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Combined Effect of Feed and Housing System Affects Free Amino Acid Content of Egg Yolk and Albumen in Brown Layer Chickens

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In recent years, the market share for cage-free eggs has gradually increased. Because commercially available cage-free eggs are often produced not only by several housing systems but also with different feed crude protein (CP) levels, there are combined effects of feed and housing systems between cage-free and cage eggs. Therefore, using field data, this study aimed to determine the combined effects of feed and housing systems on egg traits and yolk and albumen amino acids in table eggs. Brown layers (n = 40) at the middle laying stage under two feed and housing systems (cage, CP 15.5% diet; barn, CP 17.0% diet) were used. One-way analysis of variance and Pearson's correlation analysis were used to evaluate 10 egg traits, 19 yolk amino acid traits, and 20 albumen amino acid traits. We observed significant effects of feed and housing on two egg traits (yolk weight and eggshell color redness), 16 yolk amino acids (Asp, Glu, Asn, Ser, Gln, His, Arg, Thr, Ala, Tyr, Met, Cys, Ile, Leu, Phe, and Lys), and 14 albumen amino acids (Asp, Asn, Ser, Gln, Gly, His, Arg, Thr, Ala, Val, Met, Cys, Ile, and Leu). This study revealed that eggs from the barn system (CP 17.0%) contained higher levels of free amino acids in 15 yolk and nine albumen amino acid traits. Phenotypic correlations among the 49 egg traits indicated similar correlation patterns in both systems, which implies that the balance of free amino acid content in yolk and albumen is similar in each system. Although some potential confounding factors may be present for comparing egg content between cage (CP 15.5%) and barn (CP 17.0%) systems, this study suggests that commercially available cage-free eggs may be different from cage eggs not only in external egg traits but also yolk and albumen amino acid traits.

Key words: albumen, chicken, feed and housing system, free amino acid, table egg, yolk

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Introduction

In recent years, animal welfare has progressed rapidly from a concept to laws and guidelines, especially in the poultry industry in developed countries (Shimmura et al., 2018). Therefore, the market share for cage-free eggs has gradually increased in many developed countries. Enriched cage and non-cage systems, such as barns, aviaries, and free-range systems, are employed in Europe since the banning of battery cages in 2012 (EU Directive 1999/74/EC). In the United States, battery cages have been banned in six states (Kikuchi et al., 2018). New trends in housing systems have become increasingly common (van Asselt et al., 2015). In contrast, there are quite different situations in Japan. Although Japanese egg production is the fourth highest in the world, more than 90% of egg production is based on battery cage systems (Kikuchi et al., 2018). Under the situation that cage eggs constitute the majority in the Japanese market, some consumers prefer non-cage eggs. Consumers of non-cage eggs tend to prioritize not only animal welfare, but also health foods.

Eggs are one of nature's most valuable food products (Anton et al., 2006; Sparks, 2006) and are one of the perfect foods in the world (Nimalaratne et al., 2011). Eggs contain proteins, lipids, vitamins, minerals, and some components that have anti-

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bacterial, antiviral, immunomodulatory, and anti-cancer activities (Mine and Kovacs-Nolan, 2004; Kovacs-Nolan et al., 2005). Free amino acids are involved in food taste and biological regulation (Kato et al., 1989; Nimalaratne et al., 2011). However, the mechanism underlying the egg content of free amino acids has not been elucidated. Some studies have compared egg production performance between different laying systems (Ahammed et al., 2014; Yilmaz Dikmen et al., 2016). The various housing systems for layers affect not only their health and freedom to perform natural behavior but also the quality of their eggs (Abrahamsson and Tauson, 1995; Tauson, 2005). However, there is a lack of evidence regarding the differences in free amino acids of yolk and albumen among different housing systems.

In the layer industry, the idea of “phase feeding” is adopted. Phase feeding is a cost-effective method to meet the nutritional requirements of hens during the laying period (Hy-Line Brown Commercial Management Guide). Hens tend to increase feed intake and produce non-standard eggs at the late laying stages because eggs increase in size with advancing hen age. To solve this problem, the crude protein (CP) percentage of feed often decreases gradually in modern egg layer industries. Therefore, the strategy can reduce feeding costs and decrease the number of non-standard eggs. Phase feeding is often used in battery cage systems in Japanese layer companies. However, phase feeding has not yet been adopted under a barn system for layers in Japan. Therefore, the CP percentage of the feed is often constant throughout the laying stage in the barn system. According to the management guide (Hy-Line Brown Commercial Management Guide), Brown layers at the peaking period (18–36 weeks) are fed CP 17% feed. From 36 to 100 weeks (layer periods 2–5), the feed CP% is gradually decreased to 15%. Therefore, around 70% of the caged eggs in the egg industry will exhibit effects of feed CP and housing systems, in comparison with barn eggs with constant CP%. Thus, commercially available cage-free and cage eggs potentially include the combined effects of feed and housing systems.

To expand the production scale of the barn system in Japan, it is important to inform consumers of the differences in egg quality between the conventional and non-cage systems. If non-cage eggs have a higher amount of free amino acids than cage eggs, the non-cage production system will provide additional value to consumers, which may lead to an increased share of cage-free eggs in Japan. Therefore, the aim of this study was to determine, using field data, the combined effects of different feed and housing systems on egg yolk and albumen amino acids.

Materials and Methods

Animals and egg sampling

Brown layers (Boris Brown; n = 40 in total) reared in two different housing systems (battery cages and barns) by Hokuryo Co., Ltd. (Hokkaido, Japan) were used. We collected 20 eggs laid by 20 different hens from both systems (n = 20 in each group). The rearing densities were 471 and 1388 cm^2/hen in the battery cage and barn systems, respectively. The photoperiod in both housing systems was a cycle of 16 h light and 8 h dark. The barn, with a mesh floor, consisted of a nest box and perches. There were no outdoor fields in the barns. This study (authorization number 20-140) was approved by the Experimental Animal Committee of the Obihiro University of Agriculture and Veterinary Medicine.

