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<p>Ethics approval and consent to participate</p>	<p>The protocol for the current experiment was reviewed and approved by the Institutional Animal Care and Use Committee at Chungbuk National University (Approval No. CBNUA-24-0042-02).</p>

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6

7

## 8 **Abstract**

9 The objective of this study was to examine the effects of *in ovo* feeding of arginine (Arg) and breeder age on  
10 hatching performance and morphometry in broiler chickens. The experiment was performed using a completely  
11 randomized design in  $2 \times 2$  factorial arrangements, consisting of 2 breeder ages (32 and 62 weeks) and 2 *in ovo*  
12 feeding treatments (0% and 1% Arg). A total of 200 fertile eggs were obtained from Indian River broiler breeder  
13 flocks at 32 and 62 weeks of age, with average fertile egg weights of  $57.4 \pm 0.7$  g and  $70.8 \pm 0.6$  g, respectively.  
14 Eggs were randomly distributed into 4 treatments, each having 5 replicates of 10 eggs. All eggs were incubated  
15 under standardized temperature and humidity conditions of  $38.0 \pm 0.2^\circ\text{C}$  and  $75.0 \pm 3\%$ , respectively. Upon  
16 hatching, 80 chicks were randomly assigned into the same  $2 \times 2$  factorial arrangements comprising 5 replicates.  
17 Hatching performance parameters were evaluated immediately after hatching. On day 10 post-hatch, one chick  
18 per replicate with a body weight closet to the mean of each treatment group was selected for morphometric  
19 analysis, assessment of relative organ weight, and evaluation of liver characteristics. The findings revealed a  
20 significant interaction ( $p < 0.05$ ) between *in ovo* feeding of Arg and breeder age for hatchability and relative  
21 proventriculus and intestine weights. Chicks in the Arg group exhibited significantly less ( $p < 0.05$ ) relative liver  
22 and kidney weights than those in the non-injected group. With respect to the main effects of breeder age, egg  
23 weight, body weight, middle toe and tibia lengths, and liver and intestine weights were significantly greater ( $p <$   
24  $0.05$ ) in chicks from 62-week-old breeder hens than in those from 32-week-old breeder hens. In conclusion, *in*  
25 *ovo* feeding of Arg and breeder age affect both hatchability and organ development in broiler chickens. *In ovo*  
26 feeding of Arg may alleviate the age-associated reduction in hatchability and promote intestinal development in  
27 chicks from older breeder hens. These findings suggest that the efficacy of *in ovo* feeding of Arg is influenced by  
28 the maternal age of the breeder hens.

29 **Keywords:** arginine, breeder age, broiler chicken, *in ovo* feeding, post-hatch performance  
30

## 31 **INTRODUCTION**

32 The late embryonic and early post-hatch period constitutes a crucial developmental window in broiler chickens,  
33 characterized by an abrupt physiological and metabolic transition from reliance on endogenous yolk-based  
34 nutrients to dependence on exogenous feed intake (FI) [1-3]. This transition imposes considerable metabolic and  
35 functional demands on newly hatched chicks, whose gastrointestinal tract and immune system have not yet  
36 reached full maturity [4-6]. Minor nutrient insufficiencies during this interval may compromise intestinal

37 development, delay immune system maturation, and negatively impact long-term productivity and survivability  
38 [2,7]. Considering the accelerated growth pattern of modern broiler chickens, where market age is typically  
39 achieved between 35 and 42 days, the embryonic and peri-hatch periods represent over 50% of the bird's entire  
40 lifespan [8,9]. Thus, enhancing early development processes is fundamental for attaining optimal production  
41 efficiency and supporting animal welfare within commercial poultry operations.

42 Among various maternal and environmental factors that affect embryonic viability and chick quality, breeder  
43 hen age has consistently emerged as a principal determinant [10,11]. Eggs produced by older breeder hens tend  
44 to be heavier and contain larger yolk reserves, features that are often considered beneficial for embryonic growth  
45 [12-14]. Nevertheless, these advantages can be counterbalanced by a higher incidence of embryonic mortality,  
46 particularly in the later stages of incubation, along with declines in albumen quality, shell strength, and nutrient  
47 content [15,16]. As breeder flocks age, reproductive efficiency usually decreases due to hormonal fluctuations  
48 and deteriorating oocyte quality, which together reduce hatchability and hinder post-hatch growth performance  
49 [17,18]. Therefore, achieving appropriate egg size, shell integrity, and internal quality becomes increasingly  
50 challenging as breeder flocks age, particularly beyond 55 to 60 weeks.

