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Author	Yeong Bin Kim ^{1,#} , Deok Yun Kim ^{2,#} , Hyun Woo Kim ³ , Jong Hyuk Kim ^{1,*} [#] These authors contributed equally to this work as the first author [*] Corresponding author
Affiliation	¹ Department of Animal Science, Chungbuk National University, Cheongju, Korea ² Institute of Technology, SEO BU Feed Co., Ltd., Incheon, Korea ³ Department of Animal Science and Technology, Chung-Ang University, Anseong, Korea
ORCID (for more information, please visit https://orcid.org)	Yeong Bin Kim (https://orcid.org/0009-0007-9151-0135) Deok Yun Kim (https://orcid.org/0000-0001-7286-9264) Hyun Woo Kim (https://orcid.org/0000-0002-8000-1219) Jong Hyuk Kim (https://orcid.org/0000-0003-0289-2949)
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4

5 CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading, and reprints)	Fill in information in each box below
First name, middle initial, last name	Jong Hyuk Kim
Email address – this is where your proofs will be sent	Jong Hyuk Kim: jonghyuk@chungbuk.ac.kr
Secondary Email address	Jong Hyuk Kim: mrdgj7@naver.com
Address	Department of Animal Science, Chungbuk National University, Cheongju, Korea
Cell phone number	+82 – 10 – 4935 – 8583
Office phone number	+82 – 43 – 261 – 2546
Fax number	+82 – 43 – 273 – 2240

6

Abstract

The effects of different combinations of supplemental arginine (Arg) and proline (Pro) on productive performance, egg quality, relative organ weight, plasma measurements, and liver characteristics were investigated in aged laying hens. Four hundred and eighty 100-wk-old Hy-Line Brown laying hens were allotted to five dietary treatments with eight replicates of 12 individually caged hens for six weeks. The treatments consisted of a basal diet and four supplemented diets that formed a 2×2 factorial arrangement of 0.2 or 0.4% supplemented Arg and 0.2 or 0.4% supplemented Pro. A significant interaction between dietary Arg and Pro levels was observed for feed intake. Hens fed diets containing 0.2% supplemental Arg with 0.4% supplemental Pro or 0.4% supplemental Arg with 0.2% supplemental Pro had a larger ($p < 0.05$) feed intake than those fed diets containing 0.2% supplemental Arg with 0.2% supplemental Pro. For both supplemental Arg levels, hens fed diets containing 0.2% supplemental Pro had higher ($p < 0.05$) eggshell strength and enhanced yolk color than those fed diets containing 0.4% supplemental Pro. Increasing supplemental Pro from 0.2 to 0.4% was associated with lower ($p < 0.05$) relative spleen weight and liver lightness value, but higher ($p < 0.05$) relative abdominal fat weight. Among the five dietary treatments, plasma alanine aminotransferase activity was lower ($p < 0.05$) in hens fed the two mixed-level combinations or the high-level combination than in those fed the basal diet or the low-level combination. Plasma aspartate aminotransferase activity also was lower ($p < 0.05$) in hens fed the two mixed-level combinations than in those fed the basal diet. Overall, dietary Arg and Pro supplementation did not improve the productive performance of aged laying hens, although specific combinations affected feed intake and plasma measurements. Thus, the supplemental levels of Arg or Pro examined in this study provided no clear productivity advantage in aged laying hens.

Keywords: Aged laying hen, Arginine, Egg quality, Productive performance, Proline

INTRODUCTION

Extending the productive lifespan of commercial laying hens has received increasing attention as a strategy for improving resource-use efficiency and reducing the environmental burden of egg production [1,2]. However, successful extension of the laying period requires maintenance of laying persistence, egg quality, and hen health during the late laying period [1,3,4]. Eggshell quality generally deteriorates during the late phase of production, while the laying persistence and uniformity of productivity and egg quality traits may also decline with advancing age [5,6]. Advanced age is associated with changes in immune and hepatic physiology, which may increase the

susceptibility of hens to metabolic disturbances [7,8]. Therefore, dietary strategies for aged hens should be evaluated in terms of their productive performance, egg quality, and metabolic characteristics.

Arginine (Arg) is an essential amino acid (AA) in birds because the incomplete urea cycle limits endogenous Arg synthesis [9,10]. In addition to its role in protein synthesis, Arg serves as a precursor of biologically active metabolites, including nitric oxide, creatine, ornithine, and polyamines [10]. These metabolites are involved in cellular metabolism, vascular regulation, immune responses, and tissue maintenance [9,10]. Therefore, Arg availability may influence the physiological and metabolic responses of laying hens, beyond dietary protein contribution. However, the effects of supplemental Arg depend on the physiological age of the hens, adequacy of the basal diet, and availability of metabolically related AAs [11,12]. Proline (Pro) is generally classified as a nonessential AA because it can be synthesized endogenously; however, dietary Pro supplementation may influence collagen turnover and intermediary metabolism under specific physiological or nutritional conditions [13]. Pro and hydroxyproline are abundant AAs in collagen, which is a major structural component of connective tissues and eggshell membranes [13]. Thus, dietary Pro supplementation may influence tissue and eggshell membrane characteristics, although the level of response depends on overall AA balance and supplementation level. Pro also has metabolic functions that are independent of its incorporation into proteins. Its interconversion with pyrroline-5-carboxylate and glutamate links Pro metabolism to mitochondrial bioenergetics, substrate utilization, and cellular redox regulation [14,15]. These structural and metabolic roles suggest that dietary Pro supplementation influences egg quality and the physiological responses of aged laying hens.

