Chicken embryo development: metabolic and morphological basis for *in ovo* feeding technology

Patricia E. N. Givisiez ,[*,1] Alexandre L. B. Moreira Filho,[†] Maylane R. B. Santos,[*] Heraldo B. Oliveira,[‡] Peter R. Ferket,[§] Celso J. B. Oliveira,[*] and Ramon D. Malheiros[§]

[*]*Department of Animal Science, Federal University of Paraiba, Areia, Brazil;* [†]*Department of Animal Science, Federal University of Paraiba, Bananeiras, Brazil;* [‡]*Department of Animal Science, Federal University of Pernambuco, Recife, Brazil; and* [§]*Prestage Department of Poultry Science, NC State University, Raleigh, USA*

ABSTRACT Broiler embryonic development depends on the nutrients that are available in the egg, which includes mostly water, lipids, and proteins. Carbohydrates represent less than 1%, and free glucose only 0.3%, of the total nutrients. Considering that energy requirements increase during incubation and metabolism is shifted toward the use of glycogen stores and gluconeogenesis from amino acids, extensive muscle protein degradation in the end of incubation can compromise chick development in the initial days after hatch. Significant prehatch changes occur in embryonic metabolism to parallel the rapid embryonic development. Oral consumption of the amniotic fluid begins around 17 d of incubation and promotes rapid development of the intestinal mucosa, which is characterized by morphological changes and increased expression and activity of enzymes and transporters. Furthermore, ingested substrates are stored as nutritional reserves to be used during hatching and in the first week after hatch. At hatch, this limited-nutrient store is directed to the functional development of the gastrointestinal tract to enable assimilation of exogenous nutrients. *In ovo* feeding is an alternative to deliver essential nutrients to chick embryos at this critical and challenging phase. The improved nutritional status and physiological changes triggered by *in ovo* feeding can resonate throughout the entire rearing period with significant health and economic gains. The present review addresses the main changes in metabolism and intestinal development throughout incubation, and also addresses scientific advances, limitations and future perspectives associated with the use of *in ovo* feeding that has been regarded as an important technology by the poultry industry.

Key words: *in ovo* feeding, incubation, intestinal development, metabolic pathways

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INTRODUCTION

The genetic improvement of modern broiler lines has contributed to the success of the poultry industry worldwide. Since the 1950s, slaughter age has been reduced by approximately 40%, and slaughter weight has more than doubled. Current lines have been genetically improved with focus on rapid growth and maximum performance based on continuous improvement of feed conversion. Embryonic development (**ED**) accounts nowadays for more than 33% of the whole life period of commercial broiler lines. Disturbances during ED may affect the entire production cycle and cause irreversible losses to broiler chicken producers.

Bird embryos are completely dependent on the nutrient contents of the egg; therefore, during incubation, metabolism shifts in accordance with the type of substrate available and oxygen supply. In the first week, small glucose stores are used to maintain metabolism (Moran, 2007). Afterward, greater embryo development is ensured as the extraembryonic membranes are formed and lipids are used as the main energy substrate (De Oliveira et al., 2008). In the final third of incubation, the internal environment changes considerably, leading to significant shifts in embryonic metabolism. The ingestion of amniotic fluid initiates at approximately 17 d of ED (**ED17**) (Uni and Ferket, 2003; Lu et al., 2007; De Oliveira et al., 2008; Omede et al., 2017) and substrates become available to be deposited in glycogen stores (Uni and Ferket, 2003). Oxygen availability decreases with the rupture of the chorioallantoic membrane on ED19, and the use of fatty acids becomes ineffective and unable

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[1]Corresponding author: patriciagivisiez@gmail.com

to meet the embryonic energy demands (Moran, 2007). Thus, metabolism is shifted toward anaerobic catabolism of glucose, which depends on the breakdown of glycogen stores and the gluconeogenesis pathway (Uni and Ferket, 2004). As a result, glycogen stores are practically depleted at the end of incubation.

Moreover, hatchlings have to promptly adapt from the use of egg nutrients to a protein-carbohydrate exogenous diet (Uni et al., 2003a). Once nutrients are available from an exogenous diet, the remaining energy body stores are used to support the gastrointestinal tract development, both morphologically and physiologically (Uni and Ferket, 2004).

