

Effect of Thermal Manipulation during Embryogenesis on Thermotolerance and Hatched Broiler Performance

MOHAMMAD, Sh.¹ and Al SARDARY, S.

Animal Resource Department, Salahaddin University, Agriculture College-Erbil, IRAQ

¹shireen.dhahir@yahoo.com

Abstract: - This study was conducted to investigate the effect epigenetic adaptation by thermal manipulation (TM) during different stages of embryogenesis broiler breeder eggs by using an intermittent temperature treatments with amplitude of 1 °C higher than the standard incubation temperature for 4 hours daily at embryonic days (ED): T1 (1-5), T2 (8-12), T3 (14-18), T4 (19-21) and T5 was control (no TM), also through early age thermal conditioning chicks by exposing them to 38.0 ± 2 °C for 12 hours/day at 5th and 7th days of age then from the beginning of 4th week and onward the birds acclimated to elevated heat of 26±2 °C, on post-hatch broiler productive performance, body temperature and carcass traits at 35 and 42 days of broiler age. Six hundred (600) day-old chicks were randomly assigned according to treatment groups during hatching process. Chicks were distributed into five treatments with four replicates/group, with thirty chicks per each replicate (15 for each sex), all treatment groups were subjected to 38 ± 2 °C for 12 hrs/day at 5th and 7th days (d) post-hatching as thermal conditioning (TC). After each exposure body temperature of birds were measured. Live body weight, bodyweight gain, feed consumption, feed conversion ratio and mortality were recorded weekly. Production index, dressing percentage and carcass cuts percentages, were measured at 35 and 42 days of age of broiler chicks. The overall data showed that T1 and T3 showed significantly (p≤0.05) higher relative growth percentage than control group. T3 showed significantly (p≤0.05) lower rectal temperature than other thermally manipulated groups and control group at 42 days of age. T1 and T3 showed significantly better feed conversion ratio than the other thermally manipulated groups and control group during the period 1- 42 days. T2 showed significantly lowest cumulative mortality percentage than other thermally manipulated groups and control. T2 and T3 had the higher production index than other treated groups and control group at marketing age of 35 and 42 days, respectively. In conclusion, using of epigenetic adaptation to temperature lead to decrease cloacae temperature at 42 days of age than control which confirms the acquisition of thermotolerance in thermally manipulated groups.

Key-Words: - Epigenetic adaption, Thermal manipulation, Early age heat conditioning, Performance, Thermotolerance.

1 Introduction

Poultry meat consumption is expected to raise by 60 % over the next 20 years and will be the most important meat category worldwide by 2030 [1]. Genetic selection strategies which has significantly improved growth of meat-type broiler chickens during recent decades, but lack of a parallel development of the visceral systems causes significant difficulties for broiler chickens in coping with high temperature challenges, due to the large body mass and high rate of metabolism associated with rapid growth [2].

Thermotolerance can be defined as the ability of organism's to survive and overcome lethal thermal stress from a previous heat exposure [3]. For increasing the thermotolerance capacity of the birds and also inhibition of economic losses as a result of heat stress, the adaptation to ambient conditions

depends on a mechanism called epigenetic adaptation when chicken can be better conditioned to thermal stress tolerance during the pre-hatching and early post-hatching period through epigenetic mechanism by exploiting the immaturity of temperature regulation in embryos and early post-hatch birds by thermal conditioning at critical developmental phases [4] and these methods have provided some suitable results for broiler industry [5]. Under these conditions there is a period when the thermotolerance can be enhanced by thermal conditioning, without impairing the performance [6].

2 Materials and Methods

The chicks of this experiment were exposed to thermal manipulation by increasing 1 °C from standard temperature for four hours daily during

embryogenesis in different embryonic stages, the heat exposing on treatments were as follow:

T1, T2, T3 and T4 represents the thermal manipulation started from ED (1-5), ED (8-12), ED (14-18) of incubation periods and ED (19-21) of hatching period, respectively. While, **T5: Control: no TM**= exposed to standard conditions (37.7 °C and 65-70% RH) in incubator and (37.3, 37.1 and 37.0 °C during 19th, 20th and 21st days of hatching, respectively with 75-80% RH) in hatchery.