51 To address the limitations imposed by maternal nutrition and older breeder hens, *in ovo* feeding has emerged  
52 as a targeted approach for improving embryonic development. This method entails the injection of nutrients or  
53 bioactive substances into the amniotic cavity or yolk sac during mid to late incubation [4]. The central objective  
54 is to deliver direct nutritional support to the embryo at a stage when nutrient absorption mechanisms are  
55 developing but still largely rely on endogenous stores [19,20]. Substantial evidence from various studies indicates  
56 that *in ovo* feeding promotes intestinal villi development, boosts glycogen reserves, strengthens immune function,  
57 and enhances post-hatch performance [20,21]. In addition, this method is recognized as both safe and suitable for  
58 commercial applications, with recent findings highlighting its potential to influence gut microbiota and improve  
59 metabolic resilience [22].

60 Among the nutrients considered for *in ovo* administration, arginine (Arg) has attracted significant interest due  
61 to its diverse physiological functions [23-27]. Recognized as a conditionally essential amino acid in poultry, Arg  
62 serves as an important precursor for nitric oxide (NO), polyamines, proline, and creatine, all of which play vital  
63 roles in cell proliferation, protein synthesis, angiogenesis, and immune system modulation [28,29]. In avian  
64 embryos, NO produced from Arg is essential for the vascularization of the chorioallantoic membrane, which in  
65 turn enables efficient nutrient transfer to rapidly developing tissues [29]. Previous studies have shown that *in ovo*  
66 Arg administration promotes vascular development, elevates immunoglobulin synthesis, and optimizes energy

67 utilization, leading to improvements in hatchability and subsequent body weight (BW) after hatching [30,31].  
68 Nevertheless, characteristics of the egg such as yolk volume, shell porosity, and endogenous nutrient content, can  
69 differ considerably with breeder age and may affect the response to *in ovo* Arg administration. Despite  
70 encouraging results, there is a paucity of data exploring how breeder hen age interacts with *in ovo* feeding of Arg.  
71 Therefore, the aim of this study was to investigate the effects of *in ovo* feeding of Arg and breeder age on hatching  
72 performance and morphometry in broiler chickens.

73

## 74 **MATERIALS AND METHODS**

75 The protocol for this experiment was approved by the Institutional Animal Care and the Use Committee (IACUC)  
76 at Chungbuk National University (IACUC approval No. CBNUA-24-0042-02).

77

### 78 **Egg and incubation**

79 The experiment was carried out using a completely randomized design with a  $2 \times 2$  factorial arrangements,  
80 including 2 *in ovo* feeding treatments (CON, no injection group; Arg, 1% Arg injection group) and 2 breeder hen  
81 ages (32 and 62 weeks). Two hundred fertile eggs were assigned to 4 treatments with 50 eggs per treatment, each  
82 having 5 replicates (10 eggs per replicate). A total of 200 fertile eggs from 32- and 62-week-old Indian River  
83 broiler breeder flocks with an average fertile egg weight of  $57.4 \pm 0.7$  g and  $70.8 \pm 0.6$  g, respectively, were  
84 obtained from the hatchery (Isu farm, Dangjin-si, Republic of Korea). All fertile eggs were incubated under the  
85 recommended temperature and humidity conditions of  $37.8^{\circ}\text{C}$  and 70%. Before incubation, the incubators were  
86 pre-warmed at  $24^{\circ}\text{C}$  for 3 hours, after which the eggs were individually weighed and numbered. The eggs were  
87 then randomly arranged in an automatic incubator (Rcom MARU Deluxe max 200, Autoalex Co., Ltd., Gimhae-  
88 si, Republic of Korea) with controlled temperature, humidity, and ventilation settings. The eggs were turned  
89 automatically every hour until day 18.

90

### 91 **Solution and injection procedure**

92 The injection solution was prepared as a 1% (w/v) solution by dissolving 0.2 g of each L-Arg ( $\geq 98\%$ , Sigma-  
93 Aldrich Inc., St. Louis, MO, USA) in 20 mL of phosphate-buffered saline (PBS, LB004-01, Welgene Inc.,  
94 Gyeongsan-si, Republic of Korea). The solution preparation and injection procedure were conducted according  
95 to the method described by Yu et al. [32] and our previous study [33] in which the same injection protocol was

96 established, with minor modifications. After complete dissolution was achieved, the solution was autoclaved at  
97 120°C for 15 minutes and then incubated at 37°C prior to use. On embryonic day 14, eggs were candled to locate  
98 the albumen, disinfected with 70% alcohol, and injected with 100 µL of the prepared solution into the albumen at  
99 a depth of approximately 1-2 cm using a 21-gauge syringe (Korea Vaccine Co., Ltd., Ansan-si, Republic of Korea).  
100 The injection site was then sealed with surgical tape (3M™, St. Paul, MN, USA) to prevent contamination [34,35].  
101 Previous research has shown that albumen enters the amniotic cavity and mixes with the amniotic fluid during the  
102 later phases of embryogenesis [36]. This component of the amniotic fluid is subsequently ingested by the embryo,  
103 delivering crucial nutritional support [37]. Consequently, Arg was injected into the albumen, as this route allows  
104 for steady diffusion into the embryo and ensures efficient absorption, while also reducing the risk of direct damage  
105 to embryonic tissues.