Hatchlings may be fasted for up to 48 h until being delivered to rearing farms, sometimes even 72 h. Delayed feed access has been associated to increased posthatch mortality and developmental problems, including delayed gut development (Geyra et al., 2001) and increased susceptibility of the intestinal mucosa barrier against pathogens (Uni et al., 2003a). Uni and Ferket (2003) proposed providing nutrients during ED to minimize the negative effects of posthatch fasting and transition phase, thereby ensuring maximum expression of the genetic potential throughout the production cycle. The present review will address the main changes in metabolism and intestinal development throughout incubation, the current scientific information about *in ovo* feeding (**IOF**) as a means to improve chick survivability and performance after hatch, and its practical limitations and future perspectives as a potential technology to be fully explored by the poultry industry.

METABOLIC CHANGES IN CHICKEN EMBRYOS

Development of Extraembryonic Membranes

Extraembryonic membranes are crucial for embryo development and survival during embryogenesis. The yolk sac, amnion, chorion, and allantois are responsible for the nutrition, protection, respiration, and stores of metabolites derived from the embryonic metabolism, respectively.

The yolk sac contains small amounts of albumen proteins and the yolk, and holds all essential nutrients for ED, including proteins, lipids, carbohydrates, and minerals (Uni et al., 2012). In addition, the yolk sac is an important site of blood cell synthesis, especially erythrocytes (Yadgary et al., 2014). The highly vascularized yolk sac develops from the intestinal region of the embryo in first week of incubation. The yolk becomes fully enclosed approximately at ED10 and is covered by a layer of endothelial cells responsible for properly delivering nutrients to the embryo. Yolk contents are transferred to the embryonic circulation either by means of endocytosis or nutrient transporters (Yadgary et al., 2011).

Lipid transport occurs by endocytosis mediated by lipoprotein receptors present on the endothelial cell membrane (Hermann et al., 2000). Lipoproteins undergo lysosomal digestion and the resulting fatty acids and glycerol are then re-esterified into triglycerides and exported to the embryo by endothelial cells (Bauer et al., 2013). On the other hand, proteins and glucose cannot be transported by endocytosis. Thus, yolk sac membrane (**YSM**) cells are able to digest and transport nutrients similar to enterocytes. Indeed, YSM cells express nutrient transporters and digestive enzymes (Uni et al., 2012), such as transporters for cationic amino acid, dipeptides and tripeptides, glucose, and minerals, as well as the enzymes aminopeptidase and sucrose-isomaltase (Yadgary et al., 2011).

The yolk sac supports or replaces various organs that have not yet reached their full functional capacity (Yadgary et al., 2014). Similar to the liver, it synthesizes plasma proteins for triglyceride transport; similar to the bone marrow, it produces blood cells; and similar to the gut, it digests and transports nutrients to the embryo. The yolk sac is internalized around ED19 and accounts for approximately 15 to 20% of the hatchling weight (Yadgary et al., 2010), becoming crucial for survival after hatch as the only source of nutrients until exogenous feeding is provided to the chicks. It also contributes to defense mechanisms against pathogens by transferring maternal antibodies to the embryo.

The amnion encloses the embryo during development; the inner cell layer secretes amniotic fluid, which protects the embryo from mechanical and thermal shocks and prevents dehydration. Shortly before hatching (ED17), the embryo ingests amniotic fluid as a source of water and nutrients (De Oliveira, 2007; Omede et al., 2017). Amniotic fluid intake is also crucial for intestinal mucosa development and preparation for hatching because the ability to digest and absorb nutrients is poorly developed at this stage (De Oliveira, 2007).

The chorion encircles all other structures and protects both the embryo and the extraembryonic membranes. All compounds excreted during the ED are accumulated in the allantois or allantoic sac (Cesario, 2013). The allantois surrounds the amniotic sac, the yolk sac and the albumen and reaches its maximum volume on ED13, decreasing afterward as allantois membrane cells reabsorb water, sodium, and chloride (Everaert and Decuypere, 2013). The allantois then fuses with the chorion to form the chorioallantoic membrane. The chorioallantoic membrane serves as the respiratory organ and plays a key role in the acid–base balance by reabsorbing water and electrolytes from the allantoic fluid (Gabrielli and Accili, 2010). Closer to hatch, the fusion between chorioallantoic membrane and eggshell pores enables calcium removal and transportation from the eggshell to the embryo. This process weakens the eggshell as a preparation for the hatching process (Cesario, 2013).

