the thickest egg shell formation, 100% organic minerals fed group (0.40) showed significant (P< 0.05) difference from the T_1 (0.31) and T_2 (0.36) during 26-28 weeks. Dietary organic minerals increased the egg shell thickness significantly during 28-30 weeks also, from negative control, with no significant differences among organic groups. Thickness of the egg shell increased significantly (P<0.05) in all supplemented groups as compared to negative control during 30-32 weeks and from negative control as well as inorganic sources during 32-34 weeks. Increment in the egg shell thickness of layers during last two weeks (34-38) had followed the pattern of significantly increased egg shell thickness in all organic minerals fed groups (0.41 in T_6) in comparison to negative control (0.33).

Supplementation of basal diet with additional amounts of Zn had positive effect on eggshell thickness (Zamani *et al*., 2005) [17] because Zn and Mn have an important role in the activity of metalloenzymes and carbonic anhydrase enzyme required for hydrolysis of carbonic acid. These minerals affect egg shell mechanical properties by effect on calcite crystal formation and modifying crystallographic structure of eggshell through deposition of calcium carbonate.

Egg Albumin Index

Albumin index of the control groups and birds supplemented with the organic form of minerals was observed every fortnightly. Albumin index of all the birds reached the maximum value of 11.21 at the end of 30th week. The albumin index of the control groups and birds treated with organic minerals was similar (not differed significantly (P<0.05) during the whole experimental trial. Sources of organic trace minerals used individually or associated did not have any influence on haugh unit, yolk and albumin index (Scatolini, 2007; Correia *et al*., 2000) [12, 2]. On the other hand, Xavier *et al*., 2004 [16]; Rutz *et al*., 2006 [10] observed a trend of better yolk and albumin weights and a consistent improvement of albumin quality on feeding organic minerals to layers.

Table 7: Egg shell weight during progressive age (weeks) under different dietary treatments

Weeks/ Treatment	T1	T2	T3	T4	T5	T6
22-24	4.74^{b}±0.18	4.99a^{b}±0.11	5.13a^{b}±0.16	5.04a^{b}±0.03	4.90a^{b}±0.18	5.21a±0.06
24-26	4.83±0.11	4.90±0.15	4.99±0.11	4.94±0.18	5.06±0.21	5.15±0.04
26-28	4.87a^{b}±0.05	4.78a^{b}±0.11	4.69^{b}±0.20	5.0a^{b}±0.06	4.97a^{b}±0.11	5.06a±0.06
28-30	4.76±0.22	5.06±0.07	5.10±0.04	4.99±0.06	5.00±0.09	5.01±0.07
30-32	4.81±0.13	4.78±0.11	5.01±0.06	4.96±0.09	4.88±0.13	4.96±0.09
32-34	4.61^{b}±0.09	4.85a^{b}±0.07	5.19a±0.20	4.89a^{b}±0.08	5.04a±0.05	5.09a±0.05
34-36	4.80±0.11	4.95±0.12	4.66±0.19	4.75±0.12	4.88±0.12	5.03±0.04
36-38	4.80^{b}±0.11	5.03a±0.07	5.02a±0.09	5.09a±0.04	5.07a±0.02	5.13a±0.05

Means bearing different superscripts in a row differ significantly (P<0.05).

Table 8: Egg Shell Thickness (mm) during progressive age (weeks) under different dietary treatments

Treatment	22-24wks	24-26	26-28	28-30	30-32	32-34	34-36	36-38
T_1	$0.30^{c}\pm0.01$	$0.32^{c}\pm0.007$	$0.31^{c}\pm0.01$	$0.31^{c}\pm0.01$	$0.31^{c}\pm0.01$	$0.32^{c}\pm0.01$	$0.33^{b}\pm0.01$	$0.33^{b}\pm0.01$
T_2	$0.34^{b}\pm0.02$	$0.35^{b}\pm0.02$	$0.36^{b}\pm0.01$	$0.37^{b}\pm0.01$	$0.37^{b}\pm0.01$	$0.37^{b}\pm0.01$	$0.38^{a}\pm0.01$	$0.38^{a}\pm0.01$
T_3	$0.34^{b}\pm0.01$	$0.38^{ab}\pm0.01$	$0.38^{ab}\pm0.01$	$0.39^{ab}\pm0.01$	$0.39^{ab}\pm0.01$	$0.39^{a}\pm0.01$	$0.39^{a}\pm0.01$	$0.40^{a}\pm0.01$
T_4	$0.35^{b}\pm0.01$	$0.36^{b}\pm0.01$	$0.38^{ab}\pm0.01$	$0.39^{ab}\pm0.01$	$0.40^{a}\pm0.01$	$0.40^{a}\pm0.02$	$0.40^{a}\pm0.01$	$0.40^{a}\pm0.01$
T_5	$0.37^{ab}\pm0.02$	$0.38^{ab}\pm0.02$	$0.39^{ab}\pm0.01$	$0.39^{ab}\pm0.01$	$0.39^{a}\pm0.01$	$0.39^{a}\pm0.01$	$0.39^{a}\pm0.02$	$0.40^{a}\pm0.01$
T_6	$0.39^{a}\pm0.01$	$0.39^{a}\pm0.004$	$0.40^{a}\pm0.02$	$0.40^{a}\pm0.01$	$0.40^{a}\pm0.02$	$0.41^{a}\pm0.02$	$0.41^{a}\pm0.02$	$0.41^{a}\pm0.02$

Means bearing different superscripts in a column differ significantly ($P<0.05$)

Table 9: Egg albumin Index during progressive age (weeks) under different dietary treatments

Weeks/ Treatment	T_1	T_2	T_3	T_4	T_5	T_6
22-24	8.22±0.29	8.38±0.60	8.31±0.39	8.04±0.43	8.05±0.06	8.33±0.49
24-26	8.45±0.27	8.50±0.32	8.17±0.13	8.41±0.37	8.42±0.30	8.15±0.40
26-28	9.90±0.43	9.73±0.21	9.54±0.46	8.74±0.25	9.18±0.31	9.73±0.71
28-30	9.46±0.32	10.04±0.73	10.52±0.45	10.47±0.29	10.12±0.21	11.21±0.39
30-32	10.35±0.33	10.90±0.39	11.12±0.44	10.23±0.45	9.65±0.42	10.38±0.42
32-34	9.96±0.48	9.42±0.43	9.55±0.28	9.36±0.25	10.29±0.53	10.41±0.24
34-36	10.04±0.36	9.96±0.46	10.17±0.24	9.27±0.31	10.05±0.38	9.86±0.48
36-38	9.41±0.24	9.93±0.51	9.72±0.38	9.22±0.43	10.29±0.27	9.25±0.52

Means bearing different superscripts in a row differ significantly ($P<0.05$)

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