106

### 107 **Hatching performance**

108 In this study, hatching performance measurements were conducted using the method described by Muiyarakandy  
109 et al. [38] with minor modifications. Hatching performance was evaluated by measuring egg weight prior to  
110 incubation and recording the live weight of each chick immediately after hatching using an electronic scale (HS-  
111 1000A, Hansung Co., Ltd., Gwangmyeong-si, Republic of Korea). The hatching rate was determined by  
112 calculating as the percentage of chicks hatched from the total number of fertile eggs set. After hatching, the  
113 number of hatched chicks and the BW of each chick were recorded. The survival rate represented the proportion  
114 of live chicks at hatch. Chick yield was calculated by dividing the average weight of the hatched chicks by the  
115 total number of fertilized eggs.

116

### 117 **Birds, diets, and experimental design**

118 After all broiler chicks hatched, 80 sex-mixed broiler chicks were allotted to the same 2 × 2 factorial arrangements  
119 with 5 replicates. Experimental design included 2 *in ovo* feeding treatments (CON, no injection group; Arg, 1%  
120 Arg injection group) and 2 breeder hen ages (32 and 62 weeks). The 80 birds were allotted to 4 treatments with  
121 20 birds per treatment, each having 5 replicates (4 birds per replicate). The 80 birds were allotted to each cage (32  
122 cm × 31.5 cm × 30 cm = width × length × height) with a similar average BW among cages. The basal diet was  
123 formulated in accordance with Indian River broiler nutrition specifications [39] (Table 1), providing all required  
124 nutrients and energy. All diets were provided in mash form and offered ad libitum to the chicks for 10 days. The  
125 temperature and relative humidity of the room were maintained at 30°C and 60% during the first week and then

126 the temperature was gradually reduced to 26°C by the conclusion of the study as recommended by Indian River  
127 broiler pocket guide [39]. A continuous 24-hour lighting schedule was used throughout the experiment. At the  
128 end of the experiment, BW gain (BWG) and FI were recorded. The feed conversion ratio (FCR) was determined  
129 by dividing FI by BWG after adjusting FI for mortality. At the end of the experiment, one 10-day-old chick per  
130 replicate with a BW close to the mean of each treatment group was selected for the evaluation of hatching and  
131 post-hatch performance, morphometry, relative organ weight, and liver characteristics.

132

### 133 **Morphometric measurement**

134 At day 10 post-hatch, 1 male chick per replicate was humanely euthanized using CO<sub>2</sub> to evaluate a range of  
135 morphometric parameters and the weights of the breast, leg meat, and liver. The breast, leg meat, and liver tissues  
136 were carefully excised and individually weighed using a digital scale (HS1000A, Hansung, Seoul, Republic of  
137 Korea). Morphometric measurements included middle toe, tibia, radius, small intestine, and chick body lengths.  
138 The length of middle toe was measured from the base to the tip of the third toe using a 15-cm ruler. The tibia and  
139 radius lengths were recorded after the muscles were gently removed from the leg and wing, using a 15-cm ruler  
140 for each. The small intestine was gently extended without excessive tension and measured with a 30-cm flexible  
141 ruler to minimize tissue damage. The overall chick body length was measured from the tip of the beak to the base  
142 of the middle toe using a 15-cm ruler. All assessments were performed by a single trained individual to ensure  
143 consistency and minimize inter-observer variability.

144

### 145 **Relative organ weight**

146 The relative organ weights of the heart, liver, gizzard, proventriculus, kidney, small intestine, thymus, bursa of  
147 Fabricius, and spleen in broiler chicks were measured using a digital scale (HS1000A, Hansung, Seoul, Republic  
148 of Korea) and expressed relative to BW.

149

### 150 **Liver characteristics**

151 Lightness (L\*), redness (a\*), and yellowness (b\*) values of the surface of the liver were measured precisely using  
152 a colorimeter (CR-10, Konica Minolta, Tokyo, Japan). Hemorrhage liver score ranged from 0 to 5, where 0  
153 represented normal liver and 5 denoted large and massive hemorrhages. Fatty liver score ranged from 1 to 5, with  
154 1 indicating normal liver color and 5 indicating dark red to light yellowish red [40,41]. Panel of six evaluators  
155 independently assessed the color scores for hemorrhagic and fatty livers.

