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Original Article

*Effect of In Ovo Feeding of L-Glutamine to Chick Embryos**

■Author(s)

Rufino JPF[I] https://orcid.org/0000-0002-1605-5255
Cruz FGG[II] https://orcid.org/0000-0001-9076-9849
Costa VR[I] https://orcid.org/0000-0002-0904-2376
Silva AF[I] https://orcid.org/0000-0003-3343-9530
Melo LD[III] https://orcid.org/0000-0002-8243-670X
Bezerra NS[III] https://orcid.org/0000-0002-4859-1204

[I] Graduate Program in Animal Science, College of Agrarian Sciences, Federal University of Amazonas, Manaus, Amazonas, Brazil.
[II] Department of Animal and Vegetable Production, College of Agrarian Sciences, Federal University of Amazonas, Manaus, Amazonas, Brazil.
[III] Animal Science Course, College of Agrarian Sciences, Federal University of Amazonas, Manaus, Amazonas, Brazil.

*Part of master's degree dissertation of the first author.

■Mail Address

Corresponding author e-mail address
João Paulo Ferreira Rufino
Federal University of Amazonas - Dept. of Animal and Vegetable Production – Av. General Rodrigo Octávio Jordão Ramos number 3000 Neighborhood Coroado I MANAUS Amazonas, Brazil 69077-000.
Email: bernardorfnogueira@gmail.com

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ABSTRACT

In ovo feeding (IOF), injecting nutrients into the amnion of the avian embryo may enhance hatchability, gastrointestinal development and serum metabolism changes. This hypothesis was evaluated with 5 IOF solutions containing L-glutamine. Were used 315 fertile Rhode Island Red eggs were used (breeders with 32-weeks). The experimental design was completely randomized with the treatments constituted by two controls and five solutions containing L-glutamine levels with 45 replicates each. Data collected were subjected to polynomial regression at 5% of significance. Differences ($p>0.05$) were observed in hatchability and intermediary mortality, with a gradual lower of decrease in hatchability from the IOF of L-glutamine. The yolk sac was higher ($p<0.05$) after the IOF (control and L-glutamine) in the in ovo fed embryos at 0.5% L-glutamine. IOF of L-glutamine alone enhanced the blood pH and reduced the other serum parameters ($p<0.05$), which may have fuelled more embryo development, mainly vital organs how as the heart and the liver due to the larger concentration of available nutrients for the embryo. The results of this study indicate that until 0.5% L-glutamine may be supplemented in-ovo to chick embryos without negative influence on chick weight and gastrointestinal tract development, acting as serum biochemical metabolism regulator and obtaining better hatchability.

INTRODUCTION

During the incubation period and in the first hours after hatching, the birds have limited digestive functions, which reduces the nutrients availability to growth metabolism and restricts the digestive capacity that begins the development when the amniotic fluid is orally consumed at 17 d of incubation (Uni *et al.*, 2005).

And even the egg composition being considered complete, in nutritional terms, the percentages of amino acids, carbohydrates, vitamins, minerals and lipids are sufficient only in the initial phase (1 to 7 days) of the incubation period, being below the required levels in the final phase (15 to 21 days) and during the hatching (Gonzales *et al.*, 2013).

The IOF in the pre-hatching phase is a recent technology in poultry industry. This method of supplementing for oviparous species, described within the US Patent (6,592,878) of Uni & Ferket (2003), involves the administration of exogenous nutrients into the amnion of the developing embryo of chickens and turkeys at about 17 and 23 d of incubation, respectively (Foye *et al.*, 2006).

These substances as nutritional supplements aim to improve the initial development of the gastrointestinal tract and boost the digestive enzymes and a greater growth of villi (Geyra *et al.*, 2001).

In this context, the IOF using aminoacids already proved to beviable (Ohta*et al.*, 1999), improving the chick weight at birth by increasing the availability of aminoacid (Ohta*et al.*, 2001) used by the embryo (Al-Murrani, 1982). However, there are few studies on IOF regarding the use of glutamine in broiler breeder hen eggs (Salmanzadeh *et al*., 2016).

Glutamine is the principle metabolic fuel for development of the gastrointestinal tract (Andrew & Griffiths, 2002). This amino acid can generate 30 moles of ATP, source of energy almost as important as glucose for these cells (Lacey & Wilmore, 1990; Hall *et al*., 1996; Vieira *et al*., 2006). Besides, glutamine may increase intestinal villus height and consequently, improve growth performance of broilers (Bartell & Batal,2007; Yi *et al*., 2005; Jazideh *et al*., 2014)

All these beneficial actions of glutamine in particular, make it an amino acid deserving of scientific and technical attention (Salmanzadeh *et al*., 2016). Thus, the present study examined the hypothesis that IOF of L-glutamine may accelerate the development of the gastrointestinal tract, affect the hatchability and serum biochemical metabolism of chicks.