Embryo Metabolic Pathways

The avian embryonic metabolism is entirely dependent on egg nutrients. The egg is rich in proteins and

lipids, but carbohydrates account for less than 1% of total nutrients. The avian embryonic metabolism changes in accordance with the available substrates and oxygen supply. The latter is directly related to extraembryonic membrane development.

Embryonic metabolism can be divided in 3 phases (Moran, 2007): embryo implantation, embryo completion, and preparation for hatching. Initial growth occurs in low oxygen conditions due to immature blood cells and underdeveloped chorioallantoic membrane. Glucose is the main energy substrate (Moran, 2007) and glycolysis is the central metabolic pathway. Two pyruvate molecules are produced for each glucose molecule entering the pathway and pyruvate is then converted into lactate, which accumulates within cells. Once oxygen levels increase, the lactate is transported to the liver and recycled to glucose via gluconeogenesis (Christensen et al., 2003). The Cori cycle is active in the embryonic metabolism in the first week, when the chorioallantoic membrane is underdeveloped, and during hatching, when it is ruptured by the embryo (De Oliveira, 2007).

The second phase is characterized by a fully functional chorioallantoic membrane, ensuring adequate oxygen supply for the faster and energy-demanding phase of ED (De Oliveira, 2007; Moran, 2007). Yolk content decreases abruptly (65% on ED13 to 44% on ED21) (Pulikanti et al., 2010) and fatty acids stored as triglycerides and phospholipids in the yolk sac become the main energy substrates (De Oliveira et al., 2008). Triglycerides and phospholipids are transported from the yolk sac to the embryo via endocytosis by YSM endothelial cells (Herman et al., 2000). Glycerol and fatty acid molecules are released from triglyceride and phospholipid digestion. Glycerol may enter gluconeogenesis and produce glucose (De Oliveira, 2007), whereas fatty acids are exported to different tissues and undergo β-oxidation, producing acetyl-CoA (De Oliveira et al., 2008). Acetyl-CoA then enters the Krebs cycle and is oxidized to adenosine triphosphate and carbon dioxide. Because oxygen is more available in the second week of development, this becomes the main pathway for adenosine triphosphate production, but decreases drastically before hatching (De Oliveira et al., 2008).

Apart from fatty acid use through β-oxidation, other substrates can be used for glucose synthesis and energy production through gluneogenesis. The main precursors for gluconeogenesis are lactate, formed during the first week of development; glycerol, generated by hydrolysis of yolk triglycerides; and yolk proteins (Foye, 2005). The synthetized glucose is stored as glycogen in the liver, kidney, and muscles and will be used during the last week of incubation. In addition, substrates provided by the amniotic fluid after ED17 are also directed to glycogen stores (Uni and Ferket, 2004; De Oliveira et al., 2008).

Major changes occur in the internal egg environment just before hatch. Embryo movements cause the rupture of chorioallantoic membrane, decreasing oxygen availability and causing shifts in metabolism once again. Energy demands cannot be fulfilled exclusively by fatty acid oxidation because the process is no longer as efficient (Moran, 2007), causing an increase in anaerobic glucose catabolism. Glucose is provided by glycogen stores and gluconeogenesis from amino acids, glycerol, and lactate (Uni and Ferket, 2004).

Glucose mobilized from glycogen stores enters the glycolytic pathway and produces 2 pyruvate molecules. At the end of incubation, glucose-derived pyruvate is diverted from Krebs cycle to lactate synthesis due to low oxygen supply, resulting in upregulation of glycolysis-regulatory enzymes (hexokinase, phosphofructokinase, and pyruvate kinase) and downregulation of Krebs cycle enzymes (citrate synthase, isocitrate dehydrogenase, and malate dehydrogenase) (De Oliveira et al., 2013). Lactate produced by different tissues is exported to the liver to be converted into glucose once the oxygen supply is restored, that is, the Cori cycle is again effective after lung respiration starts (De Oliveira et al., 2008).

Concurrent with glycogen store depletion, there is an increase in the activity of glucose 6-phosphatase and phosphoenolpyruvate carboxykinase, which are involved in the gluconeogenesis pathway (De Oliveira et al., 2013). Most likely, gluconeogenesis from protein catabolism is the only source of glucose in the third week of incubation (Uni and Ferket, 2004; Moran, 2007) and the main source of gluconeogenic amino acids is the breast muscle (Lu et al., 2007). Significant protein catabolism in the end of incubation adversely affects growth and development, leading to lower hatchling weight (Uni and Ferket, 2004; Uni et al., 2005).