156

### 157 **Statistical analysis**

158 All data were analyzed by 2-way ANOVA as a completely randomized design using the PROC MIXED procedure  
159 of SAS (SAS Institute Inc., Cary, NC, USA). The replicate was considered an experimental unit for all  
160 measurements. Outlier data were checked using the PROC UNIVARIATE procedure of SAS. The main effects  
161 in the model included *in ovo* feeding of Arg, breeder hen ages, and their interactions. The LSMEANS procedure  
162 was used to calculate treatment means. The PDIFF option of SAS was used to separate the means if the difference  
163 was significant. Significance for statistical tests was set at  $p < 0.05$ .

164

## 165 **RESULTS**

### 166 **Hatching performance**

167 A significant interaction ( $p < 0.05$ ) between *in ovo* feeding of Arg and breeder hen age was observed for hatching  
168 rate (Table 2). Specifically, the CON group of chicks from 62-week-old breeders showed significantly decreased  
169 ( $p < 0.05$ ) hatching rate compared to those from 32-week-old breeder hens. In contrast, no significant difference  
170 in hatching rate was observed for Arg group between the breeder hen ages. Additionally, no significant  
171 interactions were observed for egg weight, live weight, survival rate, and chick yield. Considering the main effects  
172 of *in ovo* feeding of Arg, hatchability was not significantly influenced. For the main effects of breeder hen age,  
173 eggs from 62-week-old breeder hens had significantly greater ( $p < 0.05$ ) egg weight and live weight than those  
174 from 32-week-old breeder hens. Conversely, hatching rate and chick yield were significantly less ( $p < 0.05$ ) in  
175 chicks from 62-week-old breeder hens compared to those from 32-week-old breeder hens.

176

### 177 **Post-hatch performance**

178 No significant interactions between *in ovo* feeding of Arg and breeder age were observed for post-hatch  
179 performance, including BWG, FI, FCR, and mortality (Table 3). In terms of the main effects of *in ovo* feeding of  
180 Arg, post-hatch performance did not differ significantly. For the main effects of breeder age, chicks from 62-  
181 week-old breeder hens had significantly greater ( $p < 0.05$ ) BWG than those from 32-week-old breeder hens.

182

### 183 **Morphometric measurement**

184 No significant interactions between *in ovo* feeding of Arg and breeder hen age were observed for morphometric  
185 measurements, including middle toe length, tibia length, radius length, intestinal length, and chick length (Table  
186 4). Regarding the main effects of *in ovo* feeding of Arg, there were no significant differences in morphometric  
187 measurements between groups. Regarding the main effects of breeder hen age, chicks from 62-week-old breeder  
188 hens had significantly greater ( $p < 0.05$ ) middle toe and tibia lengths than those from 32-week-old breeder hens.

189

### 190 **Relative organ weight**

191 Significant interactions ( $p < 0.05$ ) were found in the relative weights of proventriculus and intestine (Table 5).  
192 Specifically, Arg group significantly decreased ( $p < 0.05$ ) the relative proventriculus weight in chicks from 32-  
193 week-old breeder hens compared to the CON group, whereas no significant difference was observed in chicks  
194 from 62-week-old breeder hens. Additionally, the Arg group of chicks from 32-week-old breeder hens exhibited  
195 significantly less ( $p < 0.05$ ) relative intestine weight than those from 62-week-old breeder hens, which were also  
196 significantly less ( $p < 0.05$ ) than those in the CON group, regardless of breeder hen age. Concerning the main  
197 effects of *in ovo* feeding of Arg, the Arg group significantly decreased ( $p < 0.05$ ) relative liver and kidney weights  
198 compared to the CON group. In terms of the main effect of breeder hen age, chicks from 62-week-old breeder  
199 hens had significantly greater ( $p < 0.05$ ) relative liver and intestine weights than those from 32-week-old breeder  
200 hens.

201

### 202 **Liver characteristic**

203 No significant interactions between *in ovo* feeding of Arg and breeder age were observed for liver characteristics  
204 (Table 6). Furthermore, there were no significant main effects of *in ovo* feeding of Arg and breeder hen age on  
205 liver characteristics.

206

## 207 **DISCUSSION**

208 Breeder hen age is widely recognized as an important factor influencing hatchability and growth performance in  
209 poultry [42,43]. In the present study, eggs from older breeder hens exhibited increased egg weight and chick  
210 weight compared to those from younger breeder hens. Conversely, eggs from younger breeder hens showed a  
211 greater hatching rate and chick yield. This result aligns with previous studies indicating that heavier eggs from  
212 older breeder hens are linked to decreased hatchability and chick growth [44,45]. In the present study, hatching