MATERIAL AND METHODS

This study was conducted at the Laboratory of Poultry Technology, Poultry Sector, Department of Animal and Vegetable Production (DPAV), College of Agrarian Sciences (FCA), Federal University of Amazonas (UFAM), South Sector at the University Campus, Manaus, State of Amazonas, Brazil.

The experimental procedures were conducted in accordance with the ethical principles for animal experimentation adopted by the Brazilian College of Animal Experimentation (COBEA) and the experimental procedures were approved by the local Committee for Ethical Animal Use (CEUA - protocol n. 016/2016) of Federal University of Amazonas, Manaus, AM, Brazil.

315 fertile Rhode Island Red eggs were used (breeders with 32-weeks). A completely randomized design was applied, with the treatments (Table 1) constituted by two controls and five solutions containing L-glutamine levels with 45 replicates (eggs) each. The L-glutamine used was manufactured by Midway International Labs Ltda© and denominated as pure and micronized.

All eggs were collected at one time, being weighed in room temperature, and distributed in trays in an incubator machine PETERSIME 168 with 37.6 °C temperature, 66% relative humidity and turn of eggs in one hour intervals. The eggs were randomly distributed, and after analysis to fertility confirmation at 16 days of incubation, they were separated in 45 units per treatment.

Table 1 – Experimental solutions with L-glutamine.

Treatments	Solutions	Osmolarity (mOsm/L)
Control	Intact egg	-
IOF Control	0.5% NaCl + 0.0 % L-glutamine	170.94
Solution 1	0.5% NaCl + 0.5 % L-glutamine	239.37
Solution 2	0.5% NaCl + 1.0 % L-glutamine	307.80
Solution 3	0.5% NaCl + 1.5 % L-glutamine	376.23
Solution 4	0.5% NaCl + 2.0 % L-glutamine	444.66
Solution 5	0.5% NaCl + 2.5 % L-glutamine	513.10

All eggs were kept out of the incubator machine for 1:30 hours due to inoculation procedures in the selected treatments. The room temperature used for inoculation was 37 °C and 65% of air humidity, in order to preserve the embryo's integrity.

Fertile eggs were sanitized and drilled in the air chamber region (avoiding to drill the inner membrane of the eggshell). The solutions (temperature of 26.7° C) were injected (0.5 mL) into the amniotic fluid (methodology previously tested) using needle syringes (7 x 2.5 mm). The holes in the egg shells were closed using melted paraffin and the eggs transferred to hatching machine PETERSIME 168 with 36.6 °C temperature, 76% relative humidity at 21 days of incubation (504±2 hours).

After birth, the % of hatchability (birth chicks/ fertile eggs), % of intermediary mortality (dead embryos among 16 and 18 days of incubation), % of late mortality (dead embryos among 19 and 21 days of incubation without pecked the eggshell), % of pipped eggs (dead embryos among 19 and 21 days of incubation that pecked the eggshell) and chick weight and chick/egg correlation were evaluated.

Regarding hatchability results, five able chicks of each treatment were selected for analysis. Blood was collected from the birds heart for analysis of biochemical serum parameters (glucose (mg/dl), triglycerides (mg/dl), cholesterol (mg/dl) and pH) using a portable biochemical analyser (Accucheck Trend, ROCHE©) and a pH meter (SENTRON, model 1001) coupled to a fine-tip penetration probe (SENTRON type LanceFET, model 1074-001).

Then, the same chicks were slaughtered by cervical dislocation for evaluation of heart (g) + gastrointestinal tract development: yolk sac (g), liver (g), pancreas (g), pro-ventricle (g), gizzard (g), digestive system length (cm), oropharynx + oesophagus (cm), duodenal loop (cm), jejunum + ileum (cm), cecum (cm) and colon + rectum (cm).

Statistical analysis was performed using the software Statistical Analysis System (2008) and estimates of treatments were subjected to polynomial regression at 5%of significance.

RESULTS

Differences (p>0.05) were observed in hatchability ($y = 106.60 + 1.566x - 2.0631x^2$; $R^2 = 0.86$) and intermediary mortality ($y = 33.717 + 17.267x$; $R^2 = 0.86$) results, with a gradual decrease in hatchability from the IOF of L-glutamine, and consequently, increase in the embryo mortality, mainly intermediate (hours after IOF procedures) (Table 2).

