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Melanin content and its correlation with weight and color of black-meat chickens at different ages

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Abstract

In Asia, black-meat chicken is popular and generally chosen and cooked as functional food because of antioxidant, immunomodulatory, and other anti-aging properties of melanin pigments in various tissues. In the present study, the melanin content was determined in the black-meat chickens in Thailand which consisted of KU-Phuphan (KU), Mongolia (MG) and Phetchabun (PB) breeds. In addition, the correlation between melanin content and weight, color of the breast, drumstick, wing and skin of black-meat chickens at different ages was estimated. The chickens used in the study had hatched at similar times and were raised under comparable management and care conditions. When the chickens were aged 30 d, 60 d, 90 d and 120 d, five chickens from each breed group were randomly collected and slaughtered and their weights and lightness (L*), redness (a*) and yellowness (b*) of the meat were measured. Melanin was extracted from breast, drumstick, wing and skin samples, and measured using an absorption spectroscopy technique. Data analysis was done using a linear model that considered breed, age and interaction between breed and age as fixed factors. Means were computed and then they were compared using t-test. Pearson's product-moment correlation coefficients (r) between the considered traits were estimated. The average melanin content of all chickens was 0.053 ± 0.031 mg/g and the range was from 0.048 ± 0.041 mg/g (wing) to 0.060 ± 0.041 mg/g (breast). MG chickens tended to have higher weights than KU and PB at all ages. The melanin content increased in PB chickens but decreased in KU and MG chickens between ages 30 d and 120 d. The melanin content was correlated ($p < 0.01$) with L* ($r = -0.330$) and b* ($r = -0.368$), but not with a* and weights. Slaughtering black-meat chickens at age 30 d produced the highest melanin content.

Introduction

Melanin pigments are normally produced through the oxidation of tyrosine in a specialized group of cells known as melanocytes (Riley, 1997). Thus, accumulation of melanin produced by melanocytes creates a dark or black color. In the market, black-meat chicken is generally chosen and cooked as functional food because the melanin isolated from various tissues exhibits antioxidant, immunomodulatory, and other anti-aging properties (Riley, 1997; Tu et al., 2009). In addition to age and weight, the dark or black color of the chicken can be used as a criterion for marketing and customer purchasing decisions.

Suwanasopee et al. (2017) provided details on native black-meat chickens in Thailand. They noted that these chickens are raised by the hilltribe people, especially the Hmong, from Northern and Central Thailand with the largest population of black-meat chickens

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being in Phetchabun province (PB). These black meat chickens originated in China and then migrated to Thailand with Hmong refugees. Traditionally, black-meat chickens are cooked with local herbs to improve the health of sick people, the elderly, women postpartum and children. Many food recipes cooked with black-meat chicken and their eggs have been created and are now popular with both Thai people and tourists so the demand has increased. However, Thai black-meat chickens are small, slow-growing and produce fewer eggs than other chickens. Thus, Thai native black-meat chickens have been crossed with Mongolian (MG) black chickens and the crossbred chickens from subsequent generations have been used to improve economically important traits (Suwanasopee et al., 2017). The KU-Phuphan (KU) breed is a Thai native black-meat-MG crossbred chicken produced and selected under a project of the Faculty of Natural Resources and Agro Industry, Kasetsart University, Thailand. These chickens have a larger body size, grow faster and produce more eggs than Thai native black-meat chickens (Phongkaew and Kumpeerawat, 2017).

Melanin pigments studies have been conducted in many varieties of black-meat or black-boned chickens, such as Taihe black-boned Silky (*Gallus gallus domesticus* Brisson, Chen et al., 2008; Tu et al., 2009), Japanese Silky (*Gallus gallus*, Ortolani-Machado et al., 2009), the Thai Royal projects' black-boned chicken (*Gallus gallus domesticus*, Nganvongpanit et al., 2020). However, none of these studies considered the correlation between meat color and weight. Thus, the objectives of this study were to determine the melanin content and estimate its correlation with the weight and color of breast, drumstick, wing and skin of the PB, MG, and KU black-meat chickens at different ages (30 d, 60 d, 90 d and 120 d). Information from this investigation would benefit not only the production but also the marketing of black-meat chickens.