Feeds

The same basic feed materials (Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan) were used for both the cage and barn groups. Along with the phase feeding for layers in the cage system, hens in cages were fed CP 16.5% feed from 17 to 49 weeks of age, and then switched to CP 15.5% from 50 weeks of age. Egg sampling points were set at 63 and 55 weeks of age (middle laying stage) for the cage and barn systems, respectively. Therefore, the feed for the cage system was CP 15.5% and metabolizable energy (ME) 11.59 MJ, whereas that for the barn system was CP 17.0% and ME 11.92 MJ at the egg sampling point. Food and water were provided *ad libitum*. Egg traits and yolk and albumen amino acids were measured at the same time and in the same manner between the cage (CP 15.5%) and barn (CP 17.0%) systems.

Egg traits

Ten egg traits, including egg weight (EW); length of the long axis of the egg (LLE); length of the short axis of the egg (LSE); eggshell weight (SW); yolk weight (YW); albumen weight (AW); eggshell thickness (ST); and eggshell color lightness (SCL), redness (SCR), and yellowness (SCY) were measured. Weight was measured using an electronic balance (EK-6000H; A&D Company Ltd., Tokyo, Japan), whereas size was measured using a digital caliper (P01 110–120; ASONE, Osaka, Japan). Eggshell color was measured using a chromameter (CR-10 Plus Color Reader; Konica Minolta Japan, Inc., Tokyo, Japan), and thickness was measured using a Peacock dial pipe gauge P-1 (Ozaki MFG Co., Ltd., Tokyo, Japan). After measuring yolk weight, the yolk was diluted 2-fold with distilled water (DW). The yolk solution was mixed thoroughly with chopsticks and kept in a tube at −30°C. After measuring the albumen weight, the albumen sample was kept in a tube at –30°C.

Free amino acid analysis of egg yolk

Five milliliters of yolk solution were mixed with 5 mL of 16% trichloroacetic acid solution (FUJIFILM Wako Chemicals, Osaka, Japan) and vortexed thoroughly. The sample was centrifuged at 1400 × *g* for 15 min using a tabletop centrifuge (model 2410; KUBOTA Corporation Co., Ltd., Tokyo, Japan) and centrifuges (RX series and RX II series; HITACHI Ltd., Tokyo, Japan). After centrifugation, the supernatant was collected with a 1 mL syringe (NIPRO Corporation, Osaka, Japan) and then filtered using a disposable cellulose acetate membrane filter unit with a pore size of 0.45 µm (DISMIC-25CS; Advantec Toyo Kaisha, Ltd., Tokyo, Japan) into a microtube. The filtered sample (40 µL) was heated at 40°C for 90 min in vacuum to dry (VOS-201SD, Eyela, Tokyo, Japan), and then 20 µL of mixing solution (ethanol:DW:triethylamine = 2:2:1) was added. The microtube was vortexed for 20 min using a micromixer E-36 (TAITEC Corporation, Saitama, Japan). The sample was heated again at 40°C for 40 min in vacuum to dry. After adding 20 µL

Table 1. **Egg traits of hens raised under two feed and housing systems**

Traits	Cage, CP 15.5% (n = 20)	Barn, CP 17.0% (n = 20)	One-way ANOVA df_{bet}	df_{res}	F value	P value
Egg weight (g)	61.3 ± 4.8	59.7 ± 3.6	1	38	2.741	0.1060
Length of the long axis of the egg (mm)	57.4 ± 2.0	56.8 ± 1.6	1	38	1.289	0.2630
Length of the short axis of the egg (mm)	43.8 ± 1.3	43.3 ± 1.1	1	38	1.375	0.2480
Yolk weight (g)	16.5 ± 1.0	15.5 ± 1.1	1	38	8.001	0.0074**
Eggshell weight (g)	7.7 ± 0.8	7.4 ± 0.6	1	38	2.099	0.1560
Albumen weight (g)	37.1 ± 3.7	36.2 ± 2.9	1	38	0.822	0.3700
Eggshell thickness (mm)	0.52 ± 0.03	0.53 ± 0.03	1	38	1.449	0.2360
Eggshell color lightness	54.8 ± 2.9	56.3 ± 2.6	1	38	2.976	0.0927
Eggshell color redness	19.1 ± 1.0	20.2 ± 0.8	1	38	13.510	0.0007***
Eggshell color yellowness	26.8 ± 1.6	26.6 ± 1.4	1	38	0.170	0.6820

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Mean ± SD. df_{bet}: between groups degree of freedom. df_{res}: residual degree of freedom.

of the mixing solution (ethanol:DW:triethylamine:phenylisothio cyanate = 7:1:1:1), the sample was vortexed for 20 min using a micromixer. The sample was spun down for a few seconds, and the sample was re-heated at 40°C for 60 min in vacuum to dry. After preprocessing, the sample tube was placed at –30°C until sample analysis.

Yolk free amino acids were analyzed using HPLC (LC-2010CHT; Shimadzu Co. Ltd., Japan) using two 4.6 μm columns (TSKgel ODS80Ts; 150 mm and 250 mm, Tosoh Corporation, Tokyo, Japan) at a flow rate of 1.0 mL/min. The UV detection value was set to 254 nm and column temperature was set to 40°C. We used a gradient release. A 60 mM acetic acid buffer solution:acetonitrile = 94:6, v/v (pH 5.60) and 60 mM acetic acid buffer solution:acetonitrile = 60:40, v/v (pH 6.95) were used as mobile phases A and B, respectively (Nishimura et al., 2021). Amino acid standards (types H and B), L-asparagine, and L-glutamine (FUJIFILM Wako Chemicals, Japan) were prepared using the same method as that used for sample preprocessing. The standards were analyzed for every 30 samples processed. The absolute concentrations of the amino acids were calculated by comparing the peaks of the samples and standards.

Free amino acid analysis of egg albumen

After thawing, the albumen was diluted 2-fold with DW. The mixture was thoroughly mixed with a hand blender (BRAUN Multiquick 5; De'Longhi Japan Corp., Tokyo, Japan) for 5 s. The albumen solution (250 μL) was mixed with 250 μL of 16% trichloroacetic acid solution and vortexed. The samples were centrifuged at 11000 × *g* for 15 min using a centrifuge. After this step, sample preprocessing and amino acid analysis were performed following the same methods as described for the yolk sample.