Six hundred (600) day-old hatched chicks (Ross-308) reared in poultry research hall of Grdarasha farm/ Animal Resources Department/ College of Agriculture/ University of Salahaddin during the period from 7/8-19/9/2013. The house was divided into 20 floor cages (2*1.7 m²). The chicks of each treatment and control groups were randomly divided in to 4 replicates / group, four cages for each treatment. The chicks were reared at standard environment temperature till 21 day old, where chicks raised under regular conditions at (32±1°C) during first week except the 5th and 7th day, all chicks were exposed to early age heat conditioning of (38±2 °C) for 12 hours/day then the ambient temperature progressively was decreased till reached 26 °C at 21 day old, after that the birds exposed to elevated temperature 26±2°C till marketing age. A constant photoperiod of 23L: 1D hours during the first week thereafter intermittent photoperiod 19L: 5D hours along rearing period was provided. Feed and water were provided *ad libitum* along the experiment period. Birds were fed three rations starter from (1-22) days, grower from (23-36) days and finisher from (36-42) days. The analyzed metabolic energy (ME kcal) and crude protein (CP %) for starter, grower and finisher was (2925 kcal, 22-23%), (3040 kcal, 20-21%) and (3100 kcal, 18-19%) respectively. Vaccination program used during the experiment was according to Intervet Schering-Plough Animal Health. The experiments executed as a complete randomized design (CRD), all data analyzed using the [7]. Duncan's multiple range tests were used to compare differences among treatment means [8].

3 Results and Discussion

Table (1) shows that T1 and T3 had significantly ($P \leq 0.05$) higher relative growth compare to T4 and control group. While T4 showed significantly ($P \leq 0.05$) lower relative growth than other thermally manipulated groups as well than control group. According to [9] development during the first week of life of a chick was important to their future performance because physiological processes such as

cell hyperplasia and hypertrophy, maturation of the thermoregulatory and immunological systems, growth and differentiation of the gastrointestinal tract will subsequently markedly influence BW until market age.

Table (2) showed that there were no significant differences in live body weight (BW) during 35 d of age among all thermally manipulated groups as compared to each other and to control group. While the results during 42 d of age explained that T3 owned significantly ($P \leq 0.05$) higher BW than T1. The results of T3 at 42 d were in agreement with the results of [10]. The non significant differences in BW at 6th week may was due to changes of kinetics of satellite cell proliferation which improved numerically BW at slaughter age. Further, in embryos as well as chicks of meat-type poultry mild heat exposure if applied during developmental 'critical periods' environmental influences can change the programming of respective body functions [11].

Accumulative BWG (1-35d) it was noted that T1 was significantly ($P \leq 0.05$) lower than control group. Whereas for accumulative BWG (1-42), T3 gained significantly ($P \leq 0.05$) higher weight than T1 (Table 2). The difference in weight gain among treated groups may be due to different environmental conditions in the hatcher, chicks hatching at different moments within the hatch window are subjected to different conditions for a variable length of time, which may lead to different chick physiology at hatch and at the typical moment of chick collection and also to different growth post-hatch [12]. Also the reduction in weight gain during and immediately after conditions to high temperatures may be due to that the chicken directs the energy used for its growth to maintain body temperature within normal range with minimal response to heat stress and ensuring the organic function of tissues within physiological limits [13]. As well as, high rearing temperature decrease in weight gain, may due to that heat stress increase serum corticosterone level (which stimulate much higher muscle breakdown, that contributed to the lower BWG observed in birds kept in heat stress than those in a thermoneutral environment [14]. In poultry it is believed that less weight gain in the heat stressed groups, due to reduction in intestinal absorption efficiency was partly explained by decreased metabolic utilization of nutrients, increased heat production, reduced protein retention, and enhanced lipid deposition [15].

Accumulative FI (1-35d) and (1-42d), T1 consumed significantly ($P \leq 0.05$) less feed than other treatment groups (Table 2). The most influential factors affecting feed consumption of chicks have been

suggested to be related to the incubation and brooding temperature [16]. The possible causes of feed intake reduction for heat treated groups may be due to the effect of metabolic heat production which is one of the factors that cause feed intake reduction in birds maintained in hot temperatures [17]. Moreover, may probably due to that chickens maintained at the higher temperatures, generally responded by increasing their respiratory rate, therefore, the period of time for their consumption of feed decreased [18]. As well as, in poultry the heat stressed groups have low appetite and lower feed intake, as it may be a defence mechanism to help reduce heat production [19].

Feed conversion is an index associated with both feed consumption and weight gain. In accumulative FCR (1-42d) T1 and T3 had significantly ($P \leq 0.05$) better FCR than T2, T4 and control. Also the results indicated that there were no significant differences among all thermally manipulated groups as compared to each other and to control group in FCR in accumulative FCR (1-35) (Table 2).