Material and Methods

Animal and management

In total, 150 black-meat chickens (50 Phetchabun Thai native [PB], 50 Mongolian [MG] and 50 KU-Phupan crossbred [KU]) that hatched on the same day were fattened until age 120 d at the Research and Employment Development Station for Farmers (15°02'50.7"N 100°47'22.7"E), Faculty of Agriculture, Kasetsart University, Lopburi province, Thailand. The black-meat chickens were raised separately by breed groups in three raised, semi-confinement cages with five birds per square meter.

The black-meat chickens were wing-tagged individually and all were raised under comparable management and care conditions throughout the study period. For ages 7–60 d, chickens from all breed groups were provided a diet that contained 21% crude protein, 5% crude fiber and 3,200 kcal/kg of metabolic energy *ad libitum*. For ages 61–120 d, chickens were fed with 19% crude protein, 5% crude fiber, and 2,900 kcal/kg of metabolic energy twice a day (0800 hours and 1700 hours). All chickens were vaccinated against Newcastle disease, infectious bronchitis and fowl pox, following the recommendations of Department of Livestock Development (2014).

Slaughter and tissue sampling

Tissue samples were taken when chicken reached ages 30 d, 60 d, 90 d and 120 d. On the sampling day, five chickens in each breed group were randomly collected and fasted for 12 hr before being weighed and then slaughtered using a conventional neck-cut, bled for 2 min, scalded at 60°C for 2 min and defeathered. The carcasses without offal were weighed and the results was recorded as the hot carcass weight. The dressing percentage of each chicken was calculated. All slaughtering processes were done according to the method described by Maurer (1972).

Hot chicken carcasses were chilled at 4°C for 24 hr followed by all carcasses being individually weighed. The breast, drumstick and wing from both the right-side and left-side of the carcass were dissected. The weights of the breast, drumsticks and wings of individual chickens were measured. The left-side parts were vacuum packed and stored at 4°C for color measurement within 24 hr, whereas the right-side parts were vacuum packed and stored in a freezer at -20°C until further analysis of the melanin content.

Color measurement

The color of the breast, drumstick, wing and skin of KU, MG and PB black-meat chickens at ages 30 d, 60 d, 90 d and 120 d were measured using a colorimeter (Spectrophotometer, CM-3500d; Minolta; Japan). Color differences on the surface of the samples were characterized using the CIE system (Nozaki and Makita, 1998) for the lightness (L*: + = lighter, – = darker), redness (a*: + = redder, – = greener), and yellowness (b*: + = yellower, – = bluer). The values of L*, a*, and b* were obtained from each sample using the average value of 10 repeated measurements taken from different locations on the surface of the samples.

Determination of melanin content

Melanin in the muscle and the skin samples were isolated following the method described by Lin and Chen (2005) with slight modification. Approximately 3 g each of the muscle and the skin samples were randomly collected from each sample for melanin extraction. The extracted melanin samples were kept at -80°C until the melanin content was determined using an absorption spectroscopy technique based on the absorbance at 490 nm and compared with the linear standard curve of synthetic melanin (Sigma No. M8631; USA). For the preparation of the standard curve, a suspension of the synthetic melanin was prepared by dissolving 1 g of synthetic melanin in 100 mL of 0.1 M NaOH, a concentration of 100 ppm. The melanin suspension was aliquoted to achieve a calibration curve in the range 0–30 ppm. The solution was determined by measuring the absorbance at 490 nm (Haywood et al., 2008) using an ultraviolet-visible spectrum spectrophotometer (Biodrop DUO; UK). The extracted melanin samples were quantitated using the equation of the linear regression of the calibration curve plotted as absorbance at 490 nm versus the concentration of synthetic melanin (in parts per million, ppm). Then, the value of the melanin content was converted to micrograms per gram.