Statistical analysis

The data (10 egg traits, 19 yolk free amino acids, and 20 albumen free amino acids) were analyzed using one-way analysis of variance (ANOVA) in RStudio 1.3.1093 (RStudio, Inc., Boston, MA, USA; http://www.rstudio.com/). The feed and housing system (cage with CP 15.5% diet and barn with CP 17.0% diet) was set as the main effect because, using field data, we could not exclude the feed effect from the housing system. Statistical significance was set at $P \leq 0.05$. For all data, Pearson's correlations were calculated using the 'corrplot' package in R ($P < 0.05$) in each feed and housing system.

Additional experiment for validation

To confirm how differences in feed CP affect egg traits, additional experiments were conducted using genetically different intercross populations in the experimental farm at the Obihiro University of Agriculture and Veterinary Medicine. Hens (n = 16) at 40 weeks of age from the intercross population from the Darumachabo and Tosa-jidori breeds (miniature breeds of Japanese indigenous chickens) were kept in individual cages (Ono et al., 2022). The same two kinds of feeds (CP 15.5% and ME 11.59 MJ and CP 17.0% and ME 11.92 MJ; Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan) were fed to two groups of hens (n = 8 in each group) from 40 to 46 weeks of age. Food and water were provided *ad libitum*. Six weeks after the change in feed CP level, eggs were collected from each hen. The egg production rate (%) was calculated as the number of eggs per 42 eggs (over 6 weeks). Egg traits and yolk and albumen amino acids were measured as described above and tested using one-way ANOVA to determine the effect of feed CP level.

Results

Because this study was based on field data, individual hen egg production and feed intake data could not be collected. However, flock averages for each feed and housing system were available. Average hen-day egg production (%) was 89.0% and 85.2% in the cage system with the CP 15.5% diet and barn system with the CP 17.0% diet, respectively. Average feed intakes were 110.2 g and 114.8 g in the cage (CP 15.5%) and barn (CP 17.0%) systems, respectively.

Egg traits

Ten egg traits from the cage (CP 15.5%) and barn (CP 17.0%) systems were analyzed (n = 20 in each group) from eggs laid in the middle laying stage (**Table 1**). The feed and housing sys-

Table 2. **Yolk amino acid levels in eggs from hens raised under two feed and housing systems**

Amino acids (μg/mL)	Cage, CP 15.5% (n = 20)	Barn, CP 17.0% (n = 20)	One-way ANOVA			
			df_{bet}	df_{res}	F value	P value
Y_Asp	15.0 ± 4.2	24.3 ± 7.0	1	38	24.720	<0.001***
Y_Glu	59.2 ± 9.9	77.3 ± 13.9	1	38	21.410	<0.001***
Y_Asn	19.1 ± 2.7	22.5 ± 2.2	1	38	17.370	<0.001***
Y_Ser	34.2 ± 4.9	38.9 ± 3.5	1	38	11.290	0.002**
Y_Gln	35.7 ± 4.7	42.1 ± 3.3	1	38	24.030	<0.001***
Y_Gly	11.7 ± 1.8	12.1 ± 1.3	1	38	0.792	0.379
Y_His	9.6 ± 1.5	10.9 ± 1.3	1	38	8.195	0.007**
Y_Arg	40.4 ± 6.3	51.4 ± 4.7	1	38	36.920	<0.001***
Y_Thr	34.3 ± 4.9	38.8 ± 3.2	1	38	11.750	0.001**
Y_Ala	19.4 ± 2.8	23.9 ± 2.6	1	38	26.800	<0.001***
Y_Pro	18.7 ± 2.7	18.2 ± 1.5	1	38	0.563	0.458
Y_Tyr	35.3 ± 5.1	43.1 ± 3.8	1	38	27.470	<0.001***
Y_Val	31.2 ± 4.3	30.8 ± 2.5	1	38	0.124	0.726
Y_Met	14.5 ± 2.1	16.4 ± 1.5	1	38	9.614	0.004**
Y_Cys	3.6 ± 0.8	5.7 ± 1.3	1	38	38.430	<0.001***
Y_Ile	33.6 ± 5.0	41.7 ± 3.8	1	38	31.000	<0.001***
Y_Leu	54.1 ± 7.5	67.2 ± 6.0	1	38	34.890	<0.001***
Y_Phe	28.8 ± 4.2	6.1 ± 0.5	1	38	535.900	<0.001***
Y_Lys	48.4 ± 7.6	58.5 ± 6.1	1	38	20.220	<0.001***

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Mean ± SD. df_{bet}: between groups degree of freedom. df_{res}: residual degree of freedom.

tems had significant effects on YW and SCR. Compared with the cage system (CP 15.5%), eggs from the barn system (CP 17.0%) showed low yolk weight and high eggshell redness. The remaining traits (EW, LLE, LSE, SW, AW, ST, SCL, and SCY) did not differ significantly between the feed and housing groups ($P > 0.05$).

Yolk amino acid traits

Egg yolk samples contained 19 free amino acids: aspartic acid (Y_Asp), glutamic acid (Y_Glu), asparagine (Y_Asn), serine (Y_Ser), glutamine (Y_Gln), glycine (Y_Gly), histidine (Y_His), arginine (Y_Arg), threonine (Y_Thr), alanine (Y_Ala), proline (Y_Pro), tyrosine (Y_Tyr), valine (Y_Val), methionine (Y_Met), cysteine (Y_Cys), isoleucine (Y_Ile), leucine (Y_Leu), phenylalanine (Y_Phe), and lysine (Y_Lys) (**Table 2**). Significant effects of feed and housing systems were revealed for 16 yolk free amino acid traits: Y_Asp, Y_Glu, Y_Asn, Y_Ser, Y_Gln, Y_His, Y_Arg, Y_Thr, Y_Ala, Y_Tyr, Y_Met, Y_Cys, Y_Ile, Y_Leu, Y_Phe, and Y_Lys. Among the 15 yolk amino acid traits, except for Y_Phe, the levels in yolk from the barn system (CP 17.0%) were significantly higher than those from the cage system (CP 15.5%). The remaining three yolk amino acid traits (Y_Gly, Y_Pro, and Y_Val) were not significantly different between the groups ($P > 0.05$).