213 rate was influenced by a interaction between *in ovo* feeding of Arg and breeder hen age. In particular, chicks from  
214 older breeder hens exhibited a lower hatching rate in the no feeding treatment compared with those from younger  
215 breeder hens. Reduced hatchability in eggs from older breeder hens is primarily due to increased embryonic  
216 mortality, especially during the late incubation period. Larger yolk size in eggs from older hens can increase  
217 endogenous heat production from day 16 of incubation [44], which may contribute to the observed reduction in  
218 hatchability. Along with an increase in water loss caused by thinner eggshells, this elevation in heat production  
219 may intensify late-stage embryonic mortality [18]. Under aging conditions, NO bioavailability is known to decline  
220 due to reduced nitric oxide synthase activity and impaired vascular function [45,46]. Consequently, the provision  
221 of Arg may stimulate NO production and partially compensate for this age-related physiological limitation [45].  
222 Under these conditions, *in ovo* feeding of Arg may partially alleviate late embryonic stress by enhancing NO-  
223 mediated blood flow, thereby improving oxygen and nutrient delivery to embryonic tissues and supporting  
224 embryonic survival during this critical period [47]. In particular, embryos from older breeder hens, which are  
225 more susceptible to late-stage embryonic stress due to elevated metabolic heat production and reduced  
226 physiological resilience, may benefit more substantially from Arg supplementation. Therefore, our current  
227 findings suggest that the beneficial effect of *in ovo* feeding of Arg on hatchability is more pronounced in embryos  
228 from older breeder hens, reflecting an age-dependent capacity of embryos to respond to Arg supplementation  
229 rather than a uniform effect across breeder ages.

230 Post-hatch performance is widely recognized as a key primary factor influencing growth efficiency, organ  
231 development, and long-term growth performance in broiler chickens [48,49]. In the present study, greater BWG  
232 was observed in chicks from older breeder hens than those from younger breeder hens, which is consistent with  
233 previous findings [50,51]. Eggs produced by older breeders generally contain larger yolks with elevated levels of  
234 lipids and proteins, promoting more advanced organ development at hatch and providing essential energy and  
235 structural components required for rapid post-hatch growth [18,52]. Therefore, the increased post-hatch BWG  
236 observed in chicks from older breeder hens likely results from increased yolk reserves, which support early organ  
237 development and provide adequate energy for accelerated growth during the starter period.

238 The skeletal system is fundamental in supporting the BW of rapidly growing broiler chickens, which undergo  
239 considerable BWG [53]. It is an essential aspect of development, as appropriate bone structure and function have  
240 a significant impact on the overall health and physical development of poultry [54]. Animal tissue development  
241 follows an ordered sequence, with bone formation preceding muscle development to provide an anatomical basis  
242 for muscle attachment and growth. Therefore, optimal skeletal development forms the basis for effective muscle

243 formation [55]. There is a well-established association between BW and tibia length, indicating that tibia length  
244 tends to increase concomitantly with BWG [56]. Furthermore, middle toe length is widely regarded as a reliable  
245 measure of chick quality and comprehensive skeletal development [57]. In the present study, middle toe length  
246 and tibia length were greater in chicks from older breeder hens than in those from younger breeder hens. Similarly,  
247 Agbehadzi et al. [48] reported that chicks from 50-week-old breeders had longer tibia lengths than those from 33-  
248 week-old breeders, further supporting the conclusion that breeder age considerably affect skeletal development in  
249 poultry. These findings may be attributed to the fact that chicks from older hens are generally larger at hatch,  
250 exhibiting increased BW and skeletal dimensions.

251 The liver is responsible for various metabolic processes in poultry, such as detoxification, protein synthesis,  
252 and nutrient storage [58]. The kidney also plays a crucial role in maintaining fluid balance, eliminating waste  
253 products, and ensuring physiological homeostasis [59]. In poultry, the urea cycle and Arg metabolism are  
254 primarily mediated through the liver and kidneys [60]. Specifically, Arg enhances NO synthesis, thereby  
255 improving blood flow and supporting more efficient delivery of nutrients and oxygen to muscle tissue.  
256 Consequently, muscle accretion may be favored over visceral organ development, leading to a reduced relative  
257 weights of visceral organs, even without a change in their absolute size [27]. In this study, *in ovo* feeding of Arg  
258 led to a notable reduction in the relative weights of the liver and kidney compared with the non-injected control  
259 group. This observation is consistent with previous studies suggesting that increased Arg supply encourages  
260 nutrient partitioning toward lean tissue deposition rather than visceral organ expansion [61].

