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Chicken Embryo Utilization of Egg Micronutrients

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Introduction

Avian embryos develop and grow from energy and nutrients stored in the egg by the hen. In this sense, breeder male contribution is not important. Follicular deposition of nutrients occurs over a wide range of time, but becomes relevant during the week prior to ovulation. Amounts, but also forms, of nutrients deposited in the egg determine success of embryo development and hatching of a healthy chick. To support the embryo during incubation, oxygen is the only nutrient not derived from the egg; however, adequate oxygen concentrations in the surrounding air and its free passage through well-established shell membrane porosities are essential. Developmental limitations and embryo mortality are aggravated when any inadequacy in these conditions prevail.

Macroscopically, the fertile egg has three components: shell, yolk, and albumen. Specific nutrients are mobilized by the embryo from each of these parts. These typically have expected compositions, which are notably affected by the hen's nutrition.

After oviposition, the egg may be submitted to changes due to *in ovo* injection using the vaccination equipment routinely used in hatcheries. However, these injections are limited to small amounts, and preferably done with more available forms of micronutrients. Research on this matter is recent and usually directed to metabolic interventions, such as the role of substances which stimulate or mediate enzyme transcription.

Egg Reserves

Macroscopically, the egg is a simple structure with three major components (shell, albumen, and yolk), demonstrating unique characteristics and playing specific roles during incubation. It is generally agreed that each egg is built with a complete capacity to produce a perfect new organism. However, due to several reasons, this is not always the case. In general, the composition of macromolecules in eggs is more constant than those elements usually found in a lower proportion, such as micronutrients. Since all egg constituents derive from the hen's organism, metabolic transformation of the hen's feed is needed to obtain adequate embryo development and viable chicks at hatching.

Egg shells consist of two main components: a matrix of protein fibers and columns of calcium carbonate crystals in a proportion of 1:50 (Romanoff & Romanoff, 1949). A glycoprotein layer, the cuticle, covers the crystal surface, and it is the last egg component to be deposited before oviposition. The cuticle has three main functions: prevention of excessive water loss (Peebles & Brake, 1986), protection from microbial infection (Roussel *et al.*, 1988), and termination of calcite crystal growth (Dennis *et al.*, 1996). The shell matrix presents two regions: a mammillary region, connected to the outer shell membrane protein fibers, and a spongy one, associated to calcite crystals deposited in layers on the mammillary matrix (Simkiss, 1968).

The shell is the primary source of calcium for the developing chick embryo (Johnston & Comar, 1955). The dissolution of minerals from the shell to be used by the embryo seems to be facilitated by the acid produced by the cells of the chorioallantoic membrane (Leeson & Leeson, 1963). Calcium is by far the largest eggshell component (98.2%), but other minerals are also likely to be supplied from the shell to the developing embryo, such as magnesium (0.9%) and phosphorus (0.9%) (Romanoff & Romanoff, 1949). Important trace minerals present in the shell are copper, zinc, manganese, and iron (Richards, 1997).

Whereas the yolk is the primary storage site of trace minerals, copper is mostly present in the eggshell and corresponding membranes (Richards, 1997). Copper main function in the egg is related to the shell structure, specifically matrix collagen synthesis and maintenance. Indeed, laying hens fed a copper-deficient diet produced eggs with abnormal shell membrane (Baumgartner *et al.*, 1978). Copper is essential for the normal maturation of collagen (Rucker & Murray, 1978). The activation of lysil oxidase also appears to involve a role of copper as cofactor, which may also act on the regulation of lysil oxidase synthesis (Harris *et al.*, 1974; Harris, 1976). A well-structured outer shell membrane is required for mineralization and strong eggshells. In addition, adequate membrane structure allows the separation between the inner and the outer shell membranes, promoting the proper formation of the air chamber immediately after oviposition. This is an important oxygen reservoir used by the embryo during pipping. Copper in the shell seems to be essential for the chick embryo metabolism, as it was shown that shell-less cultures led to a failure in accumulation of normal amounts of hepatic copper during the latter half of incubation (Richards *et al.*, 1984). Effects on the metabolism of zinc and iron were also observed (Richards *et al.*, 1984).