Albumen amino acid traits

Albumen samples contained 20 free amino acids: aspartic acid (A_Asp), glutamic acid (A_Glu), asparagine (A_Asn), serine (A_Ser), glutamine (A_Gln), glycine (A_Gly), histidine (A_His), arginine (A_Arg), threonine (A_Thr), alanine (A_Ala), proline (A_Pro), GABA (A_GABA), tyrosine (A_Tyr), valine (A_Val), methionine (A_Met), cysteine (A_Cys), isoleucine (A_Ile), leucine (A_Leu), phenylalanine (A_Phe), and lysine (A_Lys) (**Table 3**). Significant effects of feed and housing system were found for 14 albumen free amino acid traits: A_Asp, A_Asn, A_Ser, A_Gln, A_Gly, A_His, A_Arg, A_Thr, A_Ala, A_Val, A_Met, A_Cys, A_Ile, and A_Leu. Eggs from the barn system (CP 17.0%) contained higher albumen amino acid content than those from the cage system (CP 15.5%) for nine albumen amino acid traits (A_Asp, A_Asn, A_Ser, A_Gln, A_Gly, A_His, A_Arg, A_Thr, and A_Ala), whereas the content was lower in eggs from the barn system than those from the cage system for five traits (A_Val, A_Met, A_Cys, A_Ile, and A_Leu). There was no effect of the feed and housing system on the remaining six traits (A_Glu, A_Pro, A_GABA, A_Tyr, A_Phe, and A_Lys).

Phenotypic correlation analysis

Pair-wise Pearson's correlations among 49 traits in the cage system with a CP 15.5% diet and barn system with a CP 17.0% diet are shown in **Figures 1 and 2**, respectively. Similar tendencies in the phenotypic correlations were observed between the systems. There were positive correlations between the size and weight traits. Strong positive correlations among yolk free amino acids were found, whereas no or weak correlations were observed between yolk cysteine and the other yolk amino acids. Weak positive correlations were observed among albumen free amino acids. However, there were no correlations between yolk

Table 3. **Albumen amino acid levels in eggs from hens raised under two feed and housing systems**

Amino acids	Cage, CP 15.5%	Barn, CP 17.0%	One-way ANOVA			
(μg/mL)	(n = 20)	(n = 20)	df_{bet}	df_{res}	F value	P value
A_Asp	0.23 ± 0.12	0.43 ± 0.07	1	38	39.190	<0.001***
A_Glu	0.93 ± 0.15	0.97 ± 0.15	1	38	0.786	0.381
A_Asn	0.14 ± 0.03	0.18 ± 0.02	1	38	19.110	<0.001***
A_Ser	0.36 ± 0.07	0.45 ± 0.05	1	38	20.320	<0.001***
A_Gln	0.12 ± 0.04	0.15 ± 0.01	1	38	14.550	<0.001***
A_Gly	0.14 ± 0.03	0.16 ± 0.03	1	38	4.746	0.036*
A_His	0.41 ± 0.09	0.55 ± 0.08	1	38	25.680	<0.001***
A_Arg	0.88 ± 0.13	1.05 ± 0.08	1	38	23.620	<0.001***
A_Thr	0.35 ± 0.06	0.41 ± 0.05	1	38	9.554	0.004**
A_Ala	0.25 ± 0.06	0.30 ± 0.04	1	38	8.728	0.005**
A_Pro	0.35 ± 0.10	0.40 ± 0.07	1	38	3.199	0.082
A_GABA	0.15 ± 0.02	0.17 ± 0.04	1	38	1.971	0.168
A_Tyr	1.48 ± 0.50	1.94 ± 0.97	1	38	3.313	0.077
A_Val	0.93 ± 0.12	0.84 ± 0.07	1	38	9.376	0.004**
A_Met	2.88 ± 0.26	2.37 ± 0.21	1	38	43.720	<0.001***
A_Cys	1.17 ± 0.04	1.08 ± 0.03	1	38	51.530	<0.001***
A_Ile	0.65 ± 0.13	0.57 ± 0.10	1	38	5.034	0.031*
A_Leu	2.60 ± 0.44	2.06 ± 0.28	1	38	20.420	<0.001***
A_Phe	2.35 ± 0.35	2.32 ± 0.31	1	38	0.049	0.826
A_Lys	0.26 ± 0.12	0.22 ± 0.02	1	38	1.609	0.212

$*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Mean ± SD. df_{bet}: between groups degree of freedom. df_{res}: residual degree of freedom.

and albumen free amino acids.

Additional experiment for validation

Egg production rate and egg traits in the CP 15.5% and CP 17.0% groups are presented in **Table 4**. There were no significant differences in egg production rate and the 10 egg traits. Egg yolk samples contained 20 free amino acids: Y_Asp, Y_Glu, Y_Asn, Y_Ser, Y_Gln, Y_Gly, Y_His, Y_Arg, Y_Thr, Y_Ala, Y_Pro, GABA (Y_GABA), Y_Tyr, Y_Val, Y_Met, Y_Cys, Y_Ile, Y_Leu, Y_Phe, and Y_Lys (**Table 5**). No significant difference was found in the content of free amino acids in the yolk between the CP 15.5% and CP 17.0% feed groups. As listed in **Table 6**, 20 free amino acids (A_Asp, A_Glu, A_Asn, A_Ser, A_Gln, A_Gly, A_His, A_Arg, A_Thr, A_Ala, A_Pro, A_GABA, A_Tyr, A_Val, A_Met, A_Cys, A_Ile, A_Leu, A_Phe, and A_Lys) were detected. Although there were no significant differences in the levels of 19 albumen amino acids, a significant difference was observed in A_Gln ($P < 0.05$). Albumen from the CP 17.0% feed group had a higher amino acid content than that from the CP 15.5% feed group.