261 In the early post-hatch phase, chicks experience substantial structural and functional development of the  
262 gastrointestinal tract (GIT), which is critical for achieving optimal digestive efficiency and nutrient absorption [4].  
263 Eggs from younger broiler breeder hens typically have lower concentrations of proteins and lipids than those from  
264 older breeder hens, which may restrict the supply of key nutrients vital for gut maturation [36]. Although the GIT  
265 is morphologically established by around day 12 of incubation, its performance capacity remains suboptimal until  
266 just prior to hatch [62]. Thus, the gut in late-term embryos remains functionally immature and demonstrates  
267 limited ability to process and absorb proteins before hatch [63]. In the present study, the effects of *in ovo* feeding  
268 of Arg on the relative weights of the proventriculus and intestine differed between breeder hen ages, indicating a  
269 significant interaction whereby Arg on these organs depended on breeder hen age. Therefore, the physiological  
270 immaturity of the GIT during late embryogenesis may limit the efficient utilization of exogenous nutrients, such  
271 as *in ovo* feeding of Arg, resulting in age-dependent responses in gastrointestinal development [64]. Previous  
272 studies have shown that under such conditions, additional nutrient or amino acid supply in embryos from younger

273 breeders may enhance metabolic regulation and energy storage in tissues such as the liver and skeletal muscle,  
274 rather than promote gastrointestinal expansion [32]. Accordingly, *in ovo* feeding of Arg in embryos from younger  
275 breeder hens may act primarily as a modulator of early energy metabolism and nutrient partitioning, rather than  
276 as a direct stimulant of gastrointestinal development. Therefore, the reduced relative weights of the proventriculus  
277 and intestine observed in the *in ovo* feeding of Arg from younger breeders in the present study likely reflect a shift  
278 in nutrient allocation toward metabolic regulation and energy utilization, rather than impaired gastrointestinal  
279 hypertrophy.

280

281

## 282 CONCLUSION

283 *In ovo* feeding of Arg impacts hatchability and organ development in broiler chickens, with effects varying based  
284 on the age of the breeder hen. Specifically, *in ovo* feeding of Arg results in an increased relative weight of the  
285 intestine in chicks originating from older breeder hens. Eggs from older breeder hens show reduced hatching rates  
286 without *in ovo* feeding of Arg, but this decline is mitigated when Arg is provide through *in ovo* feeding. *In ovo*  
287 feeding of Arg reduces relative liver and kidney weights, whereas chicks from older breeder hens show improved  
288 hatching and post-hatch performance as well as enhanced bone development, indicating physiological advantages  
289 associated with maternal age. Collectively, these observations suggest that the effects of *in ovo* feeding of Arg on  
290 hatchability and intestinal development are dependent on breeder hen age, with age-related differences in  
291 hatchability being attenuated in *in ovo* feeding of Arg chicks. The presence of age-dependent effects further  
292 underscores that the efficacy of *in ovo* feeding of Arg is influenced by the maternal age of the breeder hens. While  
293 *in ovo* feeding of Arg offers potential advantages for specific developmental traits, careful adjustment of dose and  
294 timing is required to prevent metabolic stress and to optimize its positive effects in large-scale poultry systems.

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- 461

462 **Table 1.** Composition and nutrient content of experimental diets

Items	Basal diet (0 to 10 days)
<b>Ingredients (%)</b>	
Corn	55.10
Soybean meal (46% CP)	30.00
Corn gluten meal	7.80
Soybean oil	1.51
Salt	0.30
Monocalcium phosphate	1.85
Limestone	1.44
L-threonine (98.5%)	0.18
DL-methionine (98.5%)	0.38
L-lysine HCl (78%)	0.79
Choline (50%)	0.20
Sodium bicarbonate	0.15
Mineral premix <sup>1)</sup>	0.15
Vitamin premix <sup>2)</sup>	0.15
Total	100.00
<b>Energy and nutrient contents<sup>3)</sup></b>	
AME <sub>n</sub> (kcal/kg)	2,975
CP (%)	23.00
Digestible lysine (%)	1.32
Digestible methionine	0.69
Digestible methionine + cysteine (%)	1.00
Digestible threonine (%)	0.88
Digestible tryptophan (%)	0.21
Calcium (%)	0.95
Available phosphorus (%)	0.50

463 <sup>1)</sup>Provided per kilogram of the complete diet: Zn (as ZnSO<sub>4</sub>): 85 mg; Mn (as MnO), 90 mg; Cu (as CuSO<sub>4</sub>), 15  
464 mg; Co (as CoSO<sub>4</sub>), 250 µg; I (as Ca(IO<sub>3</sub>)<sub>2</sub>), 1.5 mg; Se (as Na<sub>2</sub>SeO<sub>3</sub>), 250 µg.

465 <sup>2)</sup>Provided per kilogram of the complete diet: vitamin A, 12,000 IU; vitamin D<sub>3</sub>, 4,000 IU; vitamin E, 80 mg;  
466 vitamin K<sub>3</sub>, 4 mg; vitamin B<sub>1</sub>, 4 mg; vitamin B<sub>2</sub>, 8 mg; vitamin B<sub>6</sub>, 6 mg; vitamin B<sub>12</sub>, 20 µg; calcium  
467 pantothenate, 20 mg; folic acid, 2 mg; biotin, 200 µg; niacin, 60 mg.