The albumen consists of four layers with similar chemical constitution. Water is the main component of albumen, with around 88% of total albumen content (Marion *et al.*, 1964). Protein is the next largest component, whereas lipids and minerals are included in minimal fractions (Romanoff & Romanoff, 1949). Egg albumen proteins present great diversity; from which ovalbumin represents 54% of the total protein content (Li-Chan *et al.*, 1995). Ovalbumin has a high biological value, and it is an important nutrient during the last stages of embryo development, as well as phosphorus, carbohydrates and iron, zinc and copper in lower amounts (Nisbet *et al.*, 1981). Ovotransferrin, which is also called conalbumin, an important albumen protein, whereas ovomucoid (anti-trypsin activity) and lysozyme (antimicrobial activity) are minor components. Important vitamin inhibitors are ovoflavoprotein (inhibits riboflavin) and avidin (inhibits biotin) (Powrie & Nakai, 1985).

Micronutrients are present in much lower concentrations in albumen as compared to the yolk. Riboflavin is the only vitamin which may present relevant concentrations in the albumen; it may reach 61% of total egg content stores in the albumen. However, riboflavin levels in albumen are very sensitive to its levels in the hens' feed, and may rapidly change. Naber & Squires (1993) observed that riboflavin in eggs was reduced to deficient levels four to seven days after hens were fed diets without the vitamin. Authors observed reduced hatchability, but no effect on egg production.

There are very few minerals of importance for the embryo in the albumen. Chlorine, magnesium, potassium, sodium, and sulfur are exceptions among minerals which have greater contents in albumen as compared to the yolk (Cook & Briggs, 1986). Albumen proteins are not a significant source of trace minerals for the embryo, despite their ability to bind them. Ovotransferrin, is a glycoprotein with two iron binding sites, but iron content in this protein is very low (Palmer & Guillete, 1991). Organic forms of trace minerals may be an exception, since they are preferentially deposited into the albumen as compared to their inorganic salts, which are mainly deposited in the yolk (Latshaw, 1975).

Nutritionally, the yolk is the egg richest fraction. It is separated from the albumen by the vitelline membrane, which has four layers: two formed in the oviduct and two in the ovary (Etches, 1996). Yolk contents derive from liver transformation of lipids and proteins, and become important five to seven days before ovulation (Etches, 1996). Most yolk components are very-light density lipoproteins, accounting for 66% of the yolk dry matter, and are solutes in a true solution (Burley & Vadehra, 1989). This fraction consists of 80% lipids, out of which 25% are phospholipids (Martin *et al.*, 1964). Cholesterol corresponds to 5.2% of total yolk lipids (Privett *et al.*, 1962). Phospholipids are predominantly phosphatidilcholine (73%) and phosphatidilethanolamine (15%). Palmitic and estearic acids are the main fatty acids (30 and 38%, respectively) of the lipid fraction (Burley & Vadehra, 1989).

Yolk proteins are usually associated to lipids. Vitellogenin, produced in the liver in response to estrogen, is the main egg yolk precursor. Oocyte accumulation of vitellogenin from the hen's circulation continues until ovulation by receptor-mediated endocytosis; however, it is immediately subjected to proteolysis, producing phosvitin and lipovitellin (Shen *et al.*, 1993). Phosvitin is a particular protein because it contains about 10% phosphorus, and an amino acid sequence with 31% of serine, in addition to iron (Taborsky, 1980). Lipovitellins have 20% lipids, out of which 60% are phospholipids. Along with phosvitin, lipovitellin accounts for 20% of yolk dry matter (Li-Chan *et al.*, 1995).