Statistical analysis

Data analysis was done using a linear model that considered breed (KU, MG and PB), age (30 d, 60 d, 90 d and 120 d) and interaction between breed and age as fixed factors. Mean and standard deviation values were computed for the melanin contents, live weight, hot carcass weight, breasts weight, drumsticks weight, wings weight, L*, a*, and b* of black-meat chickens. Then they were compared between subgroups of the considered factors using t-test at $\alpha = 0.05$. Pearson's product-moment correlation coefficients were estimated for the correlation between the melanin content and the other considered traits. To help understand the correlation between traits, line charts were created using the average phenotypic values of the sampled chickens from the different age and breed groups.

Results and Discussion

The mean and SD of weight of the KU, MG and PB black-meat chickens at different ages are shown in Table 1. At similar ages, MG and KU tended to have a greater live weight and hot carcass weight than PB. Significant difference between them was found at age 30 d and 120 d ($p < 0.05$). Similar trends were found for the breast weight, drumstick weight and wing weight. The different weights among breed groups found in this study could have been due to the genetic background of these black-meat chickens. The PB chickens are an indigenous strain and they are currently reared in mountainous areas in Phetchabun province, Thailand (Suwanasopee et al., 2017). This strain had less genetic improvement for commercial purposes when compared to MG and KU chickens, and their products were limited to the local market, while KU chicken was developed from MG for commercial purposes (Phongkaew and Kumpeerawat, 2017). Thus, MG and KU were genetically more similar than PB.

These results agreed with Jaturasitha et al. (2008), who reported that the live weight, meat color and skin color of the chickens slaughtered at age 16 wk varied among breed groups ($p < 0.05$), while the retail cuts with bones including breast, thigh, drumstick, and tenderloin were not significantly different. Differences in the live weight and breast meat color among Korean local chickens and Silky fowl were also found by Choo et al. (2014). Hot carcass percentage, edible portion (leg, wing, and breast), and color of skin and meat varied among Thai inheritance chicken breeds (Pripwai et al., 2014). Furthermore, redness (a*) and yellowness (b*) in the breast and drumstick of Yanjin black-boned chickens varied across ages ($p < 0.05$; Hu et al., 2016),

Compared to the Thai black-boned chickens raised in northern Thailand, KU and MG at age 90 d had higher live weights and proportions of retail cuts with bones than those Thai black-boned chickens slaughtered at age 16 wk (1,100 g of live weight with dressing percentage of 63.7%; Jaturasitha et al., 2008). On the other hand, PB chickens at age 120 d had a lower average live weight than those at age 14 wk of Thai black-boned chickens (1,419.73 ± 19.19 g) which were studied by Pripwai et al. (2014). However, the averages live weights of KU, MG and PB chickens at age 60 d were higher than those Silky fowl at age 59 d raised in Korea (394 g, Choo et al., 2014). The black-meat chickens at age 90 d in the current study had higher live weights than those Vietnamese AC black-boned chickens slaughtered at age 12 wk (495 g, Phuong, 2002). The breast weights of the retail cut with bones of KU, MG and PB chickens increased when age of the chickens increased from 30 d to 120 d. These results agreed with the study of Wattanachant et al. (2007) in Thai indigenous chicken reared under intensive care, where the chickens had a higher breast muscle percentage when older than 14 wk.

Changes in the hot carcass weight, breast weight, drumstick weight and wing weight of the KU, MG and PB black-meat chickens at different ages were found. The weights for hot carcass, breast, drumstick, and wing in MG were greater than ($p < 0.05$) for KU and PB at age 60 and 120 d. At ages 30 d and 60 d, the hot carcass, breast, drumstick, and wing weights of KU and PB were almost similar. At age 90 d, the weights of KU increased to be close those for MG. Then, the weights for KU were in between MG and PB, but closer to MG than PB. The rate of weight change for MG seemed to continue after age 120 d, but that for KU and PB seemed to drop after age 90 d.