In accumulative mortality percentage (1-35 and 1-42) T2, T3 and control group had significantly ($P \leq 0.05$) lower mortality percentage than T1 (Table 1). The result of cumulative mortality percentage of T3 and T4 were in agree with [20]. The lower mortality percentage during the all over rearing period in T2 and T3 could related to that broilers from conditioned embryos might have less difficulty in coping with heat stress than the birds from untreated embryos conditioning due to the correct TM stage [21], also may early age heat conditioning relieved the negative effects of heat stress on viability and ability of broilers to tolerate the high temperatures [22]. Whereas the increased mortality percentage during rearing period in T1 and T4 may be related to the negative effects of elevated temperature during embryogenesis on the cardiovascular system as heart size, heart weight and energy metabolism capabilities in cardiac tissue and this may increase the incidence of broiler problems such as sudden death syndrome and ascites may be related to metabolic disorders that are associated with cardiovascular development later in life [23].

Table (2) clarifies that T2 and control group represented significantly ($P \leq 0.05$) higher PI than T1 and T4 during the period of 1-35 days. While, at the duration of 1-42 days, the most preferable production efficiency noted in T3 which was significantly ($P \leq 0.05$) higher than T1 and T4. The improvement in production index may be related to overall improvement in average body weight and mortality percentage of broilers during both rearing period. Heat stress has a negative effect on poultry

production efficiency [24]. Many researches showed that manipulation of incubation temperature intermittently during broiler embryogenesis with low magnitude may have a long-lasting influence on improving poultry performance [25, 26, 27]. In contrast [28] demonstrated that TM adversely affected broiler performance.

Table (3) shows carcass traits at 35 d, when T2 had significantly ($P \leq 0.05$) higher live body weight than control. Breast percentage were significantly ($P \leq 0.05$) higher in T1 and T2 than T3, T4 and control group. Thigh percentage was significantly ($P \leq 0.05$) higher in T2 than T4 and control group. Main parts were significantly ($P \leq 0.05$) higher in T1 and T2 than other treatments. Secondary parts percentage were significantly ($P \leq 0.05$) higher in T4 and control group than T1, T2 and T3. As well as, at 35 d there were no significant differences among all thermally manipulated groups as compared to each other and to control group in percentages of dressing with and without giblets, edible parts and abdominal fat. The results of live body weight (LBW) at slaughtering in T2 were consistent to the results of [29]. In contrast to the result of T2 and T3 was the result of [30] for LBW at 35 days. Results of [31] were in agreement with T1 that heating the embryos improved the relative breast muscle slightly at 35 days. Also, the breast percentage results of T2 and T3 were in agreement with the results of [25, 30] at 35 days. Results of T2 and T3 were disagreed with the results of [30] of abdominal fat percentage numerically at 35 days. Results of T2 and T3 were in contrary to the results of [25] in relative weight of the abdominal fat pad was significantly lower. Results of T2 and T3 were confirmed by those of [26] who resulted that decreased abdominal fat accumulation in males and females.

At 42 d, dressing percentage without giblets was significantly ($P \leq 0.05$) higher in T1 than other treatments. T3 had significantly ($P \leq 0.05$) higher thigh percentage than T1. While control group had significantly ($P \leq 0.05$) higher abdominal fat percentage than T1. T1 had significantly ($P \leq 0.05$) lower main parts percentage than other treatments. Only T1 had significantly ($P \leq 0.05$) higher secondary parts percentage than other treatments. While there were no significant differences among all thermally manipulated groups as compared to each other and to control group in live body weight and percentages of dressing with giblets, edible parts and breast at 42 d. At 42 d the results of [27] confirmed the result of T4 that increasing incubation temperature 1 °C above the standard during the last three days of incubation numerically increased body weight at slaughter than control. The results of treated (TT) groups for

dressing without giblets percentage were confirmed by the results of [32] increased carcass percentage slightly at 42 days. While, the results of T3 for giblets and breast percentages were in agreement with the results of [33] who showed that there were no significant differences in percentages of entrails and breast in thermally manipulated chickens than control at 42 days. The thigh percentage results of T2, T3 and T4 were in agreement with the results of [32] at 42 days. Results of abdominal fat in T1 were in agreement with the results of [32]. In contrast, the results of T2, T3 and T4 at 42 days. Results of T1 was matched the results of [18] who found that TM of 38.5°C from ED4 through ED7 resulted smaller fat pads and adipocytes than controls. While, the results of T2, T3 and T4 were in agreement with those of [34] that elevating temperature during 2nd and 3rd weeks of embryogenesis did not affect abdominal fat percentage significantly at 42 days.