Except for riboflavin, all other vitamins are expected to be in higher concentrations in the yolk than in the albumen. Obviously, fat-soluble vitamins are expected to accumulate rapidly in the yolk after their levels are increased in the feed. Vitamin A contents in the egg yolk remained stable throughout egg production when hens were supplemented with 9,000 IU of vitamin A/kg of feed, whereas its levels declined and were not able to sustain egg production in non-supplemented hens (Squires & Naber, 1992). Moreover, it seems that some water soluble vitamins present similar response. For instance, vitamin B12 concentrations in egg yolks increased in parallel with increments in the feed, and are indicative of vitamin B12 status in the hen (Squires & Naber, 1992). Folate concentration, following folic acid supplementation, was 100 times higher in the yolk than in the albumen (Sherwood *et al.*, 1993).

Phosvitin and lipovitellin are metal-binding proteins, which bind iron, copper, and zinc, and are thought to regulate trace mineral supply to the embryo according to differential utilization of each protein fraction (Richards, 1997). Most iron in the yolk (90%) is bound to phosvitin (Greengard *et al.*, 1964), whereas 90% of zinc is bound to lipovitellin (Tupper *et al.*, 1954). Lipovitellin also binds copper (McFarlane, 1932). Yolk granules also accumulate manganese (Grau *et al.*, 1979).

Opposite to phosvitin and lipovitellin, which are granule proteins, livetins are proteins present in the plasma fraction. They account for 10% of yolk dry matter, and generally consist of α-, β-, and γ-livetins, which correspond to the hens' plasma albumin, α2-glycoprotein, and mixed immunoglobulins, respectively (Williams, 1962). The proteins γ-livetins are also usually called IgG or IgY (Leslie & Clem, 1969).

Yolk carbohydrates are negligible, corresponding to less than 1% of the total egg carbohydrates, out of which only 0.3% is free glucose, and the rest is mostly bound to protein and lipids (Romanoff & Romanoff, 1949). The yolk also has 1.1 % ash, in which phosphorus, calcium and potassium are the largest proportion.

Maternal nutrition transferred to the chick embryo

Transference of nutrients from the hen to the egg follows two pathways: via the ovary to the yolk or via the oviduct to the albumen, egg shells, and membranes. Chemically, the transference of nutrients from the hen to the ovary and the oviduct involves the synthesis and the export of proteins able to bind specific molecules. Inside the egg, the embryo develops specific mechanisms to mobilize previously stored vitamins and minerals by means of transport proteins. Nutrient absorption, metabolism, and deposition vary with hens' genetics (Lillie *et al.*, 1951). Marginal deficiencies can significantly affect some chickens in a flock but not others, leading to higher embryonic mortality rate at the end of incubation (Wilson, 1997). High mortality during the second week of incubation of chicken embryos suggests nutrient deficiencies in hens' diets, as normal mortality rates are very low during this period (Leeson *et al.*, 1979). Excesses, as well as deficiencies, can affect embryo development, and may interrupt egg production by the hen. As nutrient deficiency or excess advances and becomes severe, effects on embryo development aggravate, and become evident earlier (Wilson, 1997).

Severe deficiencies in broiler breeder diets are not very common or expected in today's egg and chick production. However, there are factors involved in practical chick production that may affect egg production and progeny performance. The inclusion of animal by products in broiler breeder diets is usually not recommended in order to reduce or to limit vertical transmission of microorganisms to the chick; however, the impact of all vegetable diets is usually underestimated in terms of the potential limitation of nutrient availability. Nutrients present in low concentrations, such as vitamins and trace minerals, are usually more available in animal by products as compared to vegetable meals. Soybeans are rich in phytic acid, which binds many trace minerals, and potentially affects animal performance. Savage (1968) observed that an increase in dietary phytic acid reduced zinc availability, affecting egg production and hatchability. Broiler breeder diets are supplemented with higher vitamin and mineral levels as compared to broiler diets; however, these supplementations often use cheap supplements, which quality is suspicious. Inal *et al.* (2001) observed that zinc levels in the egg yolks of supplemented hens were twice as high as compared to hens fed diets without premixes.