Table 1 Mean and standard deviation of live weight, hot carcass weight, breast weight, drumstick weight and wing weight of KU-Phupan (KU), Mongolia (MG) and Phetchabun (PB) black-meat chickens at different ages

Breed	Age	Live weight (g)	Hot carcass weight (g)	Breast weight (g)	Drumstick weight (g)	Wing weight (g)
KU	30	204.78 ± 19.98	152.62 ± 26.31	14.40 ± 2.19	16.80 ± 1.79	19.60 ± 2.97
MG	30	269.06 ± 44.72	193.62 ± 28.31	18.80 ± 3.35	22.40 ± 3.85	25.60 ± 3.29
PB	30	157.22 ± 14.15	115.64 ± 10.59	10.00 ± 3.16	13.60 ± 2.19	17.20 ± 1.79
KU	60	469.24 ± 136.96^{b}	335.92 ± 88.25^{b}	34.40 ± 10.43^{b}	42.00 ± 13.56^{b}	43.20 ± 13.46^{b}
MG	60	882.08 ± 214.92^{a}	681.20 ± 214.62^{a}	75.60 ± 27.18^{a}	94.40 ± 31.57^{a}	84.00 ± 21.95^{a}
PB	60	449.06 ± 110.44^{b}	350.12 ± 81.04^{b}	40.00 ± 12.41^{b}	47.60 ± 11.95^{b}	44.40 ± 12.12^{b}
KU	90	1,227.82 ± 334.01	948.24 ± 284.28	124.84 ± 44.40	134.82 ± 45.21	116.74 ± 31.83
MG	90	1,214.60 ± 349.65	946.34 ± 298.31	118.62 ± 36.98	133.46 ± 48.76	116.24 ± 32.00
PB	90	996.28 ± 170.08	788.16 ± 141.19	106.16 ± 20.19	111.30 ± 24.26	102.08 ± 17.85
KU	120	1,721.66 ± 401.17^{b}	1,342.18 ± 332.99^{b}	209.52 ± 38.53^{b}	187.86 ± 69.08^{b}	127.74 ± 44.63^{b}
MG	120	1,955.62 ± 370.55^{a}	1,611.30 ± 327.12^{a}	235.86 ± 36.01^{a}	223.58 ± 53.02^{a}	156.64 ± 32.20^{a}
PB	120	1,106.82 ± 222.71^{c}	890.40 ± 188.06^{c}	137.44 ± 27.38^{c}	120.94 ± 31.95^{c}	90.78 ± 23.12^{c}

Mean±SD in a column within each age group superscripted with different lowercase letters are significant different ($p < 0.05$) while those without superscripts indicate non-significant difference ($p > 0.05$) within age group.

The results revealed that KU and MG black-meat chickens had greater body development over a shorter time to reach the market weight (approximately 800–900 g) than for PB. The hot carcass percentages across ages, were in the range from 72.22 ± 2.84 to 77.75 ± 2.47% in KU, from 72.15 ± 2.43 to 82.34 ± 3.91% in MG and from 73.56 ± 2.07 to 80.30 ± 2.06% in PB. The hot carcass percentage of black-meat chickens in the current study was higher than for Thai black-boned chickens slaughtered at age 14 wk (69.4%, Pripwai et al., 2014) and 16 wk (63.7%, Jaturasitha et al., 2008) and for Silky fowls (64.3%, Choo et al., 2014). The retail breast cut percentages across ages, ranged from 9.54 ± 1.52% to 15.82 ± 1.18% for KU, 9.68 ± 0.42% to 14.80 ± 1.49% for MG and 8.52 ± 2.02% to 15.49 ± 0.83% for PB, respectively. These breast percentages at measured ages were slightly low when compared to those previous studies. The drumstick percentages were also lower than those for Thai black-boned chickens (16.7%, Jaturasitha et al., 2008). Furthermore, there was a decreasing in wing percentage as the age increased. However, the wing percentage of black-meat chickens (12.61 ± 0.75%) in the current study was higher than those for Silky fowls (8.68%, Choo et al., 2014).

The whole carcass of black-meat chicken (800–900 g) is generally preferred for cooking as stewed chicken with herbs. The current result revealed that KU and MG black-meat chickens reached the market weight of 800–900 g faster than PB by at least 30 d. Thus, raising KU and MG black-meat chickens could generate an earlier economic returns for the producers, especially when such healthy food is in high demand and the customers had a positive attitude toward purchasing black-meat chickens (Bubphaphant, 2017).