Elevating incubation temperature during embryogenesis improved productive characteristics of adult chickens because have a stimulatory effect on the proliferation of satellite cells (muscle cells) that is considered the precursor of myogenic cells in the embryo or post-hatching muscles, which affects on increased growth and development of skeletal musculature in the postnatal period of development [26] by changing the rate of myoblast proliferation resulting in increased number of cells within a muscle, enhancing myofiber diameter and/or numbers in the pectoral major muscle in the heat treated embryos [25].

Table (4) shows that no significant differences among all thermally manipulated groups as compared to each other and to control group in body temperature at hatch, 5th, 21st and 28th day of age. At 7th day control group had significantly ($P \leq 0.05$) lower body temperature than T1 and T3. At 14th day T2 had significantly ($P \leq 0.05$) lower body temperature than T1, T3 and T4. At 35th day T2 had significantly ($P \leq 0.05$) lower body temperature than T3. At 42nd day all thermally manipulated groups had significantly ($P \leq 0.05$) lower body temperature than control group. The results of T3 and T4 was in agreement with the results of [35] at the 7th day of age. In contrast [36] disagreed with T2 and T3 at 7th day of age. At 14th days the results of [36] was in agreement with T2 and T3. [37] confirm the result of T2 and T3 at 35th and 42nd days of age. In contrast [36] results were disagreed T2 and T3 at 42 days. The reduced rectal temperature in TT group compare to control at 42 days of age may be due to increased rearing temperature causes a decrease in the concentration of T₃ and T₄ hormones in blood plasma and a decrease in metabolic rate of broiler chickens

generally led to lower body temperature at normal or high temperature [38]. Nevertheless, may be thermal conditioning in broiler chickens enhanced heat tolerance [39].

4 Conclusions and Recommendations

In light of the present results of exposing the broiler breeder eggs to intermittent thermal manipulation (TM) by 1°C above the optimum incubation temperature during different embryogenesis periods, followed by early post-hatch thermal conditioning (TC) of chicks at 5th and 7th days of age, it was concluded that:

- 1- Relative growth percentage at 1st week age as an indicator for future performance.
- 2- All thermally manipulated birds showed a significant reduction in 42 days body temperature compared to control, which is an indicator for best acquisition of thermotolerance.
- 3- Production index as an indicator for birds subsequent performance, ED (8-12) and ED (14-18) groups were superior to all treated and control groups during the first and the second marketing age, respectively.
- 4- In mixed (males and females) rearing thermal manipulation had no significant effect on dressing percentage with giblets generally at 35 and 42 days, also dressing percentage without giblets at 35 days, while at 42 days thermally manipulated groups at ED (1-5) was significantly higher than other treatments.
- 5- Main parts percentage were significantly higher in both thermally manipulated groups ED (1-5) and (8-12) at 35 days, while the result reversed for thermally manipulated group ED (1-5) at 42 days.

So we recommend that:-

- 1- Determination of relative growth at 1st week is recommended in predicting the subsequent productive performance of broilers.
- 2- Thermal manipulation is recommended generally as a method for better subsequent thermotolerance.
- 3- Thermal manipulation in during ED 14-18 is recommended for better productive performance.
- 4- To obtain higher percentages of main carcass parts at 35 days it is recommended to apply thermal manipulation during both ED 1-5 and ED 8-12, while using thermal manipulation during ED (14-18), (19-21) and control are advised in getting higher percentages of main carcass parts at 42 days.
- 5- More future investigations is needed to have better idea about thermal manipulation and thermal conditioning in breeders, layers and broilers.

Table 1: Effect of thermal manipulation and early age heat conditioning on relative growth. (%) of broiler chicks during first week of age.

| RG | Treatment* | | | | | L.S. |
|----------------------|-------------------------|--------------------------|-------------------------|-------------------------|--------------------------|------|
| | T1 | T2 | T3 | T4 | Control | |
| 1 st week | 421.5 ±7.7 ^a | 405.4 ±4.2 ^{ab} | 424.7 ±4.4 ^a | 356.8 ±4.3 ^c | 396.8 ±12.0 ^b | * |

a, b, c : means within each row had the different subscript were differ significantly (P<0.05).