In this study, it was possible to determine the embryo physiological limit point from the increasing IOF of L-glutamine addition (Figure 1).

Table 2 – The effects of IOF of L-glutamine on hatchability and embryo mortality.

Treatments	Hatchability (%)	Intermediary mortality (%)	Late mortality (%)	Pipped eggs (%)	Chick weight (g)	Chick-Egg Correlation
Control	93.28	0.00	4.45	2.27	32.11	0.59
IOF Control	100.00	0.00	0.00	0.00	33.35	0.62
0.5 NaCl + 0.5 Glut.	97.82	2.18	0.00	0.00	33.07	0.66
0.5 NaCl + 1.0 Glut.	79.94	13.44	6.62	0.00	32.70	0.62
0.5 NaCl + 1.5 Glut.	19.86	69.22	9.80	1.12	31.88	0.62
0.5 NaCl + 2.0 Glut.	15.98	71.42	10.80	1.80	33.46	0.61
0.5 NaCl + 2.5 Glut.	6.62	81.20	12.18	0.00	33.60	0.61
p-value	0.01	0.01	0.01	0.57	0.70	0.01
Effect	Q	PL	PL	ns	ns	Q
CV (%)	7.02	11.68	14.75	16.64	5.77	5.95

CV – Coefficient of variation. *p*-value - Coefficient of probability. Q - Quadratic. PL–Positive Linear. ns – non significant.

However, IOF embryos (IOF Control and 0.5% L-glutamine) showed higher hatchability than the control group (without IOF), with lower (p<0.05) late mortality ($y = 1.5343 + 1.9496x$; $R^2 = 0.70$) and better (p<0.05) chick-egg correlation ($y = 0.6929 + 0.0055x - 0.0038x^2$; $R^2 = 0.79$).

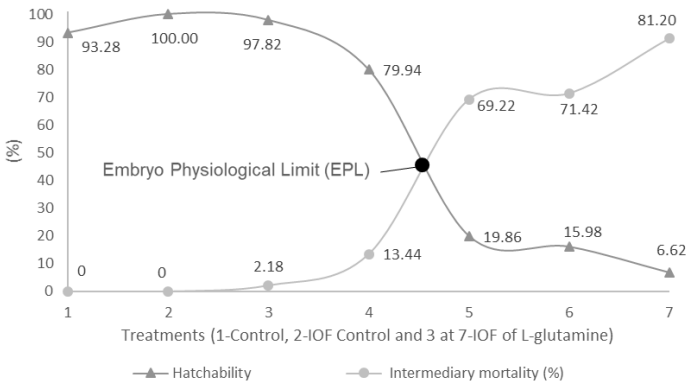

Figure 1 – The behaviour of IOF of L-glutamine on hatchability and intermediary mortality. All data represent the mean value per treatment. The meeting of curves between 1.0 and 1.5% IOF of L-glutamine determines the embryo physiological limit point.

The yolk sac ($y = 7.235 + 0.5113x - 0.3616x^2$; $R^2 = 0.85$) was higher (p<0.05) after the IOF (control and L-glutamine) in the in ovo fed embryos at 1.0% L-glutamine, whereas all IOF embryos exhibited greatest (p<0.05) abilities regarding heart development ($y = 0.2487 + 0.0209x$; $R^2 = 0.89$) compared with the control groups (Table 3).

No significant effect (p>0.05) of the IOF treatments was observed in the gastrointestinal tract development. However, there was increase in several organs and compartments of gastrointestinal tract of in ovo fed embryos (Table 3 and Table 4, respectively).

Chicks from embryos in ovo fed L-glutamine had greater (p<0.05) blood pH ($y = 7.159 + 0.02987x - 0.0202x^2$; $R^2 = 0.83$), whereas all in ovo embryos exhibited lower (p<0.05) triglycerides concentration ($y = 283.63 - 18.371x$; $R^2 = 0.82$), and significant changes (p<0.05) in glucose ($y = 225.8 + 3.846x - 3.125x^2$; $R^2 = 0.83$) and cholesterol concentrations ($y = 173.05 + 7.898x - 2.4732x^2$; $R^2 = 0.85$).

In summary, IOF of L-glutamine alone enhanced the blood pH and reduced the other serum parameters (Table 5), which may have fueled more embryo development, mainly vital organs as heart, liver and pancreas due to the larger concentration of available nutrient for embryo.