The average melanin content of all black-meat chickens in the current study was 0.053 ± 0.031 mg/g (Table 2). The ranges of melanin content in each part were from 0.048 ± 0.041 mg/g (wing) to 0.060 ± 0.041 mg/g (breast). The average melanin content from all parts (breast, drumstick, wing and skin) of PB (0.065 ± 0.032 mg/g) was higher ($p < 0.05$) than MG (0.049 ± 0.030 mg/g) and KU (0.044 ± 0.028 mg/g). In addition, it increased with age from 0.052 ± 0.012 mg/g at 30 d to 0.083 ± 0.033 mg/g at 120 d. This trend was the opposite for KU and MG, which had their highest melanin content at age 30 d, dropping at age 60 d and then decreasing further at ages 90 d and 120 d. However, the high SD values of all traits in each breed and age subclass indicated a large variation in the melanin content that might have been due to the large differences in the genetic background of individual samples taken from the same breed group.

Although the melanin content has been investigated in many strains of black-bone chicken worldwide, none of the references reported on the variation in the melanin content in subsequent ages among breed groups. The current study is the first to report age-related change in the melanin content in the three breed groups of Thai native black-meat chicken. For this reason, the age-related change in melanin content of these black-meat chickens was described in term of melanocyte distribution. The presence of melanocyte could be related to the amount of melanin pigments in the observed tissue because melanocyte development (Dorshorst et al., 2010) and melanocyte proliferation (Halaban, 2000) influence the melanin content in chicken.

Nishimura et al. (2006) reported that the melanocyte distribution decreased with growth in skeleton muscles (*pectoralis* and *postacetabular* as part of the *iliotibialis lateralis*) of Japanese white-feather Silky. The melanocyte areas in the muscles when the chicken reached ages 1 wk, 3 wk, 5 wk, 10 wk, 15 wk, 20 wk and 30 wk were significantly different between these two muscles, especially for the first 10 wk. In addition, Hirano (1990) reported different pigmentation in bones between ages 3 wk and 2 yr for Silky fowls, with the number of pigment-containing cells of young fowls lower that in the adult fowls. In young fowls, the melanin pigments could be detected in all layers of the periosteum and bone; however, the pigments were present in small amounts. In contrast, most of cells in the bones of the adult fowls contained melanin pigments.

In particular, the melanin contents of various tissues were investigated in Silky fowls by Muroya et al. (2000) at three different ages (0 d, 30 d and 90 d). The melanin contents of the heart, liver and gizzard were in the range 0.039 ± 0.004 mg/g to 0.124 ± 0.033 mg/g,

Table 2 Mean and standard deviation of melanin content in breast, drumstick, wing, skin and overall average from all parts of KU-Phupan (KU), Mongolia (MG), and Phetchabun (PB) black-meat chickens at different ages

Breed Group	Age (d)	Melanin in breast (mg/g)	Melanin in drumstick (mg/g)	Melanin in wing (mg/g)	Melanin in skin (mg/g)	Average melanin in all parts (mg/g)
KU	30	0.086 ± 0.049	0.090 ± 0.102	0.043 ± 0.028	0.097 ± 0.073	0.079 ± 0.032[a]
MG	30	0.113 ± 0.070	0.075 ± 0.057	0.076 ± 0.072	0.079 ± 0.088	0.086 ± 0.027[a]
PB	30	0.062 ± 0.041	0.056 ± 0.015	0.038 ± 0.016	0.050 ± 0.023	0.052 ± 0.012[b]
KU	60	0.031 ± 0.010	0.013 ± 0.009	0.028 ± 0.016	0.025 ± 0.012	0.024 ± 0.002[b]
MG	60	0.034 ± 0.015	0.032 ± 0.008	0.019 ± 0.011	0.025 ± 0.013	0.028 ± 0.009[b]
PB	60	0.052 ± 0.027	0.067 ± 0.049	0.068 ± 0.079	0.048 ± 0.023	0.059 ± 0.029[a]
KU	90	0.044 ± 0.024	0.027 ± 0.019	0.021 ± 0.013	0.022 ± 0.010	0.029 ± 0.012[b]
MG	90	0.026 ± 0.012	0.024 ± 0.015	0.046 ± 0.037	0.026 ± 0.004	0.030 ± 0.011[b]
PB	90	0.099 ± 0.165	0.040 ± 0.009	0.062 ± 0.039	0.061 ± 0.036	0.066 ± 0.045[a]
KU	120	0.055 ± 0.031	0.044 ± 0.023	0.043 ± 0.022	0.035 ± 0.019	0.044 ± 0.012[b]
MG	120	0.048 ± 0.026	0.041 ± 0.023	0.043 ± 0.018	0.077 ± 0.065	0.052 ± 0.026[b]
PB	120	0.074 ± 0.068	0.085 ± 0.087	0.087 ± 0.046	0.085 ± 0.057	0.083 ± 0.033[a]
Overall		0.060 ± 0.061	0.050 ± 0.048	0.048 ± 0.041	0.053 ± 0.048	0.053 ± 0.031