Liver is considered an essential organ for embryonic metabolism and maintenance of glucose homeostasis in the final incubation period because it is known to be the main site for glycogen stores and gluconeogenesis from noncarbohydrate substrates (Uni et al., 2005). However, Yadgary and Uni (2012) reported that glucose storage in the form of glycogen is greater in the yolk sac compared with the liver. Glycogen synthesis is similar in both sites; glycogen synthetase expression in the yolk sac increases around ED13–17, coinciding with increased glycogen levels (Yadgary and Uni, 2012). Besides glycogen stores, the yolk sac is also an important site for gluconeogenesis, as shown by increased activity of regulatory enzymes, particularly fructose 1,6 bisphosphatase; glucose 6-phosphatase; and phosphoenolpyruvate carboxykinase (Yadgary and Uni, 2012). The main gluconeogenic substrates in the yolk sac are presumably glycerol resulting from triglyceride hydrolysis and lactate generated from glucose released from the glycogen stores (Yadgary et al., 2013). Yolk sac glycogen is depleted as hatching approaches and the generated glucose is transferred to the embryo to maintain glucose homeostasis (Yadgary and Uni, 2012).

Thus, rather than the liver, yolk sac is the main source of nutrients during the last week of ED and therefore the main site of glucose synthesis and glycogen stores. It has been suggested that the yolk sac has the potential to store 20 times more glycogen (Yadgary and Uni, 2012) and to transfer 10 times more glycogen-derived glucose to the embryo than the liver (Uni et al., 2012).

Hormonal Changes in Chicken Embryo

The endocrine system develops early in the chicken embryo (ED3 to ED8), with the emergence of pituitary, hypothalamus, and pancreas cells (Sunny, 2008). According to Zhou et al. (2007), the main hormones controlling embryonic metabolism are insulin, glucagon, thyroxine (**T4**), triiodothyronine (**T3**), and insulin-like growth factors (**IGF-I** and **IGF-II**).

Pancreas development in the embryo and secretion of insulin and glucagon are critical for embryonic metabolism control. Insulin and glucagon are protein hormones that act antagonistically in cellular metabolism. Insulin secretion is triggered by increased blood glucose and amino acid concentrations. On the other hand, glucagon secretion is triggered in response to low glucose levels, especially in the first 10 d of incubation and during the last week before hatching (Lu et al., 2007). Insulin is important for the control of amino acid and glucose concentrations in the plasma, amniotic fluid, and allantoic fluid in poultry embryos (Lu et al., 2007), promoting synthesis of glycogen, lipids and proteins, as well as inhibition of glycogenolysis, lipolysis, and proteolysis. Glucagon is directly related to embryo glucose demands; it increases plasma glucose levels through glycogenolysis and gluconeogenesis in the liver.

Lu et al. (2007) reported that plasma glucose levels increase from ED10 on and stabilizes at the beginning of the hatching process (ED19). Similarly, insulin levels in the embryonic plasma increased from ED10 (130 pg/mL) to one day after hatch (717 pg/mL). In the first week of development, lactate and glycerol originated from triglyceride hydrolysis and entering gluconeogenesis probably cause the increased glucose levels after ED10 (Foye, 2005). High glucose levels until the beginning of the hatching process are maintained by amniotic fluid ingestion from ED17 to ED19 (Uni and Ferket, 2003).

Increased plasma glucose levels in the embryo triggers insulin release and, consequently, glycogen synthesis in the liver and muscle. Glycogen accumulation is crucial for the final development of the embryo (Moran, 2007). In addition, insulin seems to be as important for embryo growth, promoting muscle protein deposition, especially during the intense muscle development occurring in the second week of incubation. This period is characterized by increased levels of plasma insulin and the mobilization of proteins stored in yolk and albumen (Lu et al., 2007).

As hatching approaches, the low oxygen supply impairs energy production from fatty acid oxidation. Thus, glycogen reserves are mobilized to maintain glucose homeostasis (De Oliveira et al., 2013). Indeed, glucagon plasma levels increase continuously after ED15 (Lu et al., 2007), promoting glycogenolysis and gluconeogenesis from muscle proteins in the end of incubation (Christensen et al., 2001).