Discussion

In this study, using field data, we aimed to investigate the combined effects of feed and housing systems on 10 egg traits, 19 yolk amino acids, 20 albumen amino acids, and phenotypic correlations. We observed significant feed and housing effects on two egg traits (YW and SCR), 16 yolk amino acids (Y_Asp, Y_Glu, Y_Asn, Y_Ser, Y_Gln, Y_His, Y_Arg, Y_Thr, Y_Ala, Y_Tyr, Y_Met, Y_Cys, Y_Ile, Y_Leu, Y_Phe, and Y_Lys), and 14 albumen amino acids (A_Asp, A_Asn, A_Ser, A_Gln, A_Gly, A_His, A_Arg, A_Thr, A_Ala, A_Val, A_Met, A_Cys, A_Ile, and A_Leu). This study revealed that eggs from the barn system (CP 17.0%) contained higher levels of free amino acids in 15 yolk and nine albumen amino acid traits at the middle laying stage. Phenotypic correlations among the 49 egg traits indicated similar correlation patterns in the systems, which implies that the balance of free amino acid content in yolk and albumen is basically the same in both feed and housing systems. This study demonstrated that commercially available cage-free eggs are different from cage eggs not only in external egg traits, but also yolk and albumen amino acid traits.

In recent years, interest in animal welfare has increased, particularly in developed countries. Many studies have demonstrated how various housing systems (battery cage, enriched-cage, barn, aviary, and free-range) affect egg traits. Using the brown layer, the present study revealed significant effects of feed and housing systems on YW and SCR. Brown eggshell pigmentation is affected by several factors, including aging, nutrients, stress, the environment, and disease (Lu et al., 2021). Although higher redness (not lightness and yellowness) was observed in the barn system than in the cage, Samiullah et al. (2017) reported that brown egg-laying hens reared in cages lay eggs with a darker eggshell color than hens in free-range systems. Lu et al. (2021) summa-

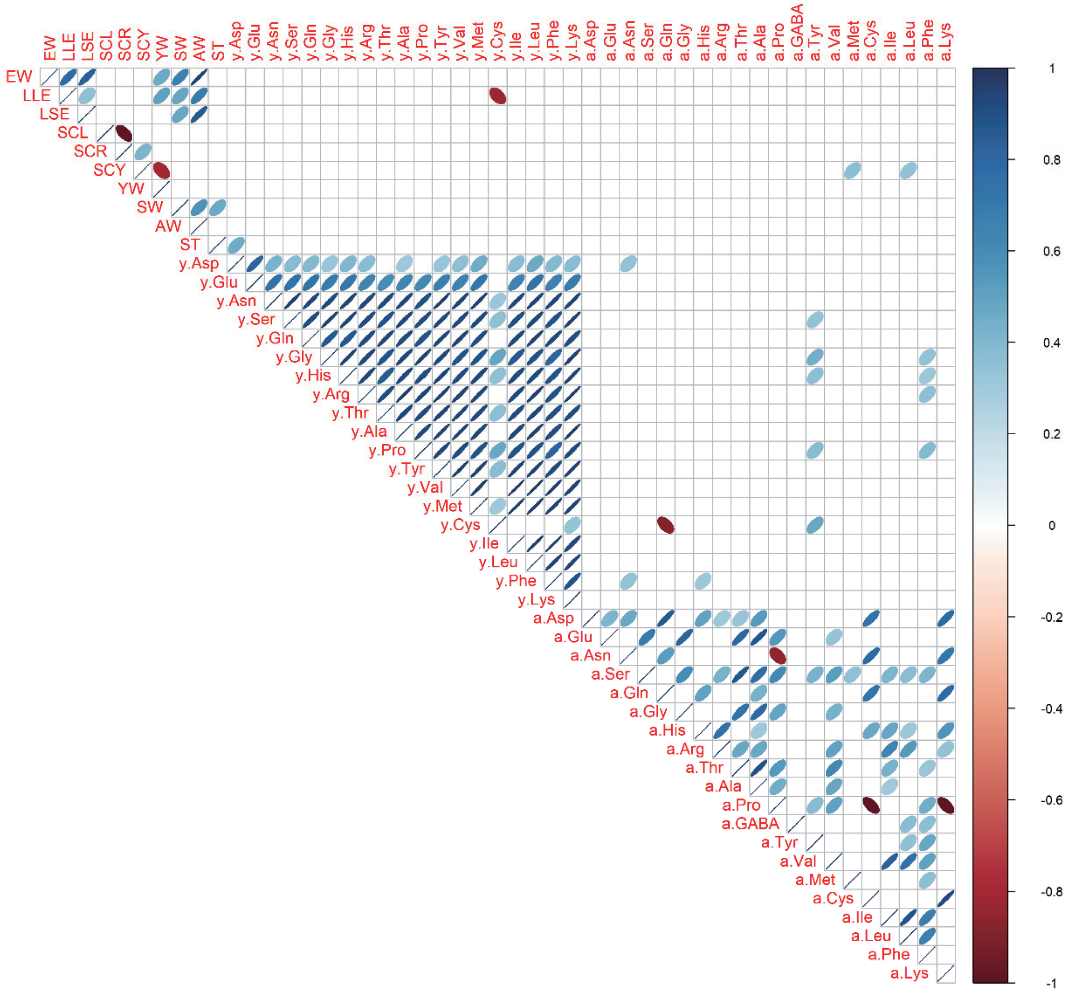

Fig. 1. **Phenotypic correlations of eggs from a cage system with hens fed a CP 15.5% diet**

Ten egg traits, 19 yolk amino acid traits, and 20 albumen amino acid traits of 20 eggs laid by 20 different hens raised under the cage system (CP 15.5%) were evaluated. Pearson's correlations are expressed as ellipses. Blue ellipses indicate positive correlations and red ellipses indicate negative correlations for each pair ($P < 0.05$). Blank cells indicate no correlation.