468 <sup>3)</sup>Calculated values from Aviagen (2022).

469

ACCEPTED

470 **Table 2.** Effect of *in ovo* feeding of arginine (Arg) and breeder age on hatching performance of broiler chickens<sup>1)</sup>

<i>In ovo</i> feeding <sup>3)</sup>	Age	Hatching performance <sup>2)</sup>				
		Egg weight (g)	Body weight (g)	Hatching rate (%)	Survival rate (%)	Chick yield (%)
Control	32 week	57.4	44.5	82.0	100.0	77.1
	62 week	70.7	53.8	50.0	100.0	75.5
Arg	32 week	57.4	44.6	67.5	98.0	77.3
	62 week	70.7	51.4	66.0	100.0	72.8
SEM (n = 5)		0.18	0.71	7.41	1.00	0.69
Main effect						
<i>In ovo</i> feeding						
Control		64.0	49.1	66.0	100.0	76.3
Arg		64.1	48.0	66.8	99.0	75.1
SEM (n = 10)		0.11	0.45	4.97	0.71	0.56
Age						
32 week		57.4	44.5	74.8	99.0	77.2
62 week		70.7	52.6	58.0	100.0	74.1
SEM (n = 10)		0.11	0.47	4.97	0.71	0.56
<i>p</i> -value						
<i>In ovo</i> feeding		0.857	0.080	0.917	0.332	0.121
Age		<0.001	<0.001	0.032	0.332	0.001
<i>In ovo</i> feeding × Age		0.946	0.067	0.048	0.332	0.077

471 <sup>a,b</sup>Means with different superscripts within a column differ ( $p < 0.05$ ).

472 <sup>1)</sup>Each value represents the mean of 5 replicates per each treatment.

473 <sup>2)</sup>Hatching rate = (number of eggs hatched / number of fertile eggs) × 100; survival rate = (number of live  
474 chick / number of fertile eggs) × 100; chick yield = (average hatched chick weight / average fertile egg weight)  
475 × 100.

476 <sup>3)</sup>Control, without *in ovo* feeding; Arg, *in ovo* feeding of 1% Arg.

477

**Table 3.** Effects of *in ovo* feeding of arginine (Arg) and breeder age on post-hatch performance of broiler chickens<sup>1)</sup>

<i>In ovo</i> feeding <sup>3)</sup>	Age	Post-hatch performance <sup>2)</sup>			
		BWG (g)	FI (g)	FCR (g/g)	Mortality (%)
Control	32 week	170.8	237.7	1.40	20.0
	62 week	208.6	309.4	1.49	5.0
Arg	32 week	175.2	300.0	1.72	5.0
	62 week	208.7	308.6	1.50	5.0
SEM (n = 5)		15.69	26.38	0.127	7.50
Main effect					
<i>In ovo</i> feeding					
Control		189.7	273.6	1.44	12.5
Arg		192.0	304.3	1.61	5.0
SEM (n = 10)		10.52	17.70	0.090	5.30
Age					
32 week		173.0	268.8	1.56	12.5
62 week		208.7	309.0	1.50	5.0
SEM (n = 10)		10.52	17.70	0.090	5.30
<i>p</i> -value					
<i>In ovo</i> feeding		0.877	0.226	0.224	0.332
Age		0.026	0.119	0.646	0.332
<i>In ovo</i> feeding × Age		0.882	0.214	0.251	0.332

479 <sup>1)</sup>Each value represents the mean of 5 replicates per each treatment.

480 <sup>2)</sup>BWG, body weight gain; FI, feed intake; FCR, feed conversion ratio.

481 <sup>3)</sup>Control, without *in ovo* feeding; Arg, *in ovo* feeding of 1% Arg.

482

483 **Table 4.** Effect of *in ovo* feeding of arginine (Arg) and breeder age on morphometric measurement of broiler  
 484 chickens<sup>1)</sup>

<i>In ovo</i> feeding <sup>2)</sup>	Age	Morphometric measurement (cm)				Chick length <sup>3)</sup>
		Middle toe	Tibia	Radius	Intestine	
Control	32 week	3.36	5.96	4.40	83.3	30.04
	62 week	3.48	6.22	4.54	84.4	29.96
Arg	32 week	3.36	5.80	4.24	69.3	29.52
	62 week	3.60	6.28	4.52	82.7	30.42
SEM (n = 5)		0.070	0.161	0.120	4.35	0.451
Main effect						
<i>In ovo</i> feeding						
Control		3.42	6.10	4.47	83.9	30.00
Arg		3.48	6.04	4.38	76.0	29.97
SEM (n = 10)		0.050	0.114	0.085	3.07	0.319
Age						
32 week		3.36	5.88	4.32	76.3	29.78
62 week		3.54	6.25	4.53	83.5	30.19
SEM (n = 10)		0.050	0.114	0.085	3.07	0.319
<i>p</i> -value						
<i>In ovo</i> feeding		0.404	0.760	0.464	0.089	0.948
Age		0.021	0.035	0.100	0.117	0.376
<i>In ovo</i> feeding × Age		0.404	0.504	0.568	0.174	0.293

485 <sup>1)</sup>Each value represents the mean of 5 replicates per each treatment.