Arg and Pro metabolism is connected through ornithine and pyrroline-5-carboxylate. Studies in chickens have shown that Arg contributes to Pro synthesis through this pathway, although the contribution appears to be limited under the experimental conditions examined [11,16]. Conversely, Pro catabolism may contribute to shared ornithine and pyrroline-5-carboxylate pools, further supporting the metabolic connection between Arg and Pro [17]. Consequently, changes in dietary supplementation of Arg and Pro may affect the utilization of these metabolically connected AAs; however, the physiological responses to supplementation may depend on the combination and relative levels of Arg and Pro, rather than on the level of either AA separately. Nevertheless, limited information is available on the responses of aged laying hens to graded combinations of supplemental Arg and Pro, particularly when the basal diet provides adequate energy and nutrients. Based on their metabolic connections, we hypothesized that the responses of aged laying hens to Arg and Pro supplementation would vary according to the combination and relative supplementation levels of the two AAs. Therefore, the present study

evaluated the effects of different combinations of supplemental Arg and Pro on productive performance, egg quality, relative organ weights, plasma measurements, and liver characteristics of aged laying hens.

MATERIALS AND METHODS

Diets, animals, and experimental design

Four hundred and eighty 100-wk-old Hy-Line Brown laying hens were allotted to one of five dietary treatments in a completely randomized design. Each treatment had eight replicates, with 12 individually caged (width × length × height = 30 × 37 × 40 cm) hens per replicate. Although hens were housed in individual cages, the 12 hens assigned to each replicate collectively constituted one experimental unit for statistical analysis. Energy and all other nutrients in the basal diet were formulated to meet or exceed the estimated requirements (Table 1) for Hy-Line Brown laying hens [18]. The calculated concentrations of Arg and Pro in the basal diet were 0.75% and 1.03%, respectively. Four additional diets were prepared by supplementing the basal diet with two levels each of Arg (0.2 and 0.4%; ≥ 98.5% purity; CJ Bio) and Pro (0.2 and 0.4%; ≥ 99% purity; Ajinomoto). Corn starch, celite (Celite 545, Samchun Pure Chemical Co., Ltd.), and alanine (≥ 98.5% purity; Ajinomoto) were adjusted as needed among the experimental diets. Alanine was used as a nitrogen-balancing AA because Arg, Pro, and alanine differ in their nitrogen contributions, whereas corn starch and celite were adjusted to maintain a constant total formulation. This formulation resulted in comparable calculated dietary CP concentrations among treatments. Total Arg and Pro concentrations in the five experimental diets are presented in Table 2. Diets and water were provided ad libitum to the laying hens for 6 weeks. The averages of ambient temperature and relative humidity were 20.7 ± 2.29°C and 69.1 ± 6.57%, respectively. A 16-h lighting schedule was applied throughout the experiment.

Productive performance and egg quality

Productive performances, including hen-day egg production, egg weight, egg mass, and broken and shell-less egg production rates, were recorded daily. Feed intake (FI) and feed conversion ratio (FCR) were calculated weekly for each replicate, with FCR calculated as FI divided by egg mass. Productive performance data from the 12 hens within each replicate were summarized for statistical analysis. Egg quality was measured using randomly collected samples of 12 eggs per replicate throughout most of the experiment, and four eggs per day during the last three days of the experiment. Egg quality was determined using the method described by Kim et al. [19]. Briefly,

eggshell strength was measured using an eggshell strength tester (Fujihara Ltd.); eggshell thickness was measured in three regions (top, middle, and bottom) using a dial-pipe gauge (model 7360; Mitutoyo Co., Ltd.); and egg yolk color was measured using a Roche color fan (Hoffmann-La Roche Ltd.; 1 = light pale, 15 = dark orange). An eggshell color fan (Samyangsa; where 1 = very light and pale, and 15 = very dark brown) and a color reader (CR-10, Konica Minolta Optics Inc.) were used to determine the eggshell color score and CIE Lab value, respectively. The Haugh unit (HU) values were calculated from egg weight (W) and albumen height (H) using the following equation: $HU = 100 \log (H - 1.7 W^{0.37} + 7.6)$, as demonstrated by Eisen et al. [20].