rized that it is plausible that cage systems may provide hens with more appropriate temperature and humidity, which might be beneficial with respect to pigment synthesis. Further experimental studies are needed to understand the differences in eggshell coloration between the cage and barn systems in terms of stress and environmental conditions. In terms of YW, the yolk from the cage system is heavier than that from the non-cage system using brown and white layers (Basmacioglu and Eegul, 2005), which supports the results of this study. Samiullah et al. (2017) also reported that hens from cage systems produced heavier eggs than those from the barn systems in the brown layer. However, Singh et al. (2009) reported the opposite results using brown and white layers. No significant effect of housing systems on yolk weight has been reported for the brown layer (Pistekova et al., 2006). As these results using modern layers are variable depending on the study, further analyses will be necessary to determine the factors that affect yolk weight. In addition, Nii et al. (2021) reported that yolk size is decreased by slight disruptions in the intestinal environment, which indicates that the microbiota affects yolk size. As the gut microbiota is modified by physical exercise (Mika et al., 2015; Donati Zeppa et al., 2020), the intestinal bacterial flora between the cage and barn may be different. Investigation of the relationship between microbiota and yolk size is required in the future.

The present study suggests that yolk and albumen free amino acids are affected by feed and housing systems in commercially available eggs. In the present study, eggs from the barn system (CP 17.0%) contained higher levels of 15 yolk and nine albumen amino acids than those from the cage system. As consumers in Japan can select both types of eggs, this evidence may be useful

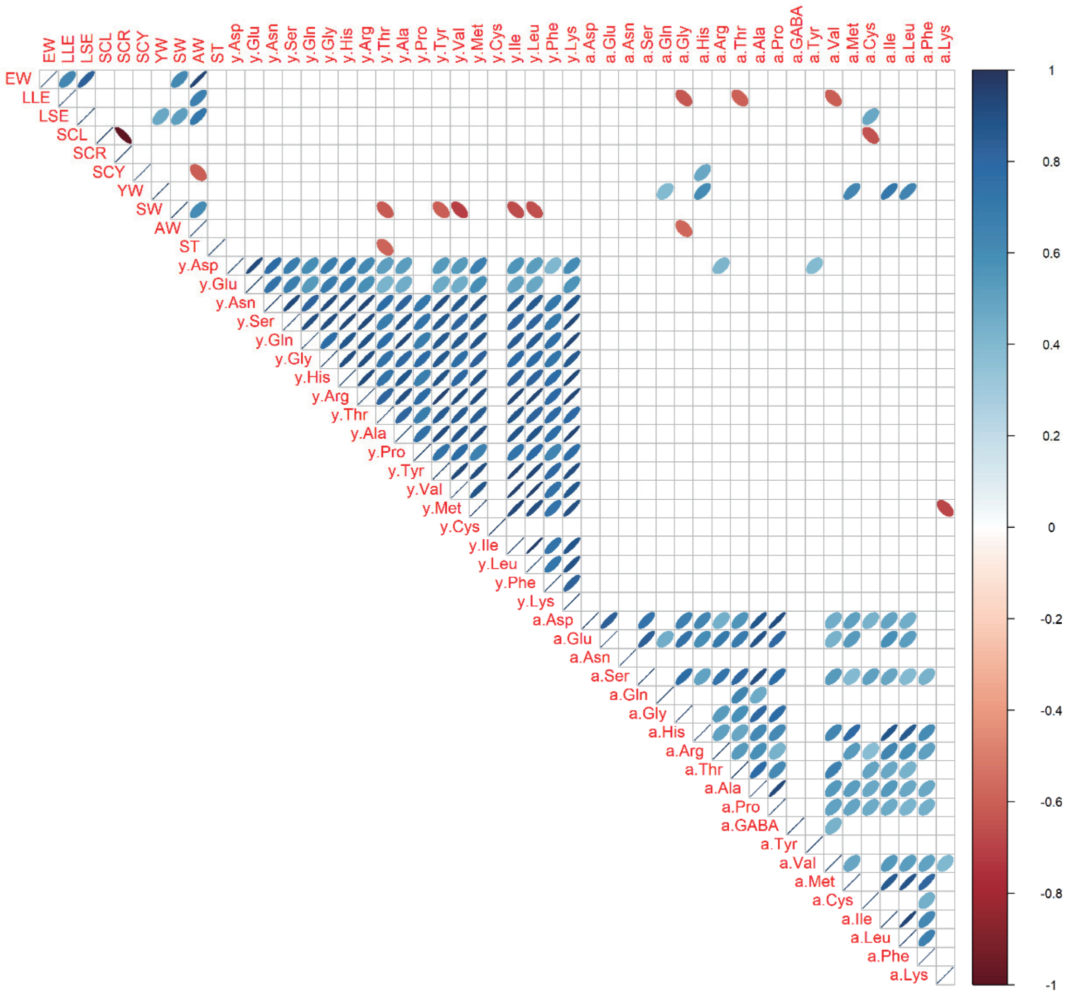

Fig. 2. **Phenotypic correlations of eggs from a barn system with hens fed a CP 17.0% diet**
Ten egg traits, 19 yolk amino acid traits, and 20 albumen amino acid traits from 20 eggs laid by 20 different hens raised under the barn system (CP 17.0%) were evaluated. Pearson's correlations are expressed as ellipses. Blue ellipses indicate positive correlations and red ellipses indicate negative correlations for each pair ($P < 0.05$). Blank cells indicate no correlations.

for increasing the share of non-cage eggs in the Japanese market. To date, few reports have focused on the effects of rearing systems on yolk and albumen components. Karsten et al. (2010) reported that pastured hens supplemented with commercial feed produce eggs with significantly more vitamin E and total omega-3 fatty acids than those in eggs from cage hens. Regarding folate (various forms of B-vitamins) content of eggs, no difference between cage and barn systems has been reported (Czarnowska-Kujawska et al., 2021). Further research targeting the effects of housing on yolk and albumen components will provide beneficial information on the nutritional (primary function), sensory (secondary function), and physiological (tertiary function) functions of functional foods (Shimizu, 2003) for potential consumers.