Thyroid hormones are also important during embryo development and initial posthatch phase. Triiodothyronine (T3) and thyroxine (T4) are related to heat production, muscle growth, glycogen mobilization, and fat mobilization (Christensen et al., 2003). Thyroid hormones are also involved in the development of the hatching muscle and preparation for eggshell rupture (Christensen et al., 2003). Lu et al. (2007) reported that T3 levels were practically constant throughout the incubation process, with a sudden increase at hatch that lasts for the first week of life, whereas T4 levels increased significantly from ED15 and reached a peak at ED19, decreasing after hatch.

The establishment of pulmonary respiration provides increased oxygen supply, concomitant with increased T3 plasma levels at hatching (Lu et al., 2007). Oxygen consumption and heat production are stimulated by thyroid hormones and may compensate for the immature thermoregulatory system during the first week of life. Increased T4 levels after ED15 possibly stimulate increased amino acid uptake during the final period of incubation to ensure proper development of the embryo. In addition, it is possible that T4 acts synergistically with glucagon in promoting glycogenolysis (Lu et al., 2007).

Insulin-like growth factors (IGF-I and IGF-II) have been detected in the amniotic and allantoic fluids, but their role in chicken embryo metabolism remains unclear. The mobilization of amino acids from the amnion and allantoic fluid is likely regulated by IGF-I (Karcher et al., 2005). It has also been suggested that IGF-I and -II promote glycogen and protein synthesis in the liver (De Oliveira, 2007). According to Decuypere and Buyse (2005), IGF-I increases amino acid uptake and protein synthesis in muscle cells and negatively regulates muscle protein degradation in broilers. Plasma IGF-I and IGF-II levels increase significantly from ED10 to ED14, and then decrease slowly until hatching (Lu et al., 2007). Thus, it is possible that IGFs play a critical role as modulators of tissue maturation, directly influencing embryo growth and development after the mid-incubation period.

INTESTINAL CHANGES DURING EMBRYOGENESIS

The functional capacity of the gastrointestinal tract improves due to extensive morphological and physiological changes following amniotic fluid intake after ED17 (Uni et al., 2003b; Uni and Ferket, 2003; Dibner and Richards, 2004). Glycogen synthesized from amnion-derived substrates is vital during hatching and posthatch development (De Oliveira et al., 2008). Thus, amniotic fluid volume and nutritional composition are crucial during the physiological and metabolic posthatch transition (Uni et al., 2005).

In the last week of incubation, intestinal weight increases at a higher rate than body weight. Uni et al. (2003b) reported that intestine–to–body weight ratio changed from 1% at ED17 to 3.5% on hatching day. Morphological changes of the intestinal mucosa start on ED15, with the presence of rudimentary villi; at ED17, villi differ in length and shape in accordance with the developmental phases (mainly **V1** and **V2**)

(Uni et al., 2003b). Higher percentage of the short and pointy V2 villi is seen at ED17 (65%), whereas the longer and digitiform V1 villi prevail as hatching nears (Uni et al., 2003b). Expansion of the surface area for digestion and absorption results from intestinal villus growth and, consequently, higher density of cells involved in defense (goblet cells), digestion and absorption (enterocytes), and regulation of gastrointestinal function (enteroendocrine cells) (Dibner and Richards, 2004).

The functional development of the intestinal mucosa also starts during the final third of incubation. During embryogenesis, brush border enzyme activity is minimal. Activity increases as the hatching process nears and is possibly affected by ingestion of amniotic fluid. Greater activity of brush border disaccharidases, dipeptidases, and tripeptidases has been reported between ED16 and ED20 (Uni et al., 2003b). In broilers, isomaltase was detected after ED15, and sucrase and maltase activities increased rapidly between the prehatching period and 2 d after hatch (Uni et al., 2003b). Aminopeptidase expression in broilers starts around ED15 and increases as hatching nears (Uni et al., 2003b; Speier et al., 2012).

Monosaccharides, amino acids, dipeptides, and tripeptides released from enzymatic reactions are then transported into enterocytes by protein carriers (Speier et al., 2012). Similar to brush border enzymes, greater expression of intestinal transporters in broilers is detected between ED16 and ED20, showing an increase of 15 to 40 times (Uni et al., 2003b). There are specific transporters for anionic, cationic, or neutral amino acids (Hyde et al., 2003). Dipeptides and tripeptides are transported by the peptide transporter 1 (**PepT1**) (Gilbert et al., 2008) and glucose is transported by the sodium-glucose cotransporter (**SGLT1**) (Uni et al., 2003b), both of which are sodium-dependent transporters. The expression of *PEPT1* in broiler embryos was detected on ED15 and increased closer to hatch (Speier et al., 2012). Furthermore, *PEPT1* expression was higher in the duodenum and proportionally lower in the jejunum and ileum (Gilbert et al., 2007). In broiler embryos, *SGLT1* was initially expressed in low levels from ED15 to ED17 and increased more than 3 times by ED21 (Uni et al., 2003b).