Sample collection and chemical analysis

At the conclusion of the experiment, one hen with a body weight (BW) close to the replicate mean BW was selected from among the 12 individually caged hens in each replicate (i.e., eight hens per treatment). Selected hens were euthanized by CO₂ asphyxiation and immediately dissected for sample collection. The relative weights of the liver, spleen, kidney, and abdominal fat were measured and expressed as percentages of BW. Blood samples were collected from each hen via heart puncture into a 10 mL sodium heparin tube (Becton, Dickinson and Company), and then immediately centrifuged at $3,000 \times g$ at 4°C for 20 min to separate the plasma. The concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, and total protein in the plasma were analyzed using a HITACHI automatic analyzer 7020 (Hitachi Ltd.). The liver samples were analyzed to measure the objective liver color score and photographed to determine the subjective liver scores, such as fatty liver score and hemorrhage. Objective CIE Lab values were measured using a color reader (CR-10, Konica Minolta Optics Inc.). In addition, photographs of the liver attached to the body were visually scored to determine fatty liver scores (1 = dark red and 5 = yellowish red) [21] and liver hemorrhage scores (0 = no hemorrhage and 5 = severe hemorrhage) [22]. Liver samples were analyzed for acid-hydrolyzed ether extract (method 996.01) to measure fat concentration and total fat in the liver [23].

Statistical analysis

Statistical analyses were performed using the PROC MIXED procedure in SAS software (SAS Institute Inc.). All five dietary treatments were analyzed by one-way ANOVA, with dietary treatment included as a fixed effect. In addition, data from the four Arg and Pro supplemented treatment were analyzed in a 2×2 factorial arrangement, with supplemental Arg level (0.2 or 0.4%), supplemental Pro level (0.2 or 0.4%), and their interaction included as fixed effects. Each replicate was considered an experimental unit for statistical analysis. Productive

performance data were calculated for each replicate, and egg quality measurements were averaged within each replicate before statistical analysis. For relative organ weights, plasma measurements, and liver characteristics, one hen with a BW close to the replicate mean BW was selected from each replicate. Outlier data were checked using the UNIVARIATE procedure in SAS [24]. Least-squares means were calculated using the LSMEANS procedure. When the treatment effect in the one-way ANOVA was significant, means among the five dietary treatments were separated using the PDIFF option. When a significant Arg × Pro interaction was detected, pairwise comparisons among the four supplemented treatment means were performed using the LSMEANS procedure with the PDIFF option. Statistical significance was set at $p < 0.05$.

RESULTS

Productive performance

There was a significant interaction ($p < 0.05$) between dietary Arg and Pro concentrations for FI in aged laying hens (Table 3). FI was higher in hens fed diets supplemented with 0.2% Arg + 0.4% Pro and 0.4% Arg + 0.2% Pro than those fed diets supplemented with 0.2% Arg + 0.2% Pro. However, no interactions were observed for other productive performances.

Egg quality

No significant interactions were observed between dietary Arg and Pro concentrations and egg quality in aged laying hens (Table 4). Regarding the main effects of dietary Pro concentration, hens fed diets supplemented with 0.4% Pro had lower ($p < 0.05$) eggshell strength and yolk color than hens fed diets supplemented with 0.2% Pro. Among the five dietary treatments, hens fed diets supplemented with 0.2% Arg + 0.2% Pro and 0.4% Arg + 0.2% Pro had higher ($p < 0.05$) eggshell strength than those fed the basal diet or diets supplemented with 0.2% Arg + 0.4% Pro. Additionally, yolk color scores were higher ($p < 0.05$) in hens fed diets supplemented with 0.2% Arg + 0.2% Pro and 0.4% Arg + 0.2% Pro than in those fed diets supplemented with 0.2% Arg + 0.4% Pro or 0.4% Arg + 0.4% Pro.

Relative organ and abdominal fat weight

There were no significant interactions between dietary Arg and Pro concentrations and relative organ and abdominal fat weights in aged laying hens (Table 5). Regarding the main effects of dietary Pro concentration,

hens fed diets supplemented with 0.2% Pro had a higher ($p < 0.05$) relative spleen weight than those fed diets supplemented with 0.4% Pro, whereas hens fed diets supplemented with 0.4% Pro had a higher ($p < 0.05$) relative abdominal fat weight than those fed diets supplemented with 0.2% Pro.

Plasma measurements

There was a significant interaction ($p < 0.05$) between dietary Arg and Pro concentrations and ALT activity in aged laying hens (Table 6). Hens fed diets supplemented with 0.2% Arg + 0.4% Pro or 0.4% Arg + 0.2% Pro had lower ALT activity than those fed diets supplemented with 0.2% Arg + 0.2% Pro. Among the five dietary treatments, hens fed diets supplemented with 0.2% Arg + 0.4% Pro, 0.4% Arg + 0.2% Pro, or 0.4% Arg + 0.4% Pro had lower ($p < 0.05$) ALT activity than those fed the basal diet or diets supplemented with 0.2% Arg + 0.2% Pro. In addition, hens fed diets supplemented with 0.2% Arg + 0.4% Pro and 0.4% Arg + 0.2% Pro had lower ($p < 0.05$) AST activity than those fed the basal diet.