Free amino acids in eggs can be altered by breed and feed (Goto et al., 2019; 2021a; 2021b; 2022; Attia et al., 2020; Mori et al., 2020; Nishimura et al., 2021). Free amino acids are known to be related to the taste of foodstuffs (Kirimura et al., 1969; Schiffman and Dackis, 1975). In the present study, yolk in eggs from the barn system (CP 17.0%) had significantly higher levels of 15 amino acids than those from the cage system. Of these, Asp, Glu, Asn, Ser, Gln, Arg, Thr, Ala, Met, Cys, and Lys are related to umami, sweet, sour, bitter, and salty taste. Therefore, the yolk from the barn eggs may have a stronger taste than the cage eggs (CP 15.5%). Albumen in eggs from the barn system (CP 17.0%) also had significantly higher levels of nine amino acids than those from the cage system. Because these nine amino acids (Asp, Asn, Ser, Gln, Gly, His, Arg, Thr, and Ala) are related to sweet, sour, and bitter taste, albumen from the barn eggs may have a stronger taste than the cage eggs similar to the yolk. In contrast, albumen in eggs from the cage system (CP 15.5%) had significantly higher

Table 4. **Egg traits of hens fed feed containing CP 15.5% and CP 17.0%**

Traits	CP 15.5% (n = 8)	CP 17.0% (n = 8)	One-way ANOVA df_{bet}	df_{res}	F value	P value
Egg production rate (%)	72.6 ± 7.1	64.6 ± 12.4	1	14	2.208	0.159
Egg weight (g)	26.9 ± 2.9	27.4 ± 2.8	1	14	0.076	0.787
Length of long axis of the egg (mm)	43.3 ± 2.3	43.3 ± 2.4	1	14	0.000	1.000
Length of short axis of the egg (mm)	34.0 ± 1.0	34.0 ± 1.3	1	14	0.000	1.000
Yolk weight (g)	9.3 ± 0.8	9.5 ± 0.9	1	14	0.161	0.695
Eggshell weight (g)	3.3 ± 0.4	3.7 ± 0.7	1	14	1.760	0.206
Albumen weight (g)	14.3 ± 2.0	14.1 ± 2.0	1	14	0.020	0.889
Eggshell thickness (mm)	0.3 ± 0.1	0.3 ± 0.1	1	14	2.618	0.128
Eggshell color lightness	80.7 ± 1.5	78.9 ± 2.5	1	14	2.956	0.108
Eggshell color redness	2.5 ± 2.2	3.9 ± 2.0	1	14	1.755	0.207
Eggshell color yellowness	8.0 ± 4.2	10.6 ± 3.3	1	14	1.664	0.218

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Mean ± SD. df_{bet}: between groups degree of freedom. df_{res}: residual degree of freedom.

Table 5. **Yolk amino acid levels in eggs from hens fed feed containing CP 15.5% and CP 17.0%**

Amino acids (μg/mL)	CP 15.5% (n = 8)	CP 17.0% (n = 8)	One-way ANOVA df_{bet}	df_{res}	F value	P value
Y_Asp	30.8 ± 3.7	32.1 ± 5.5	1	14	0.279	0.606
Y_Glu	147.0 ± 10.8	144.5 ± 10.8	1	14	0.181	0.677
Y_Asn	39.5 ± 4.1	40.7 ± 3.3	1	14	0.317	0.582
Y_Ser	60.1 ± 4.9	59.7 ± 3.8	1	14	0.037	0.850
Y_Gln	67.1 ± 4.9	68.5 ± 5.5	1	14	0.241	0.631
Y_Gly	21.2 ± 2.5	22.0 ± 1.9	1	14	0.485	0.498
Y_His	26.7 ± 3.5	28.5 ± 2.9	1	14	1.154	0.301
Y_Arg	78.4 ± 10.4	79.3 ± 6.7	1	14	0.039	0.845
Y_Thr	78.3 ± 12.0	81.7 ± 14.8	1	14	0.217	0.648
Y_Ala	33.2 ± 3.0	32.2 ± 2.4	1	14	0.473	0.503
Y_Pro	36.0 ± 3.3	38.1 ± 2.4	1	14	2.005	0.179
Y_GABA	0.5 ± 0.2	0.6 ± 0.4	1	14	0.184	0.674
Y_Tyr	66.9 ± 7.4	68.0 ± 4.6	1	14	0.100	0.757
Y_Val	58.8 ± 5.3	59.4 ± 4.0	1	14	0.057	0.814
Y_Met	27.2 ± 3.5	28.5 ± 2.8	1	14	0.594	0.454
Y_Cys	2.8 ± 0.4	2.8 ± 0.4	1	14	0.178	0.680
Y_Ile	53.3 ± 7.4	54.0 ± 5.2	1	14	0.036	0.852
Y_Leu	94.6 ± 11.3	96.5 ± 8.6	1	14	0.124	0.730
Y_Phe	54.9 ± 6.1	54.8 ± 5.0	1	14	0.004	0.954
Y_Lys	103.8 ± 13.5	104.9 ± 7.2	1	14	0.035	0.855

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Mean ± SD. df_{bet}: between groups degree of freedom. df_{res}: residual degree of freedom.

levels of five amino acids than those from the barn system (CP 17.0%). Of these, three (Val, Met, and Cys) are related to bitter taste, which indicates that albumen from cage eggs may have a different taste than that of barn eggs. Although the present study did not perform sensory evaluation, the relationship between free amino acids and the taste of sensory components was analyzed using egg yolk and albumen (Goto et al., 2021a). Further analyses including sensory evaluation are required to understand the taste differences between cage and barn eggs. In addition, amino acids have many functions, such as antioxidant, cell signaling, and immune functions (Wu, 2009). Therefore, taste- and function-enhanced designer eggs (Surai and Sparks, 2001) could be created in the future by selecting genetic, nutritional, and rearing factors.