Mean±SD in a column within each age group superscripted with different lowercase letters are significant different ($p < 0.05$) while those without superscripts indicate non-significant difference ($p > 0.05$) within age group.

whereas the melanin content in the pectoralis muscle or breast was 0.050 ± 0.014 mg/g. The average melanin content in the breast of Silky fowls was close to the overall melanin content of the breast for KU (0.054 ± 0.036 mg/g) and MG (0.055 ± 0.050 mg/g), but lower than for PB (0.072 ± 0.087 mg/g) chickens. In contrast, the melanin contents of skin in the current study were low compared to other studies. Muroya et al. (2000) reported that the melanin content of skin was 0.944 ± 0.289 mg/g. The higher level of melanin pigments in the skin could darken the skin because the skin color was primarily controlled by the melanin content (Yamaguchi et al., 2007).

Color differences of black-meat chicken varied highly significantly ($p < 0.01$) across breed, age and interaction between breed and age for L* and b*, but not for a*. The darkest color (lowest L*: 18.80 ± 3.61 in PB to 31.38 ± 4.67 in KU) was at age 30 d. As the fowls grew older, the color tended to be lighter (Fig. 1). The PB (L* = 29.52 ± 7.63; b* = -0.96 ± 0.74) had darker and bluer colors than KU (L* = 36.61 ± 5.33; b* = -0.01 ± 1.08) and MG (L* = 37.46 ± 7.01; b* = 0.19 ± 1.65) black-meat chickens.

For breast color, the averages lightness across age was 37.30 ± 4.08 in KU, 37.44 ± 8.76 in MG and 26.25 ± 8.95 in PB chickens. The current results revealed that the L* values of the breast were lower than reported in other studies as L* values of the breast were in the range 50.14–50.7 in Thai black-boned chickens (Jaturasitha et al., 2008; Pripwai et al., 2014), while, the L* of breast meat was 47.4 in Silky fowls (Choo et al., 2014). Likewise, the results of yellowness of the breast in the current study were lower than the other referred studies. For skin color, Thai black-boned chickens had higher L* (46.7–71.7) and b* (2.5–6.2) values than in the current study (31.98 ± 6.29 to 39.19 ± 6.80 for L* and -0.22 ± 0.37 to -0.37 ± 0.29 for b*). The PB, MG and KU chickens had darker breast meat and skin than the Thai black-boned chickens in the other referred studies.

No significant correlation between the melanin content and weight of black-meat chicken was found in the current study. The melanin content had a highly significant negative correlation with L* (-0.33, $p < 0.01$) and b* (-0.37, $p < 0.01$), but not a* (0.14, $p = 0.29$). The correlation coefficient estimates implied that black-meat chicken with darker (lower L*) and bluer (lower b*) colors would have more melanin content. These results confirmed the finding of Boulianne and King (1998) that color characteristics (L*, a*, and b*) were significantly different in dark-colored meat compared to normal-colored meat. The dark-colored chicken meat had higher total pigment, myoglobin, iron and redness, but lower lightness and yellowness.