Liver parameters

No significant interactions were observed between dietary Arg and Pro concentrations and liver parameters in aged laying hens (Table 7). Regarding the main effects of dietary Pro concentration, hens fed diets supplemented with 0.4% Pro had lower ($p < 0.05$) liver lightness (L*) values than those fed diets supplemented with 0.2% Pro.

DISCUSSION

In this study, dietary Arg and Pro supplementation did not affect most measures of productive performance, including hen-day egg production, egg weight, broken and shell-less eggs, egg mass, or FCR in aged laying hens. The lack of a productive performance response may be explained by the fact that the basal diet was formulated to meet or exceed the recommended nutrient levels for Hy-Line Brown laying hens, thereby limiting the potential benefits of additional Arg and Pro supplementation when neither AA is nutritionally limiting [12]. A significant interaction between supplement levels was observed for FI, where hens fed diets containing 0.2% supplemental Arg and 0.4% supplemental Pro, or a diet containing 0.4% supplemental Arg and 0.2% supplemental Pro, had a larger FI than those fed diets containing 0.2% supplemental Arg and 0.2% supplemental Pro. This interaction may be partially related to metabolic connections between Arg and Pro in avian species. In chickens, Arg contributes to Pro synthesis through the ornithine and pyrroline-5-carboxylate pathways; however, the magnitude of this

conversion appears to be limited [11,16]. Therefore, differences in the relative dietary supply of Arg and Pro may have affected AA utilization or metabolic demand, potentially contributing to the observed differences in FI. However, the higher FI observed with the mixed level combinations was not accompanied by corresponding increases in egg mass or improvements in FCR. Moreover, no consistent dose-dependent pattern was observed. Therefore, although the Arg $\times$ Pro interaction for FI was statistically significant, its biological and practical relevance appears limited and should be interpreted cautiously.

Among the Arg- and Pro-supplemented diets, increasing supplemental Pro from 0.2 to 0.4% was associated with changes in egg quality, particularly eggshell strength and yolk color. The decrease in eggshell strength at the higher supplemental Pro level was not accompanied by a change in eggshell thickness, indicating that shell thickness alone did not explain this response. Eggshell strength is also influenced by ultrastructural characteristics and organic matrix composition [25,26]. Pro is an important component of collagen and may therefore be related to the organic matrix of eggshell membranes [13]. However, because eggshell ultrastructure, mineral organization, collagen content, and organic matrix composition were not measured in the present study, any involvement of these factors, including a collagen-related mechanism, remains hypothetical. Further studies incorporating structural, mineral, and molecular analyses of the eggshell and shell gland are required to clarify this response.

Among the Arg- and Pro-supplemented diets, increasing supplemental Pro from 0.2 to 0.4% lowered the yolk color score. Because corn and corn gluten meal levels, the major pigment-containing ingredients in the experimental diets, were identical among the dietary treatments, the observed difference is unlikely to be explained solely by differences in the formulated supply of dietary pigments. Carotenoid deposition in egg yolk is influenced by intestinal absorption, lipoprotein-mediated transport, and subsequent deposition into the yolk, as well as by dietary pigment intake [27]. However, whether the lower yolk color score was related to altered carotenoid absorption, transport, or deposition could not be determined from the present study, as these components were not measured. Further studies are required to determine whether the observed response reflects differences in carotenoid intake, absorption, transport, or yolk deposition. Although the yolk color fan is widely used to evaluate yolk pigmentation, it is a visual scoring method that is less sensitive than instrumental color analysis or direct carotenoid quantification. Therefore, the relatively small difference in yolk color scores should be interpreted with caution, particularly with respect to practical significance.

Among the Arg- and Pro-supplemented treatments, higher Pro supplementation was associated with lower relative spleen weight. The spleen is a major secondary lymphoid organ in birds that plays an important role in lymphocyte activation and systemic immune responses [28]. However, relative splenic weight alone does not

provide direct evidence of impaired immune function because splenic size is not consistently associated with immunocompetence [29]. Therefore, a lower relative splenic weight should be interpreted as a morphometric response rather than as direct evidence of suppressed immune function. Age-related alterations in splenic immune cell populations have been reported in laying hens [8], but whether such changes contributed to the present response cannot be determined. Therefore, the lower relative spleen weight should be interpreted as an isolated morphometric response of uncertain immunological significance, rather than as evidence of impaired immune competence in these aged laying hens.