486 <sup>2)</sup>Control, without *in ovo* feeding; Arg, *in ovo* feeding of 1% Arg.

487 <sup>3)</sup>The chick length was measured from the tip of the middle toe to the tip of the beak.

**Table 5.** Effect of *in ovo* feeding of arginine (Arg) and breeder age on relative organ weight of broiler chickens<sup>1)</sup>

<i>In ovo</i> treatment <sup>2)</sup>	Age	Relative organ weight (%)								
		Heart	Liver	Gizzard	Proventriculus	Kidney	Intestine	Thymus	BF <sup>3)</sup>	Spleen
Control	32 week	2.16	10.6	13.4	2.79	2.23	17.2	0.21	0.59	0.24
	62 week	2.13	12.7	11.6	2.44	2.20	17.6	0.68	0.54	0.34
Arg	32 week	1.94	8.9	11.0	2.22	2.07	13.8	0.56	0.50	0.23
	62 week	1.98	10.2	11.6	2.58	1.36	18.9	0.73	0.60	0.36
SEM (n = 5)		0.127	0.63	1.03	0.154	0.197	1.04	0.088	0.051	0.057
Main effect										
<i>In ovo</i> feeding										
Control		2.14	11.6	12.5	2.61	2.22	17.4	0.74	0.57	0.29
Arg		1.96	9.6	11.3	2.41	1.72	16.4	0.64	0.55	0.30
SEM (n = 10)		0.090	0.45	0.73	0.109	0.140	0.74	0.066	0.036	0.041
Age										
32 week		2.05	9.7	12.2	2.51	2.15	15.5	0.68	0.55	0.24
62 week		2.05	11.5	11.6	2.51	1.78	18.3	0.70	0.57	0.35
SEM (n = 10)		0.090	0.45	0.73	0.109	0.140	0.74	0.062	0.036	0.041
<i>p</i> -value										
<i>In ovo</i> feeding		0.167	0.005	0.264	0.194	0.022	0.334	0.290	0.681	0.891

Age	0.975	0.015	0.568	0.969	0.079	0.018	0.808	0.654	0.069
<i>In ovo</i> feeding × Age	0.781	0.577	0.279	0.033	0.106	0.037	0.120	0.182	0.784

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489 <sup>a,b</sup>Means with different superscripts within a column differ ( $p < 0.05$ ).

490 <sup>1</sup>Each value represents the mean of 5 replicates per each treatment.

491 <sup>2</sup>Control, without *in ovo* feeding; Arg, *in ovo* feeding of 1% Arg.

492 <sup>3</sup>BF, bursa of Fabricius.

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**Table 6.** Effect of *in ovo* feeding of arginine (Arg) and breeder age on liver characteristics of broiler chickens<sup>1)</sup>

<i>In ovo</i> feeding <sup>3)</sup>	Age	Liver characteristics <sup>2)</sup>				
		L*	a*	b*	Hemorrhage liver score	Fatty liver score
Control	32 week	22.22	15.66	3.00	0.48	1.24
	62 week	23.24	15.30	2.86	0.44	1.20
Arg	32 week	22.58	15.88	3.56	0.40	1.08
	62 week	22.24	15.08	3.16	0.60	1.16
SEM (n = 5)		0.421	0.606	0.340	0.192	0.097
Main effect						
<i>In ovo</i> feeding						
Control		22.73	15.48	2.93	0.44	1.16
Arg		22.41	15.48	3.36	0.52	1.18
SEM (n = 10)		0.298	0.428	0.228	0.136	0.069
Age						
32 week		22.40	15.77	3.28	0.46	1.22
62 week		22.74	15.19	3.01	0.50	1.12
SEM (n = 10)		0.298	0.428	0.228	0.136	0.069
<i>p</i> -value						
<i>In ovo</i> feeding		0.458	1.000	0.190	0.683	0.839
Age		0.431	0.352	0.402	0.838	0.318
<i>In ovo</i> feeding × Age		0.126	0.721	0.684	0.542	0.545

494 <sup>1)</sup>Each value represents the mean of 5 replicates per each treatment.

495 <sup>2)</sup>L\*, lightness; a\*, redness; b\*, yellowness.

496 <sup>3)</sup>Control, without *in ovo* feeding; Arg, *in ovo* feeding of 1% Arg.