Table 2: Effect of thermal manipulation and early age heat conditioning on accumulative body weight (g), body weight gain (g/bird), feed consumption, feed conversion ratio, mortality percentage and production index during two rearing period (1-35d) and (1-42d) of broiler chicks.

| Accumulative | Treatment* | | | | | L.S. |
|----------------------------|--------------------------|---------------------------|--------------------------|---------------------------|---------------------------|------|
| | T1 | T2 | T3 | T4 | Control | |
| BW (1-35d) | 2093±25 ^a | 2160±32 ^a | 2136±33 ^a | 2130±26 ^a | 2134±14 ^a | N.S |
| BW (1-42d) | 2732±12 ^b | 2817±37 ^{ab} | 2845±32 ^a | 2792±29 ^{ab} | 2809±25 ^{ab} | * |
| BWG (1-35d) | 2055.6±7.8 ^b | 2120.0±35.6 ^a | 2115.4±30.2 ^a | 2092.6±24.8 ^{ab} | 2115.5±22.9 ^a | * |
| BWG (1-42d) | 2696.6±11.7 ^b | 2781.0±36.5 ^{ab} | 2808.5±32.1 ^a | 2754.0±28.7 ^{ab} | 2769.2±25.2 ^{ab} | * |
| FI (1-35 d) | 2878.0±56.3 ^b | 3088.7±62.7 ^a | 3089.8±53.8 ^a | 3031.7±31.3 ^a | 3044.9±10.8 ^a | * |
| FI (1-42 d) | 3919.8±65.6 ^b | 4206.9±71.1 ^a | 4180.1±63.6 ^a | 4146.9±41.8 ^a | 4168.9±26.9 ^a | * |
| FCR (1-35 d) | 1.400±0.02 ^a | 1.456±0.01 ^a | 1.461±0.02 ^a | 1.449±0.01 ^a | 1.439±0.01 ^a | N.S |
| FCR (1-42 d) | 1.454±0.03 ^b | 1.513±0.03 ^a | 1.443±0.02 ^b | 1.506±0.02 ^a | 1.505±0.04 ^a | * |
| Mortality(1-35d) & (1-42d) | 9.16±3.69 ^a | 0.83±0.83 ^c | 1.66±1.66 ^b | 7.49±2.84 ^{ab} | 2.49±0.83 ^b | * |
| PI 35 d | 407.6±15.8 ^b | 445.1±9.2 ^a | 439.4±17.8 ^{ab} | 408.5±13.5 ^b | 440.7±3.5 ^a | * |
| PI 42 d | 427.3±11.7 ^b | 465.5±8.6 ^{ab} | 472.9±19.9 ^a | 429.5±10.5 ^b | 458.4±4.6 ^{ab} | * |

a, b, c : means within each row had the different subscript were differ significantly (P<0.05).

Table 3: Effect of thermal manipulation and early age heat conditioning on carcass traits of mixed broilers at 35 and 42 days