Relative abdominal fat weight was larger in hens fed diets containing 0.4% Pro than in those fed diets containing 0.2% Pro. The main effect of Pro on relative abdominal fat weight was not accompanied by changes in FI, egg mass, or FCR, suggesting that greater fat deposition was not simply attributable to increased FI or improved nutrient utilization for egg production. A previous study showed a response with valine rather than with Pro, supporting the broader concept that a disproportionate supply of individual AAs can influence nutrient partitioning and hepatic lipid metabolism in laying hens [30]. In the present study, greater abdominal fat deposition at higher supplemental Pro levels, in the absence of corresponding changes in FI, egg mass, or FCR, is consistent with a possible shift in nutrient partitioning toward lipid storage. In birds, the liver is the primary site of de novo fatty acid synthesis, and newly synthesized lipids are transported through circulating lipoproteins to peripheral tissues, including the abdominal adipose tissue [31]. More abdominal fat may, therefore, reflect altered lipid production and peripheral storage; however, the metabolic basis of this response could not be determined from the present measurements. This finding may be physiologically relevant for aged laying hens, which are more susceptible to excessive fat accumulation and lipid-related metabolic disturbances during the later stages of egg production [32]. Thus, a higher relative abdominal fat weight represents a potentially unfavorable response to higher supplemental Pro levels, particularly because it was not accompanied by improvements in productive performance.

An important consideration in the interpretation of these responses is that alanine was used as a nitrogen-balancing AA and therefore its inclusion decreased as supplemental Arg and Pro increased. Although this formulation maintained comparable calculated dietary CP concentrations, alanine is metabolically active and participates in intermediary metabolism. Therefore, a contribution of the differing dietary alanine concentrations to the observed plasma aminotransferase activities or lipid-related responses cannot be completely excluded. Accordingly, these responses should be interpreted within the context of the overall dietary AA formulation rather than attributed exclusively to Arg and Pro. A significant interaction between dietary Arg and Pro levels was observed for plasma ALT activity, indicating that the response depended on the combination and relative levels

of the two supplemental AAs, rather than on a consistent main effect of either AA. Plasma ALT activity was lower when Arg or Pro was increased to 0.4%, compared to that in hens fed diets containing 0.2% supplemental Arg and 0.2% supplemental Pro. However, increasing both AAs to 0.4% did not further reduce ALT activity beyond the levels observed with the two mixed-level combinations. This non-additive response may be related to the metabolic connection between Arg and Pro. In birds, Arg and Pro metabolism are connected through ornithine and pyrroline-5-carboxylate, and differences in their relative dietary supplies may affect AA catabolism and nitrogen metabolism [10,16]. Thus, the metabolic connection between Arg and Pro may influence AA catabolism and nitrogen handling, which may partly explain the combination-dependent changes in the circulating ALT activity and cellular enzyme release [33]. Plasma AST activity showed a less consistent pattern. Hens fed diets containing 0.2% supplemental Arg with 0.4% supplemental Pro or 0.4% supplemental Arg with 0.2% supplemental Pro showed lower AST activity than those fed the basal diet. However, AST activity was not affected by the main effects of Arg, Pro, or their interactions, a less consistent response than that of ALT. Although circulating ALT and AST activities can provide information on tissue integrity and metabolic status, neither enzyme is liver-specific in birds because both are distributed across multiple tissues [34,35]. Moreover, the lower aminotransferase activities were not accompanied by corresponding changes in relative liver weight, liver color, hemorrhage scores, plasma albumin, or total protein. Therefore, the observed differences in circulating aminotransferase activities should not be interpreted as direct evidence of improved hepatic function [35]. The absence of changes in plasma albumin and total protein also indicates that these differences were not accompanied by detectable changes in circulating protein status [36]. Rather, these findings indicate combination-dependent differences in circulating enzyme activities whose tissue origin and physiological significance cannot be determined from the present measurements. Overall, the physiological responses to supplemental Arg and Pro were dependent on the dietary combination and the measured plasma parameters, rather than showing a consistent response to increasing levels of either AA.

Hens fed diets containing 0.4% supplemental Pro had a lower liver L* value than those fed diets containing 0.2% supplemental Pro. Liver L* is influenced by hepatic lipid and glycogen content and the resulting differences in tissue composition [37]. Pro metabolism is linked to mitochondrial energy and redox processes through pyrroline-5-carboxylate and glutamate, suggesting that differences in Pro supply may influence hepatic substrate utilization and metabolic status [15,38]. Accordingly, the lower liver L* at the higher supplemental Pro level may reflect changes in hepatic substrate metabolism or tissue composition that influence light reflected from the liver surface. The greater relative abdominal fat weight observed at higher supplemental Pro levels also suggests altered

lipid partitioning and peripheral storage. However, increased abdominal fat deposition does not necessarily indicate increased hepatic lipid accumulation [32,39]. Collectively, the lower liver L* value, together with the higher relative abdominal fat weight, suggests that a higher supplemental Pro level may have influenced lipid or substrate partitioning without producing overt alterations in the gross liver condition.