Accessory glands also have greater functional development in the last third of incubation. Pancreatic enzymes are secreted by the end of incubation and the activity of carboxypeptidase A, chymotrypsin, and pancreatic lipase gradually increases from ED16 to hatching (Marchaim Kulka, 1967, cited by Uni et al., 2003b). Pancreatic amylase and trypsin were detected on ED18 and significantly increased shortly after hatching (Moran, 1985, cited by Noy and Sklan, 1997).

Although morphological and functional development of the gut starts before hatching, greater changes occur mostly after feeding in the posthatch period. Higher rates of allometric growth are seen in the intestine compared with the body weight, as a result of the rapid enterocyte proliferation and differentiation (Geyra et al., 2001). Limited nutrient stores in the hatchlings stimulate the functional development of the gastrointestinal tract, improving digestion processes, nutrient assimilation, and chick survivability. Furthermore, intestinal mucosa growth is affected by metabolic hormones, physical and chemical characteristics of the diet, and intestinal microbiota (Maiorka et al., 2000). *In ovo* feeding can be an interesting approach to accelerate the ability to utilize dietary nutrients, enabling hatchlings to better express their genetic potential and resist pathogenic microorganisms (Uni and Ferket, 2004).

INFLUENCE OF *IN OVO* FEEDING ON INTESTINAL DEVELOPMENT AND PRACTICAL ASPECTS

In ovo feeding (USA Patent # 6.592.878 B2) provides nutrient solutions in the amniotic fluid of broiler and turkey embryos (Uni and Ferket, 2003). The technique was optimized in a series of studies and differs from previous approaches because nutrients are injected exclusively in the amniotic fluid, ensuring the ingestion and contact of these nutrients with the developing intestinal mucosa surface.

Several studies have assessed the effects of IOF on morphological and functional development of the intestinal mucosa, showing that IOF efficiently improves posthatch intestinal development in broilers, turkeys, and quails. Studies have focused mostly on *in ovo* supplementation of amino acids and carbohydrates, but prebiotics and hormones have also been tested.

Carbohydrate solutions injected *in ovo* increased villus surface area at hatch and 3 d after hatch, goblet cell counts, and mucin (***MUC2***) gene expression, suggesting higher resistance against pathogens (Smirnov et al., 2006). Maltose provided by IOF improved jejunum villus development, increased hatchling weight and contributed to better nutrient absorption (Jia et al., 2011). Supplementation with glucose increased interleukin 6 (***IL-6***) and *IL-10* expression, whereas supplementation with ribose increased *IL-2* and *IL-12* expression (Bhanja et al., 2014a).

In ovo injection of a 0.5 g/L zinc-methionine solution increased the expression of zinc-methionine transporter, the activity of brush border enzymes (aminopeptidase and isomaltase), and the villus surface area (Tako et al., 2005). Threonine (**Thr**) supplemented *in ovo* improved feed conversion and weight gain from 14 to 21 d and from 21 to 28 d and increased the activity of proventricular pepsin and pancreatic amylase (Kadam et al., 2008). Moreira Filho et al. (2018a) showed that *in ovo* Thr increased final weight and weight gain and decreased feed conversion from 1 to 21d. Threonine levels improved villus height, villi:crypt ratio and villus area on day of hatch and at 21 d. At hatch, all Thr levels increased the expression of *MUC2* and *PEPT1* compared with the control group. Aminopeptidase (***APN***) expression also increased, but for the lowest and the highest Thr levels (1.75 and 7%). At 21 d, there was no effect of Thr on *MUC2*, *PEPT1,* and *APN* expression. Dai et al. (2020) showed that IOF increased duodenum

weight linearly in 3-day-old chicks and jejunum weight in hatchlings and 14-day-old chicks. Arginine levels improved villus height and reduced crypt depth in the duodenum of 14-day-old chicks. In the jejunum, the *in ovo* administration of arginine increased villus height and villus height: crypt depth ratio on 3-day- and 14-day-old chicks (Dai et al., 2020).