| Carcass traits | Age | Treatment* | | | | | L.S. |
|--------------------------------------|-----|---------------------------|--------------------------|---------------------------|---------------------------|--------------------------|------|
| | | T1 | T2 | T3 | T4 | Control | |
| Live body weight (gm) | 35 | 2136.5±65.6 ^{ab} | 2244.0±73.3 ^a | 2185.3±51.7 ^{ab} | 2141.2±59.6 ^{ab} | 2105.6±56.1 ^b | * |
| | 42 | 2721.8±89.7 ^a | 2679.6±93.0 ^a | 2730.9±79.2 ^a | 2792.1±86.7 ^a | 2784.0±97.3 ^a | N.S |
| Dressing% with giblets | 35 | 76.7±0.3 ^a | 77.5±0.3 ^a | 77.6±0.4 ^a | 78.1±0.8 ^a | 78.7±2.4 ^a | N.S |
| | 42 | 80.1±0.4 ^a | 79.0±0.4 ^a | 78.8±0.2 ^a | 79.9±1.2 ^a | 78.6±0.4 ^a | N.S |
| Dressing% without giblets | 35 | 71.6±0.3 ^a | 72.0±0.3 ^a | 72.0±0.4 ^a | 72.4±0.9 ^a | 72.9±2.3 ^a | N.S |
| | 42 | 74.7±0.4 ^a | 73.6±0.4 ^b | 73.4±0.1 ^b | 73.5±0.4 ^b | 73.2±0.4 ^b | * |
| Giblets % (liver, heart and gizzard) | 35 | 7.1±0.2 ^a | 7.6±0.2 ^a | 7.8±0.2 ^a | 8.0±0.9 ^a | 8.0±0.4 ^a | N.S |
| | 42 | 7.2±0.4 ^a | 7.3±0.1 ^a | 7.4±0.1 ^a | 8.6±1.4 ^a | 7.4±0.4 ^a | N.S |
| Breast % | 35 | 36.0±0.3 ^a | 35.4±0.4 ^a | 34.0±0.4 ^b | 34.1±0.4 ^b | 34.1±0.6 ^b | * |
| | 42 | 34.3±0.5 ^a | 35.0±0.5 ^a | 35.2±0.4 ^a | 35.4±0.5 ^a | 35.6±0.5 ^a | N.S |
| Thighs % | 35 | 28.3±0.3 ^{ab} | 28.6±0.3 ^a | 28.2±0.2 ^{ab} | 27.4±0.3 ^{bc} | 27.1±0.4 ^c | * |
| | 42 | 28.1±0.4 ^b | 29.1±0.3 ^{ab} | 29.4±0.4 ^a | 29±0.3 ^{ab} | 28.8±0.4 ^{ab} | * |
| Abdominal fat% | 35 | 1.5±0.1 ^a | 1.5±0.1 ^a | 1.6±0.1 ^a | 1.7±0.1 ^a | 1.5±0.1 ^a | N.S |
| | 42 | 1.7±0.1 ^b | 1.8±0.1 ^{ab} | 2.0±0.1 ^{ab} | 1.9±0.1 ^{ab} | 2.1±0.1 ^a | * |
| Main parts % | 35 | 64.3±0.4 ^a | 64.0±0.5 ^a | 62.2±0.5 ^b | 61.5±0.4 ^b | 61.2±0.6 ^b | * |
| | 42 | 62.4±0.4 ^b | 64.1±0.4 ^a | 64.6±0.4 ^a | 64.4±0.3 ^a | 64.4±0.5 ^a | * |

| | | | | | | | |
|--------------------------|-----------|-----------|-----------|-----------|-----------|-----------|---|
| Secondary parts % | 35 | 35.7±0.4b | 36.0±0.5b | 37.8±0.5b | 38.5±0.4a | 38.8±0.6a | * |
| | 42 | 37.6±0.4a | 35.9±0.4b | 35.4±0.4b | 35.6±0.3b | 35.6±0.5b | * |

^{a, b, c} : means within each row had the different subscript were differ significantly (P≤0.05).

* Main parts= (breast & thighs), Secondary parts= (back, wing & neck).

Table 4: Effect of thermal manipulation and early age heat conditioning on body temperature (°C).

| Age (d) | Treatment* | | | | | L.S. |
|-----------------|--------------------------|--------------------------|-------------------------|--------------------------|--------------------------|------------|
| | T1 | T2 | T3 | T4 | Control | |
| At hatch | 39.31±0.11 ^a | 39.46±0.10 ^a | 39.53±0.09 ^a | 39.31±0.09 ^a | 39.31±0.11 ^a | N.S |
| 5 | 40.95±0.06 ^a | 40.99±0.06 ^a | 41.03±0.07 ^a | 40.90±0.06 ^a | 40.90±0.05 ^a | N.S |
| 7 | 40.28±0.12 ^a | 40.09±0.13 ^{ab} | 40.27±0.11 ^a | 40.18±0.13 ^{ab} | 39.87±0.11 ^b | * |
| 14 | 40.60±0.12 ^a | 40.10±0.12 ^b | 40.59±0.10 ^a | 40.48±0.14 ^a | 40.30±0.11 ^{ab} | * |
| 21 | 40.01±0.22 ^a | 40.03±0.13 ^a | 40.15±0.15 ^a | 39.86±0.29 ^a | 39.78±0.13 ^a | N.S |
| 28 | 40.70±0.16 ^a | 40.76±0.13 ^a | 40.78±0.11 ^a | 40.71±0.13 ^a | 40.93±0.09 ^a | N.S |
| 35 | 40.89±0.18 ^{ab} | 40.70±0.20 ^b | 41.29±0.15 ^a | 40.87±0.13 ^{ab} | 40.88±0.13 ^{ab} | * |
| 42 | 40.96±0.12 ^b | 40.65±0.13 ^b | 40.10±0.13 ^c | 40.76±0.15 ^b | 41.55±0.16 ^a | * |

^{a, b, c} : means within each row had the different subscript were differ significantly (P≤0.05).

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