Phenotypic correlation analyses of all 49 egg traits were performed for both the systems. No remarkable differences were

Table 6. **Albumen amino acid levels in eggs from hens fed feed containing CP 15.5% and CP 17.0%**

Amino acids	CP 15.5%	CP 17.0%	One-way ANOVA			
(μg/mL)	(n = 8)	(n = 8)	df_{bet}	df_{res}	F value	P value
A_Asp	0.35 ± 0.09	0.41 ± 0.12	1	14	1.291	0.275
A_Glu	0.91 ± 0.17	0.91 ± 0.23	1	14	0.000	0.991
A_Asn	0.13 ± 0.05	0.13 ± 0.04	1	14	0.003	0.959
A_Ser	0.26 ± 0.08	0.29 ± 0.08	1	14	0.353	0.562
A_Gln	0.15 ± 0.02	0.21 ± 0.06	1	14	6.995	0.019*
A_Gly	0.08 ± 0.02	0.09 ± 0.04	1	14	0.330	0.575
A_His	0.48 ± 0.07	0.51 ± 0.04	1	14	0.954	0.345
A_Arg	0.62 ± 0.12	0.67 ± 0.10	1	14	0.637	0.438
A_Thr	0.27 ± 0.06	0.29 ± 0.06	1	14	0.326	0.577
A_Ala	0.18 ± 0.04	0.21 ± 0.07	1	14	0.928	0.352
A_Pro	0.25 ± 0.06	0.29 ± 0.07	1	14	1.673	0.217
A_GABA	0.08 ± 0.05	0.13 ± 0.05	1	14	3.555	0.080
A_Tyr	1.09 ± 0.14	1.20 ± 0.20	1	14	1.315	0.271
A_Val	0.36 ± 0.09	0.39 ± 0.09	1	14	0.513	0.485
A_Met	1.22 ± 0.09	1.28 ± 0.12	1	14	1.419	0.253
A_Cys	0.41 ± 0.01	0.41 ± 0.00	1	14	0.000	1.000
A_Ile	0.33 ± 0.15	0.42 ± 0.11	1	14	1.491	0.242
A_Leu	1.37 ± 0.32	1.47 ± 0.25	1	14	0.467	0.506
A_Phe	3.15 ± 0.52	3.40 ± 0.52	1	14	0.836	0.376
A_Lys	0.25 ± 0.04	0.26 ± 0.05	1	14	0.125	0.729

$^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Mean ± SD. df_{bet}: between groups degree of freedom. df_{res}: residual degree of freedom.

observed between the feed and housing systems. Strong positive correlations were found among egg yolk amino acid traits, whereas weak positive correlations were found among egg albumen amino acid traits. There were no phenotypic correlations of amino acids between the yolk and albumen. These results were consistent with those of a previous study (Goto et al., 2021b), suggesting that free amino acids accumulate differently in the yolk (in ovarian follicles) and albumen (in the oviduct ampulla).

Although the present study suggested that eggs from the barn system (CP 17.0%) contained higher levels of free amino acids in the yolk and albumen than those of the cage system (CP 15.5%), there are some potential confounding factors for comparing egg content between cage and barn systems. First, physical activity and energy consumption differ between them. The number of steps is higher in the non-cage system than in the cage system (Knowles and Broom, 1990). Different housing systems change both the activity level and energy consumption. Second, microbes residing in the intestines of hens are potentially different between caged and barn hens. Microbially synthesized amino acids can be reabsorbed by the host (Vispo and Karasov, 1997) and the total absorption of glucose and vitamins is decreased by microbes in germ-free chickens (Ford and Coates, 1971). Although the mechanism of interaction between microbial colonization and nutrient absorption in birds remains unclear, it may increase the absolute energy extraction for the host (Kohl, 2012). Finally, CP levels differed among them because of phase feeding. As the present study investigated commercially available eggs at the middle laying stage in cage and barn systems, the feed CP levels were different (15.5% and 17.0% in the cage and barn systems, respectively). Under these conditions, the egg amino acid content in the barn system (CP 17.0%) was higher than that in the cage system (CP 15.5%), which suggests that feed CP may affect the free amino acid content in eggs. When comparing eggs fed different feeds (corn and wheat), Nimalaratne et al. (2011) reported that several yolk amino acids were significantly altered. However, yolk amino acids were comparable between the mixed (CP 17.5%) and fermented (CP 20.6%) feeds, whereas albumen in eggs from the mixed feed system had significantly higher levels of 15 amino acids than those in eggs from the fermented feed system, even with a low CP percentage (Goto et al., 2021a). Moreover, the validation experiment of the present study indicated that there were no differences in almost all yolk and albumen amino acid levels between the CP 15.5% and CP 17.0% groups, except for A_Glu, which indicates that the housing system itself has substantial power to change the amino acid levels in the yolk and albumen. Ideally, digestibility, absorption, metabolism, and total physical activity should be normalized to determine the effect of the housing system alone. However, removing it entirely for comparison purposes is difficult when using field data. Therefore, evidence of the main factors with minor confounding factors should be accumulated to further understand the factors that influence free amino acids in yolk and albumen.

Consumers tend to require high quality and taste in cage-free eggs as well as animal welfare (Pires et al., 2021). Given that many poultry farms adopt phase feeding following the management guide (Hy-Line Brown Commercial Management Guide), approximately 70% of the caged eggs in the egg industry will exhibit the effects of feed CP and housing system, compared to barn eggs with constant CP%. The present results suggest that commercially available cage-free eggs may add value to animal welfare as well as yolk and albumen components. However, the present study only analyzed eggs in the middle laying stage. Comparing the eggs at several time points during the entire laying period is necessary. Because there is a lack of evidence of age-dependent changes in the free amino acid content of yolk and albumen, further investigations should be conducted using both cage and barn systems.

In conclusion, we found significant effects of feed and housing on yolk weight, eggshell color redness, and many free amino acid traits in the yolk and albumen. Further experimental studies should be conducted to characterize cage-free eggs.

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Author Contributions

Nonoka Kawamura, Masahiro Takaya, and Tatsuhiko Goto conducted the experiments; Reo Yokoyama prepared the samples; Nonoka Kawamura, Masahiro Takaya, Ryoko Ono, and Tatsuhiko Goto analyzed the data; Tatsuhiko Goto designed the experiments; Nonoka Kawamura and Tatsuhiko Goto wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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