CONCLUSION

Dietary supplementation with Arg and Pro did not improve the overall productive performance of the aged laying hens. Supplemental Arg and Pro interactively affected FI and plasma ALT activity, indicating that these factors depend on specific dietary combinations. Across the two supplemental Arg levels, increasing supplemental Pro from 0.2 to 0.4% resulted in lower eggshell strength, yolk color, relative spleen weight, and objective liver L*, but higher relative abdominal fat weight, without improving productive performance. Responses to supplemental Arg and Pro appear to depend on the combination and the specific outcomes measured, rather than uniformly beneficial at higher supplemental levels. Increasing supplemental Pro from 0.2 to 0.4% provided no productive advantage and was associated with several changes in egg quality and physiological indices among the co-supplemented treatments. Increasing supplemental Arg from 0.2 to 0.4% also provided no consistent benefit, although its effects on FI and plasma ALT activity varied relative to the supplemental Pro levels. Therefore, increasing either Arg or Pro to 0.4% did not provide an overall advantage for aged laying hens. These findings also provide a useful basis for selecting appropriate hen age and supplemental AA levels in future studies of Arg and Pro supplementation during extended laying cycles.

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Items	Dietary treatments				
	Basal diet	0.2% Arg		0.4% Arg	
		0.2% Pro	0.4% Pro	0.2% Pro	0.4% Pro
Ingredients (%)					
Corn	62.74	62.74	62.74	62.74	62.74
Soybean meal (46% CP)	11.73	11.73	11.73	11.73	11.73
Wheat	5.89	5.89	5.89	5.89	5.89
Corn gluten meal	4.74	4.74	4.74	4.74	4.74
Limestone	11.09	11.09	11.09	11.09	11.09
Monocalcium phosphate	1.37	1.37	1.37	1.37	1.37
54% L-lysine H ₂ SO ₄	0.30	0.30	0.30	0.30	0.30
88% DL-methionine	0.23	0.23	0.23	0.23	0.23
20% L-tryptophan	0.14	0.14	0.14	0.14	0.14
98.5% L-threonine	0.08	0.08	0.08	0.08	0.08
50% choline	0.02	0.02	0.02	0.02	0.02
NaCl	0.12	0.12	0.12	0.12	0.12
Sodium bicarbonate	0.20	0.20	0.20	0.20	0.20
Mineral premix ¹⁾	0.10	0.10	0.10	0.10	0.10
Vitamin premix ²⁾	0.10	0.10	0.10	0.10	0.10
Corn starch ³⁾	0.00	0.10	0.00	0.29	0.18
Celite	0.00	0.07	0.13	0.10	0.17
Arginine	0.00	0.20	0.20	0.40	0.40
Proline	0.00	0.20	0.40	0.20	0.40
Alanine	1.15	0.58	0.42	0.16	0.00
Total	100.00	100.00	100.00	100.00	100.00
Energy and nutrient contents ⁴⁾					
AME _n (kcal/kg)	2,693	2,693	2,693	2,693	2,693
CP (%)	15.18	15.18	15.18	15.18	15.18
Lysine (%)	0.75	0.75	0.75	0.75	0.75
Methionine + cysteine (%)	0.70	0.70	0.70	0.70	0.70
Tryptophan (%)	0.17	0.17	0.17	0.17	0.17
Threonine (%)	0.57	0.57	0.57	0.57	0.57
Arginine (%)	0.75	0.95	0.95	1.15	1.15

Proline (%)	1.03	1.23	1.43	1.23	1.43
Calcium (%)	4.43	4.43	4.43	4.43	4.43
Available phosphorus (%)	0.33	0.33	0.33	0.33	0.33

Arg, arginine; Pro, proline; CP, crude protein; NaCl, sodium chloride; AME_n, nitrogen-corrected apparent metabolizable energy.

¹Provided per kilogram of the complete diet: Zn (as ZnO), 88 mg; Mn (as MnSO₄·H₂O), 88 mg; Fe (as FeSO₄·7H₂O), 55 mg; Cu (as CuSO₄·5H₂O), 55 mg; Co (as CoCO₃), 1,700 µg; I (as Ca(IO₃)₂·H₂O), 1.25 mg; Se (as Na₂SeO₃), 300 µg.

²Provided per kilogram of the complete diet: vitamin A (from vitamin A acetate), 13,000 IU; vitamin D₃, 4,000 IU; vitamin E (from DL- α -tocopheryl acetate), 30 mg; vitamin K₃, 3 mg; vitamin B₁, 3 mg; vitamin B₂, 7 mg; vitamin B₆, 5 mg; vitamin B₁₂, 20 µg; calcium pantothenate, 12 mg; folic acid, 1.5 mg; biotin, 150 µg; niacin, 50 mg.