In ovo Thr and arginine increased *MUC2* gene expression, whereas Thr and methionine + cysteine increased *IL-2*, *IL-6,* and tumor necrosis factor alpha expression in the intestinal mucosa (Bhanja et al., 2014b). The supplementation with **Thr** and arginine increased the jejunum and ileum length and increased villus height in the ileum, but not in the jejunum (Tahmasebi and Toghyani, 2015).

In a recent study, Moreira Filho et al. (2018b) observed that *in ovo* Thr supplementation reduced *Salmonella* Enteritidis colonization 6 d after inoculation and reduced the negative effects associated with *Salmonella* infection on intestinal morphology and performance, with results similar to those of the sham-inoculated birds. *In ovo* Thr supplementation increased *MUC2* expression at hatch and *MUC2* and *IgA* expression at 2 d of age and 6 d after inoculation in the ileum.

Supplementation of prebiotics and synbiotics *in ovo* has also been studied. Prebiotic compounds are nondigestible carbohydrates that act primarily as modulators of the intestinal microbiota, favoring the growth of beneficial bacteria (Ricke et al., 2020) and reducing colonization of the gastrointestinal tract by pathogenic bacteria, thereby improving intestinal mucosa integrity and health (Santos et al., 2013).

Mannanoligosaccharide supplemented *in ovo* has been associated with improved intestinal mucosa development, including increased villus height, villus area, and goblet cell counts, as well as greater crypt development (Cheled-Shoval et al., 2011). The authors also reported upregulation in *APN*, isomaltase, and *MUC2* expression at hatch, which was no longer present at 3 d of age.

Pruszynska-Oszmalek et al. (2015) reported that *in ovo* injected prebiotics and synbiotics increased overall pancreatic enzyme activity, including amylase, lipase, and trypsin. *In ovo* synbiotics treatment increased villus height:crypt depth ratio in the jejunum, which might result in improved nutrient absorption and weight gain (Mista et al., 2016). *In ovo* synbiotic administration significantly increased the concentration of higher and wider intestinal villi, and the absorbent surface of the duodenum on the first and 42nd of life (Sobolewska et al., 2017). Increasing doses of *in ovo* raffinose resulted in linear increase of villus height at hatch and 21 d, as well as increased villus height:crypt depth ratio on d 21 (Berrocoso et al., 2017).

Recent studies have also investigated the effect of vitamin supplementation *in ovo* (El-Senousey et al., 2018; Zhang et al., 2019; Araújo et al., 2019; Fatemi et al., 2020). Ascorbic acid *in ovo* supplementation improved both the antioxidant and the immune systems in hatchlings (El-Senousey et al., 2018). Different doses of ascorbic acid (3 to 12 mg per egg) have lasting positive effects on posthatch growth, leg muscle development, and systemic antioxidant capacity of broilers, whereas higher doses (36 mg per egg) may also improve broiler meat quality (Zhang et al., 2018). *In ovo* vitamin E improved hatchling quality and the oxidative status of chicks, resulting in better performance (Araújo et al., 2019). Late embryo mortality, feed intake, and feed conversion ratio were lower when 25-hydroxycholecalciferol was injected *in ovo* as compared with injection of diluent or vitamin D3 (Fatemi et al., 2020).

In terms of hormonal stimulation *in ovo*, Moosavinasab et al. (2015) reported that *in ovo* IGF-I increased small intestine levels of alkaline phosphatase on day 21 and small intestine sucrase concentrations on days 21 and 42 after hatch. However, no significant effects were observed on leucine amino peptidase activity in the duodenum, jejunum, or ileum at 21 and 42 d.

Overall, *in ovo* supplementation with different nutrients and substances seems to promote earlier development of the digestive tract and muscle tissues, improve digestive efficiency, and enhance the immune system of hatchlings, thus contributing to the maintenance of glycogen stores and posthatch survivability.

Interestingly, chick response to IOF may depend not exclusively on the injected nutrient, but also on factors that are not completely understood, such as breeder genetics and age, egg size, and incubation conditions, among others.