DISCUSSION

Concerning hatchability and gastrointestinal development, the IOF Control and 0.5% L-glutamine presented better results. Based on positive preliminary studies with aminoacids, the IOF solution formulation was developed to increase the available nutrients

Table 3 – The effects of IOF of L-glutamine on heart and gastrointestinal development of post-hatch chicks.

Treatments	Yolk sac (g)	Heart (g)	Liver (g)	Pancreas (g)	Pro-ventricle (g)	Gizzard (g)
Control	4.10	0.25	0.73	0.03	0.32	1.84
IOF Control	4.99	0.31	0.77	0.01	0.30	1.76
0.5 NaCl + 0.5 Glut.	7.16	0.32	0.76	0.02	0.28	1.83
0.5 NaCl + 1.0 Glut.	5.33	0.33	0.71	0.01	0.26	1.60
0.5 NaCl + 1.5 Glut.	3.85	0.35	0.97	0.01	0.32	1.93
0.5 NaCl + 2.0 Glut.	3.61	0.37	0.90	0.03	0.31	1.67
p-value	0.05	0.05	0.29	0.23	0.77	0.76
Effect	Q	PL	ns	ns	ns	ns
CV (%)	18.45	18.80	14.59	13.26	15.06	11.62

CV - Coefficient of variation. *p*-value - Coefficient of probability. Q - Quadratic. PL – Positive Linear. ns – non significant.

Table 4 – The effects of IOF of L-glutamine on gastrointestinal development of post-hatch chicks.

Treatments	Gastrointestinal tract (cm)	Oropharynx + oesophagus (cm)	Duodenal loop (cm)	Jejunum + ileum (cm)	Cecum (cm)	Colon + rectum (cm)
Control	47.00	6.80	7.00	22.52	6.96	5.28
IOF Control	47.40	6.80	7.20	27.90	7.38	3.96
0.5 NaCl + 0.5 Glut.	42.30	6.40	6.42	17.84	6.40	3.78
0.5 NaCl + 1.0 Glut.	43.40	6.10	6.00	25.20	6.20	5.14
0.5 NaCl + 1.5 Glut.	43.10	5.80	5.60	22.60	6.28	4.14
0.5 NaCl + 2.0 Glut.	44.80	6.30	6.50	23.50	7.66	4.92
p-value	0.61	0.35	0.29	0.07	0.29	0.11
Effect	ns	ns	ns	ns	ns	ns
CV (%)	12.53	19.84	18.18	11.07	17.72	17.58

CV - Coefficient of variation. *p*-value - Coefficient of probability. ns – non significant.

Table 5 – The effects of IOF of L-glutamine on serum biochemical parameters of post-hatch chicks.

Treatments	Glucose (mg/dl)	Triglycerides (mg/dl)	Cholesterol (mg/dl)	pH
Control	194.50	292.00	163.50	6.91
IOF Control	185.50	242.50	169.00	6.94
0.5 NaCl + 0.5 Glut.	221.00	199.50	178.00	7.12
0.5 NaCl + 1.0 Glut.	209.50	194.50	178.00	7.08
0.5 NaCl + 1.5 Glut.	197.50	194.00	163.00	6.99
0.5 NaCl + 2.0 Glut.	191.50	193.50	160.00	7.01
p-value	0.05	0.01	0.01	0.01
Effect	Q	NL	Q	Q
CV (%)	8.34	8.40	2.04	0.47

CV - Coefficient of variation. *p*-value - Coefficient of probability. Q - Quadratic. NL – Negative Linear.

concentration, providing a better development for the chicks (Foye *et al.*, 2007).

On the other hand, Pedroso *et al*. (2006) and Dos Santos *et al*. (2010) did not report the effect of IOF of glutamine in the amniotic fluid of embryos on hatchability. However, Salmanzadeh*et al*. (2016) showed that the hatchability was significantly reduced when injecting glutamine at days 7 of incubation.

Uni *et al*. (2005) comments that IOF to chicken embryos at the final stage of incubation may provide better effects than at initial stages, including the increase in hatchability. Besides the significant difference in hatchability among the in ovo injected groups observed in this study, other studies (Salmanzadeh *et al*., 2016), indicated that nutrient specificity and varied osmolarity of the injection might result in differing response of embryos.

Another important question about IOF, is the so-called embryo physiological limit, that corresponds to the meeting of graphic curves of hatchability and intermediate embryo mortality. This point represents the limit of the embryo organism in accepting the exogenous nutrient, substance or solution, mainly due to the clash among their osmolarities.

According to Uni and Ferket (2003), solutions at high exogenous nutrients concentrations may affect egg osmotic balance, and consequently embryo development, suggesting that the lower hatchability and high intermediary mortality of the high level amino acid-injected eggs, as was verified in the results obtained in this study, could be caused by osmotic balance changes.