³Supplemental Arg and Pro were added at inclusion levels of 0.2 or 0.4%, and the inclusion levels of corn starch, celite, and alanine were adjusted to maintain a constant total formulation and comparable calculated dietary CP concentrations.

⁴Calculated values from Hy-Line [18].

402 **Table 2.** Calculated and analyzed arginine and proline concentrations in experimental diets.

Dietary treatments	Calculated concentrations (%)		Analyzed concentrations (%)	
	Arg	Pro	Arg	Pro
Basal diet	0.75	1.03	0.86	1.06
0.2% Arg + 0.2% Pro	0.95	1.23	1.11	1.21
0.2% Arg + 0.4% Pro	0.95	1.43	1.06	1.45
0.4% Arg + 0.2% Pro	1.15	1.23	1.25	1.26
0.4% Arg + 0.4% Pro	1.15	1.43	1.29	1.45

403 Arg, arginine; Pro, proline.

Table 3. Effects of dietary supplementation with arginine and proline on productive performance of aged laying hens.

Added Arg	Added Pro	Productive performance					
		Hen-day egg production (%)	Egg weight (g)	Broken and shell-less eggs (%)	Egg mass (g)	Feed intake (g/hen/d)	Feed conversion ratio (g/g)
0.0%	0.0%	77.1	65.2	2.3	50.3	120	2.40
0.2%	0.2%	77.3	64.9	1.3	50.1	118	2.37
	0.4%	76.8	65.6	2.9	50.4	122	2.43
0.4%	0.2%	76.4	65.3	1.9	49.9	122	2.45
	0.4%	79.2	65.0	1.7	51.4	120	2.35
SEM (n = 8)		1.93	0.45	0.52	1.20	0.96	0.05
Main effect							
Arg level							
	0.2%	77.1	65.2	2.1	50.3	120	2.40
	0.4%	77.8	65.1	1.8	50.6	121	2.40
SEM (n = 16)		1.33	0.30	0.35	0.81	0.72	0.04
Pro level							
	0.2%	76.9	65.1	1.6	50.0	120	2.41
	0.4%	78.0	65.3	2.3	50.9	121	2.39
SEM (n = 16)		1.33	0.30	0.35	0.81	0.72	0.04
<i>p</i> -value							
1-way ANOVA		0.859	0.806	0.289	0.907	0.057	0.647
2-way ANOVA							
	Arg	0.704	0.821	0.607	0.734	0.405	0.995
	Pro	0.537	0.632	0.204	0.439	0.231	0.705
	Arg × Pro	0.380	0.224	0.080	0.550	0.019	0.102

Arg, arginine; Pro, proline; SEM, standard error of the mean.

407 **Table 4.** Effects of dietary supplementation with arginine and proline on egg quality in aged laying hens.

		Egg quality				Eggshell color			
Added Arg	Added Pro	Eggshell thickness (μm)	Eggshell strength (kg/cm ²)	Haugh unit	yolk color (color fan)	Eggshell color (color fan)	L* (CIE value)	a* (CIE value)	b* (CIE value)
0.0%	0.0%	377	3.1 ^b	79.2	7.2 ^{bc}	10.7	54.9	20.0	29.4
0.2%	0.2%	363	3.4 ^a	79.6	7.5 ^a	10.7	55.3	20.2	29.2
	0.4%	378	3.0 ^b	78.4	7.1 ^c	10.5	55.5	19.8	29.4
0.4%	0.2%	378	3.4 ^a	77.2	7.4 ^{ab}	10.7	55.4	20.2	29.5
	0.4%	377	3.2 ^{ab}	80.9	7.0 ^c	10.8	55.1	20.1	29.4
SEM (n = 8)		4.52	0.10	1.28	0.09	0.20	0.40	0.27	0.19
Main effect									
Arg level									
0.2%		370	3.2	79.0	7.3	10.6	55.4	20.1	29.3
0.4%		377	3.3	79.0	7.2	10.8	55.2	20.0	29.5
SEM (n = 16)		3.20	0.06	0.96	0.06	0.14	0.28	0.18	0.14
Pro level									
0.2%		377	3.4	78.4	7.5	10.7	55.4	20.2	29.4
0.4%		370	3.1	79.6	7.1	10.7	55.3	20.0	29.4
SEM (n = 16)		3.20	0.06	0.96	0.06	0.14	0.28	0.18	0.14
p-value									
1-way ANOVA		0.155	0.028	0.332	0.002	0.779	0.814	0.856	0.898
2-way ANOVA									
Arg		0.144	0.172	0.972	0.585	0.414	0.662	0.663	0.517
Pro		0.113	0.008	0.354	<0.001	0.918	0.918	0.396	0.867
Arg × Pro		0.156	0.333	0.080	0.867	0.309	0.510	0.515	0.477

408 Arg, arginine; Pro, proline; L*, lightness; a*, redness; b*, yellowness; SEM, standard error of the mean.

409 ^{a-c}Means within a variable with no common superscript differ significantly ($p < 0.05$).