IN OVO FEEDING AND EMBRYO METABOLISM

Embryo metabolism was shown to be affected by IOF. Uni et al. (2005) evaluated glycogen reserves in embryos injected with a nutrient solution containing maltose (25 g/L), sucrose (25 g/L), dextrin (200 g/L), and calcium β-hydroxy-β-methylbutyrate (**HMB-calcium**, 1 g/L) diluted in saline (0.5% NaCl) and reported increased hepatic and muscular glycogen reserves at the end of the incubation process, and higher yield of pectoral muscle at 25 d of age in broilers. *In ovo* HMB-calcium also increased hepatic glycogen stores on ED19 and ED20, and on hatch day (Kornasio et al., 2011). Similarly, glycogen stores in the liver and pectoral muscle increased linearly after *in ovo* administration of carnitine, and the glycogen status of hatched chicks was improved by higher doses of carnitine (Shafey et al., 2010).

In ovo leptin administration increased the hepatic content and serum concentration of leptin in newly hatched chickens, and upregulated hepatic mRNA genes of enzymes regulating lipid metabolism, such as sterol regulatory element binding protein 1c and 2, hydroxy-3-methylglutaryl coenzyme A reductase and cholesterol 7α-hydroxylase 1 (Hu et al., 2012). According to the same study, triglyceride and total cholesterol contents decreased in the liver and increased in the serum, together with apolipoprotein B levels.

Neves et al. (2016) and Dal Pont et al. (2019) evaluated the effect of glycerol supplementation *in ovo*. A quadratic effect of glycerol levels on blood glucose and a linear effect on liver glycerol kinase activity were observed (Neves et al., 2016). In embryos from young breeders, greater deposition of liver glycogen and better performance were seen in response to *in ovo* glycerol administration (Dal Pont et al., 2019).

Arginine *in ovo* increased both glycogen and glucose concentrations in the liver and pectoral muscle of broilers at hatch. Plasma glucose and insulin levels were higher in the arginine-supplemented group. L-arginine supplementation increased hepatic glucose-6-phosphatase, phosphoenolpyruvate carboxykinase, and fructose 1,6-bisphosphatase mRNA expressions at hatch. In addition, broilers in the Arg group had higher mRNA expression of glycogen synthase and a lower expression of glycogen phosphorylase in the liver and pectoral muscles than control birds at hatch (Yu et al., 2018).

Zhao et al. (2018) observed that IOF of creatine pyruvate increased the concentrations of glucose and glycogen in liver at ED19 and at hatch. Concentrations of creatine and phosphocreatine, and creatine kinase activity in embryos were enhanced in creatine pyruvate treatments at ED19, regardless of dosage. The same authors also reported that this treatment increased the activity of glucose-6-phosphatase in the liver at ED19.

The effects of *in ovo* supplementation with creatine pyruvate on the energy metabolism in thigh muscle of embryos and neonatal broilers were studied (Yang et al., 2019). *In ovo* creatine pyruvate increased glucose and creatine concentrations in the thigh muscle of broilers 2 d after injection. Chicks in the creatine pyruvate group showed higher mRNA expressions of glucose transporter 3 (***GLUT3***) and *GLUT8* on the day of hatch and increased the activities of hexokinase and pyruvate kinase 2 d after injection and on the day of hatch (Yang et al., 2019).

In a recent study, *in ovo* supplementation with vitamin E increased protein total concentration in the liver and pectoral muscle, indicating higher level of tissue protection against protein oxidation in broiler chicks (Araújo et al., 2019).

CONCLUSIONS

Historically, avian breeding programs have focused on obtaining more precocious birds with enhanced performance parameters. Currently, IOF technology provides an alternative to maximize productivity at early stages of the production cycle, contributing to improve profitability in the broiler industry. Immunocompetence seems to be partially compromised in fast-growing lines and, because the use antibiotics as growth promoters tend to be banned in farming animals, IOF technology can play a strategic role in the future of broiler production in this aspect. Putative benefits of IOF might not be restricted to improved performance and resistance of birds against traditional avian pathogens, but also in terms of public health, because the emergence of antimicrobial resistance in pathogenic bacteria has become a global threat. Therefore, antimicrobial stewardship in animal production is necessary to mitigate antimicrobial resistance and warrant that efficient drugs will be still available for therapeutic purposes.

Although nonantibiotic alterative products have been extensively tested after hatch, information on their effects on the embryo development is still scarce, particularly that involving early modulation of gut microbiome. Understanding how IOF affects the intestinal microbiota dynamics and also embryo metabolism will help to elucidate the factors that may change the resident microbiota. Manipulating the microbiota beneficially might help to ensure intestinal health and improve production results in the absence of antibiotic growth promoters.

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DISCLOSURES

The authors declare no conflicts of interest.

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