At the same time, the IOF amino acids stimulate the structural development, providing heavier chicks with greater development of gastrointestinal tract and yolk sac, mainly in the intestinal area (villi and cryptes) to increase the nutrients absorption area. Hence, the in ovo fed avian neonate may have a greater capacity to digest and absorb nutrients from an exogenous diet relative to the conventional hatchling (Tako *et al.*, 2004).

Depending of the profile of the exogenous nutrients supplied to the embryo, there will be different embryo answers reflected in this post-IOF development. The IOF of CHO, from results of several studies, may accelerate gut development and maturity, serving as a tool to overcome growth constraints imposed by limited digestive capacity in hatchlings by enhancing intestinal function and maturation prior to hatching (Tako *et al*., 2004; Foye *et al*., 2006; Leitão *et al*., 2010; Leitão *et al*., 2014).

These amino acids would have been absorbed by the muscles due to the action of insulin and incorporated into protein (Foye *et al*., 2006). Studies have shown that dietary amino acids are important signalling mediators in pancreatic β-cell insulin secretion in vitro and release of insulin-like growth factors in vivo (Xu *et al.*, 1998), and that the amino acid reserves present in the yolk sac are insufficient to meet the requirements for growth process of birds, especially in the final period of embryonic development and first 72 h post-hatch (Ohta *et al*., 2004).

Previous studies reported by Chen *et al*. (2009) and Salmanzadeh *et al*. (2016) presented that IOF of glutamine may be seen as an effective tool to improve the mean bodyweights of newly hatched chickens. The IOF of glutamine present a positive relationship between protein synthesis and amino acid concentration (Jepson *et al*., 1988; Welborne, 1995, Maiorka *et al.*, 2000; Silva *et al.*, 2007), and affinity with growth hormone (Ray *et al*., 2003) that synthesis begins in the embryonic stage (Harvey *et al*., 2001). Vieira & Moran (1998) affirm that yolk sac contains approximately 150 mg of glutamine, but this amount is not enough to meet the embryo requirements for ideal development.

In this context, Al-Murrani (1982) and Ohta *et al*. (2001) observed that the injection of amino acids increased the available of the amino acid contents for the embryos, increasing the yolk sac, and the embryonic body weight. The same authors also affirm that the benefits of adding external nutrients to hatching eggs illustrate the limitations of avian species, which, unlike mammals, do not have a continuous energy supply from a maternal source to support embryonic and neonatal growth. However, Al-Murrani (1982), John *et al*. (1988), Ohta *et al*. (2001), and Uni *et al*. (2005) comment that injected nutrients are not directly exposed to embryonic intestine when it is most needed, near the end of incubation, being firstly allocated in attached structures, as the yolk sac, amniotic fluid and others, and only after will be moved to embryo's organism.

The IOF, that is fundamentally feeding to the avian embryo, may circumvent the growth constraints imposed by limited gut function in the avian neonate by the oral consumption of enteric modulators (compounds that stimulate development or metabolism of the cells of the digestive system) administered in the amnionic fluid of the avian embryo, which may enhance the gut capacity to absorb and digest dietary nutrients during late-term embryonic development (Uni & Ferket, 2003; Tako *et al*., 2004; Foye *et al*., 2007).

Our data also implies that L-glutamine in ovo supplementation may act as an enteric modulator that enhances intestinal absorption, and as a serum metabolism regulator due to the significant heart development and changes in blood parameters. Numerous studies have demonstrated that the intestinal amino acid (Karasov *et al.*, 1987; Torras-Llort *et al.*, 1998) and glucose transporters (Diamond & Karasov, 1987; Karasov *et al*., 1987; Solberg & Diamond, 1987; Buddington & Diamond, 1989; Ferraris & Diamond, 1989; Ferraris *et al*., 1992) are upregulated in the presence of increasing concentrations of their specific dietary substrate(s).

The increase of these glucose transporters, once there is osmotic equilibrium between the inoculated solution and the organism of the embryo, will provide an increase in glucose concentration in the bloodstream (Lu *et al*., 2005; Uni *et al*., 2005), as was verified in our results for inoculation of 0.5% glutamine. This same relation applies to other transporters and nutrients that act directly on energy metabolism (Uni *et al*., 2005).

CONCLUSIONS

The results of this study indicate that until 0.5% L-glutamine may be supplemented in ovo to chick embryos without negative influence on chick weight and gastrointestinal tract development, acting as a biochemical metabolism serum regulator and obtaining better hatchability.

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