The efficiency of dietary vitamin transference to the egg is considered very high for vitamin A; high for riboflavin, pantothenic acid, biotin, and vitamin E; intermediate for cholecalciferol; and low for vitamin K, thiamin, and folic acid (Naber, 1993). Vitamin utilization by the chick embryo, however, depends on the active form present in the supplement. For instance, Dersch & Zile (1993) suggested that trans-retinoic acid is the most effective form of vitamin A for chick cardiovascular development, and needed in this form before incubation. Increasing pyridoxine supplementation to 18 ppm in turkey diets did not influence chick hatchability or embryo mortality (Robel, 1992).

Biotin and riboflavin are vitamins with important characteristics due to the presence of the inhibitors avidin and ovoflavoprotein in egg albumen, which affects their availability to the embryo. Deficiencies of these two vitamins are not uncommon, and may affect egg hatchability in just a few days after the hens are fed deficient diets (Leeson *et al.*, 1979). Embryo responses to supplementation of these vitamins in hen diets, as well as after *in ovo* injection, are very rapid (Couch *et al.*, 1949; Robel & Christensen, 1987). Vitamin B12presents different binders in the yolk and in the albumen, which could explain the differential vitamin B12 absorption from the egg yolk and the albumen, respectively (Levine & Doscherholmen, 1983).

Pyridoxine concentration in egg yolk remains stable in response to incremental levels in turkey breeder diets, whereas it increases in albumen. Average pyridoxine levels in the albumen is only 4% of that present in the yolk (Robel, 1992).

Vitamin B12 is not present in plants; therefore, breeder diets without animal by products require higher enrichment of this vitamin. Niacin has very low availability in plants, particularly in corn, and its utilization also varies as a function of chicken genetics (Leeson *et al*, 1979). Folic acid is a critical vitamin for all animals during reproduction, and its requirement for hatchability is higher as compared to egg production (Taylor, 1947). Egg folate content is maximized when dietary folic acid reaches 2 mg/kg, as the pool of folate precursors is saturated after this level (Hebert *et al.*, 2005). Long-term storage of eggs leads to folic acid deficiency (Whitehead *et al.*, 1985).

Vitamin E concentration in egg yolks is a direct function of its concentration in the feed. Vitamin E levels in the embryo are similar to the level found in the yolk at the early stages of incubation. The highest alpha-tocopherol levels are found in the liver, but they are rapidly depleted after hatching (Surai *et al.*, 1997). Vitamin E has been related to breeding efficiency in several species, and with immune response in poultry. Haq *et al.* (1994) observed higher progeny antibody titers after vitamin E supplementation of breeder diets. Heat stress increases metabolic peroxidation in poultry, and hens exposed to heat stress lay eggs with lower vitamin E concentrations in the yolk (Kirunda *et al.*, 2001). Maternal supplementation of vitamin E levels as high as 160 IU in the feed may be required by chicks exposed to oxidative stress (Lin *et al.*, 2005). In fact, poults from young breeders presented lower mortality when these were fed 300 IU/kg vitamin E (Siegel *et al.*, 2006).

Vitamin D is preferably deposited in the yolk in the form of vitamin D3 as compared to D2. The efficiency of Vitamin D3 deposition in the yolk is around 2.2 times higher than D3 (Mattila *et al.*, 2004).

Interactions between vitamins may affect the expression of their functions. Vitamin E, ascorbic acid, and carotenoid liver levels of hatching chicks are affected when excessive levels of vitamin A are fed to breeders, which potentially compromise the antioxidant status of the hens' progeny (Surai *et al.*, 1998).

Mineral deficiencies can also be quickly induced in developing embryos when breeders are not correctly supplemented. In general, egg production is not affected, but egg mineral content may limit embryo development (Richards & Steele, 1987). Trace mineral deficiencies lead to reduced growth rate, abnormal organ development, and embryo death in extreme cases (Savage, 1968). Trace mineral levels deposited in the eggs are variable and dependant on the quantity and chemical form present in the hens' diet (Naber, 1979).