410 **Table 5.** Effects of dietary supplementation with arginine and proline on relative organ and abdominal fat weight
411 of aged laying hens.

		Relative organ and abdominal fat weight (%)				
Added Arg	Added Pro	Liver	Spleen	Thymus	Kidney	Abdominal fat
0.0%	0.0%	2.50	0.12	0.10	0.27	4.55
0.2%	0.2%	2.63	0.12	0.11	0.24	3.75
	0.4%	2.41	0.09	0.11	0.23	5.16
0.4%	0.2%	2.71	0.13	0.09	0.27	4.85
	0.4%	2.48	0.11	0.10	0.26	5.22
SEM (n = 8)		0.17	0.01	0.02	0.02	0.42
Main effect						
Arg level						
0.2%		2.52	0.11	0.11	0.24	4.45
0.4%		2.59	0.12	0.09	0.27	5.03
SEM (n = 16)		0.12	0.01	0.01	0.01	0.30
Pro level						
0.2%		2.67	0.12	0.10	0.26	4.30
0.4%		2.45	0.10	0.10	0.25	5.19
SEM (n = 16)		0.12	0.01	0.01	0.01	0.30
p-value						
1-way ANOVA		0.725	0.159	0.857	0.730	0.119
2-way ANOVA						
Arg		0.679	0.237	0.272	0.168	0.181
Pro		0.196	0.025	0.794	0.617	0.044
Arg × Pro		0.979	0.961	0.797	0.913	0.229

412 Arg, arginine; Pro, proline; SEM, standard error of the mean.

413 **Table 6.** Effects of dietary supplementation with arginine and proline on plasma measurements of aged laying hens.

Added Arg	Added Pro	Plasma measurements			
		ALT (U/L)	AST (U/L)	Albumin (g/dL)	Total protein (g/dL)
0.0%	0.0%	1.23 ^a	249 ^a	1.57	5.84
0.2%	0.2%	0.94 ^a	218 ^{ab}	1.57	6.18
	0.4%	0.36 ^b	205 ^b	1.62	6.15
0.4%	0.2%	0.49 ^b	177 ^b	1.64	6.29
	0.4%	0.53 ^b	212 ^{ab}	1.57	5.90
SEM (n = 8)		0.16	14.91	0.05	0.16
Main effect					
Arg level					
0.2%		0.65	211	1.59	6.17
0.4%		0.51	194	1.61	6.10
SEM (n = 16)		0.10	10.44	0.03	0.11
Pro level					
0.2%		0.71	198	1.61	6.24
0.4%		0.44	208	1.60	6.02
SEM (n = 16)		0.10	10.44	0.03	0.11
<i>p</i> -value					
1-way ANOVA		<0.001	0.030	0.689	0.198
2-way ANOVA					
Arg		0.331	0.257	0.749	0.649
Pro		0.075	0.477	0.816	0.183
Arg × Pro		0.044	0.112	0.181	0.243

414 Arg, arginine; Pro, proline; ALT, alanine aminotransferase; AST, aspartate aminotransferase; SEM, standard error
 415 of the mean.

416 ^{a,b}Means within a variable with no common superscript differ significantly ($p < 0.05$).

417 **Table 7.** Effects of dietary supplementation with arginine and proline on liver parameters of aged laying hens.

		Liver parameters				
Added Arg	Added Pro	Liver score		Liver color		
		Color	Hemorrhage	L* (CIE value)	a* (CIE value)	b* (CIE value)
0.0%	0.0%	2.3	2.1	27.3	16.9	7.9
0.2%	0.2%	2.3	1.8	28.5	16.7	8.2
	0.4%	2.6	2.2	26.6	16.9	6.7
0.4%	0.2%	2.5	2.1	28.2	18.0	7.7
	0.4%	2.5	2.5	26.6	16.8	6.7
SEM (n = 8)		0.33	0.44	0.81	1.61	0.89
Main effect						
Arg level						
		2.5	2.0	27.6	16.8	7.4
		2.5	2.3	27.4	17.4	7.2
SEM (n = 16)		0.22	0.31	0.54	1.08	0.61
Pro level						
		2.4	2.0	28.4	17.4	7.9
		2.6	2.4	26.6	16.8	6.7
SEM (n = 16)		0.22	0.31	0.54	1.08	0.61
<i>p</i> -value						
1-way ANOVA		0.964	0.842	0.320	0.979	0.658
2-way ANOVA						
Arg		0.841	0.505	0.845	0.697	0.812
Pro		0.659	0.347	0.027	0.727	0.154
Arg × Pro		0.659	1.000	0.858	0.668	0.790

418 Arg, arginine; Pro, proline; SEM, standard error of the mean.