There was a positive correlation of the melanin content across ages between MG and KU (r = 0.99, $p < 0.01$), while negative correlations of the melanin content across ages were found between MG and PB (r = -0.55, $p < 0.01$) and KU and PB (r = -0.54, $p < 0.01$). Similar correlations between MG, KU and PB were found for L* and b*. The variation in the genetic background among these breed groups could have been a major reason for these differences. As described earlier, KU was developed from MG (Phongkaew and Kumpeerawat, 2017), while PB originated from China and was separately raised in a restricted area (Suwanasopee et al., 2017). Genetic difference

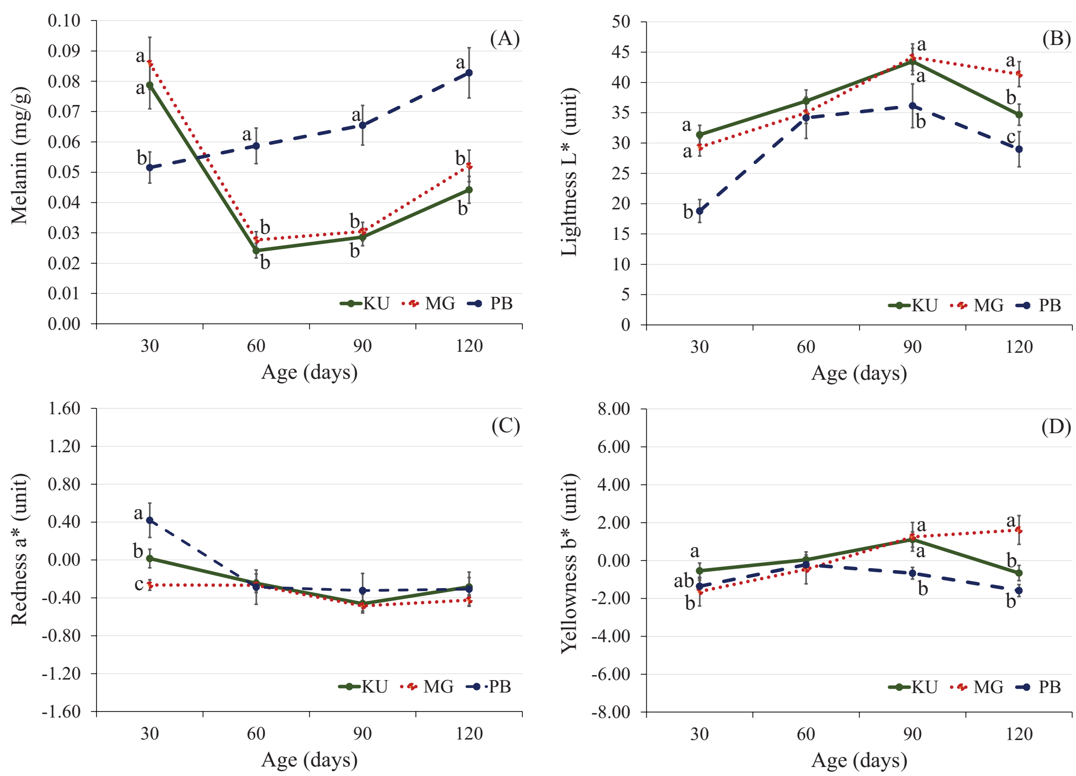

Fig. 1 KU-Phupan (KU), Mongolia (MG), and Phetchabun (PB) black-meat chickens at different ages: (A) melanin content; (B), lightness; (C) redness; (D) yellowness. Means with different alphabets (a, b, or c) indicated significant difference ($p < 0.05$).

is a key factor affecting poultry meat color and pigment deposition (Mugler and Cunningham, 1972). Moreover, the variation in the genetic background within a breed can also influence muscle color; for example, the expression of *RAB29*, a novel protein, was associated with variation in Muchuan black-boned chicken breast muscle color phenotypes (Yu et al., 2018). Thus, it could be possible to improve meat color in black-meat chicken by using marker-based selection.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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