Important differences exist among breeders from the beginning to the end of the egg production cycle. These differences account for the higher mortality rates of embryos from young breeders. At the end of incubation, young breeders' embryos accumulate residual lipids in the yolk sac, whereas in older breeders' embryos the highest amount of lipids is found in the liver (Yafei & Noble, 1990). This reduced utilization of lipids by young breeders' embryos possibly influences their vitamin and trace mineral uptake.

In ovo nutrition

The introduction of nutrients directly into the embryo was performed in several occasions. Experimentally, this was carried out using a manual device in different moments. *In ovo* vaccination technique is currently proposed as practical and safe to carry out this supplementation. However, *in ovo* injection is performed when eggs are transferred from the incubator to the hatcher later in incubation and, therefore, the correction of nutrient deficiencies is limited. Eggs from breeders fed biotin-deficient diets produced healthy embryos after the eggs were injected with biotin between 72 and 96 hours of incubation (Couch *et al.*, 1948).

In ovo injection of turkey eggs with biotin, folic acid, and pyridoxine improved hatchability of fertile eggs (Robel, 2002). This supports the higher biotin requirement for hatchability of aging hens. Because turkey hens were fed recommended or higher levels of these vitamins, and a vitamin deficiency related to hatchability was not apparent, it was considered that the transport of vitamin from the egg to the chick was responsible for the failure in embryo development. This reasoning reinforces that the practice of *in ovo* injection of vitamins, as well as other nutrients, may become a routine in poultry production.

The introduction of nutrients using *in ovo* vaccination equipment assumes nutrient utilization during the period the embryo swallows amniotic fluid. Therefore, nutrients enter in a direct contact with the enterocyte, and may affect its differentiation prior to hatching. Tako *et al*. (2004) observed that the administration of nutrients into the amnion stimulated the intestinal development of hatching chicks, as determined by increase in villi length and disaccharide digestion improvement. In another study, Tako *et al*. (2005) observed that zinc methionine injected *in ovo* at 17 days of incubation improved enterocyte function. These authors observed improvement in the biochemical activity of enzymes and transporters, as well as in villi surface at hatching. Embryos injected with zinc methionine originated broilers with improved weight gain, which lasted for 14 days after hatching.

Maternal nutrition and progeny performance

It has been stated that breeder diets are fed energy and nutrients to optimize egg production, and to eventually maximize chick numbers from every hen. In the sequence of events following embryo development, protein and energy are first obtained from the yolk, and derive from the albumen only after 14 days of incubation. Minerals are also primarily obtained from the yolk and from the shell only after the chorioallantoic circulation is established. Bone mineralization process requires phosphorus, derived from yolk phosvitin, which reacts with calcium from the shell. Intense metabolism, involving carbonic acid and enzymes, is in effect for mineral removal from the egg. During this process, it is very likely that other important minerals for the embryo, such as magnesium, copper and zinc, are also solubilized.

Optimal mineral nutrition of the breeder does not necessarily require higher levels of mineral sources. Actually, increasing mineral salt contents in feeds potentially leads to individual competition between mineral salts with similar electronic charges. Zinc requirements for egg production are lower than those for hatchability (Savage, 1968). Hudson *et al.* (2004) observed that egg specific gravity improved, the number of cracked eggs was reduced, and the number of hatching eggs per hen increased when broiler breeders were fed zinc methionine during the egg production cycle. These authors also found a significant increase in the amount of zinc deposited in the eggs, and reduced embryo mortality during the first week of incubation. Immune response also improved when breeders were fed zinc methionine. Hudson *et al*. (2004) observed that Newcastle Disease titers and humoral response, as measured by phytohemagglutination, improved when breeders were fed zinc methionine. Zinc is recognized as a nutrient involved in the expression of immunity (Kidd *et al*., 1996). Virden *et al*. (2003) observed that broiler chicks from breeders fed zinc- and manganese-amino acid presented lower early mortality. Virden *et al*. (2004) also found a positive influence on heart functional capacity in the progeny of breeder hens fed zinc- and manganese-amino acid. In this latter study, it was also observed that birds had better immunity.

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