MOLECULAR AND CELLULAR BIOLOGY
Non-Invited Review

Recent advances in avian egg science: A review

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ABSTRACT

Eggs and egg products form an integral part of the food chain. As such, research into egg structure, function, and production has made an important contribution to the field of poultry science. The past decade has seen significant advances in avian egg science research, with work supplementing our understanding of the nature of the avian egg, and its biological, chemical, and physical properties.

Eggshell color, strength, and chemical composition, poultry nutrition, and genetics have all been intensively studied recently, with significant progress being made in a number of these areas. Indeed, with the prevalence of robust theoretical techniques, it is now commonplace to combine experimental investigations with theory, providing a balanced and interdisciplinary perspective.

Key words: eggshell structure, eggshell function, eggshell formation, laying hen

INTRODUCTION

The avian egg is a chemical and mechanical powerhouse, containing within it the ingredients for life, and is protected by a unique crystalline barrier. In poultry, the yolk and albumen are formed in the ovary and oviduct, respectively, and subsequently coated in the uterine fluid of the hen. This secretion contains organic and inorganic materials, with a high concentration of calcium carbonate (CaCO3). The calcium carbonate in an ionic form binds to membrane proteins on the outer albumen, and begins to crystallize. In healthy fowl, this process continues until an even, protective shell is formed, whereby the terminal organic layer, or cuticle, is deposited, and acts most notably as a bacterial barrier.

The main focus of this review will be on poultry eggs, examining factors influencing their color, structure, and properties. Eggs and derived products form one of the most popular classes of foodstuffs. Indeed, according to the UK Department for Environment, Food, and Rural Affairs (2017) in the fourth quarter of 2016, more than 24.9 thousand tonnes of egg products were produced in the UK alone. The inherent commercial interests in poultry egg production have a profound impact on research; most notably, work is ongoing in finding new ways to improve poultry feed for egg and meat production, developing our theoretical understanding of the biological processes of fowl, and considering the effects of the environment on laying hens.

Herein is presented a concise review of progress in the field of avian egg research, with an emphasis on species of poultry. Understanding the molecular basis of eggshell formation can have broad impact on poultry feed production, as well as impact the fields of solid state chemistry and crystallization. This review describes recent research on the factors affecting the quality of eggs and eggshells in particular, beginning with a discussion on pigmentation, moving on to nutritional and genetic factors influencing eggshell production, and finishing with a discussion on the impact of medication on eggshell formation.

EGG PIGMENTATION: AN OVERVIEW

Eggshell color has provided a great deal of material for research. Indeed, it has been shown in numerous studies (Fulton et al., 2012) that the color of eggs significantly affects commercial retail demand. Originally, the brown pigment extracted from eggshells by Sorby (1875), was called oorhodene; however, it was one hundred years later that protoporphyrin IX was in fact found responsible for the color (Kennedy and Vevers,
The pigment is thought to be deposited throughout the formation process; however, 50 to 74% is deposited in the last 5 h before lay (Warren and Conrad, 1942). Hormones and other factors such as the mineral content in the shell gland are responsible for the cessation of cuticle deposition, thereby affecting the incorporation of the pigment. Indeed, a number of factors have been identified, which when combined have significant bearing on the concentration of pigment in the cuticle layer, most notably diet and mineral intake (Nys et al., 1991).

### RELATIONSHIP BETWEEN NUTRITION AND EGG COLOR

Improving the color of eggs has long been of commercial and exhibition interest. With consumers now searching for quality over quantity, following the premise that free-range flocks provide superior eggs (Wang et al., 2009), it has been suggested that maintaining egg color in free-range flocks is more difficult than caged layers (Samiullah and Roberts, 2013).

Nutrition is a contributing factor to the quality and color of the egg, both internally and externally (Sekeroglu and Duman, 2011). Indeed, Hooge (2007) found that feeding probiotics to layers could improve eggshell color, particularly in brown-shelled eggs. Supplements containing *Bacillus subtilis* were administered to 63-week-old Lohman Brown commercial hybrids, resulting in increased pigmentation for up to 2 wk after first delivery. It is not yet clear how this affects the intensity of the color, although the relationship between certain amino acids and mediation of metal incorporation into polyporphyrin has been discussed. Indeed, the enzyme ferrochelatase catalyzes the insertion of iron into protoporphyrin IX. Site-specific mutagenesis on ferrochelatase from *Bacillus subtilis* identified 2 residues in the active site: histidine 183 and glutamic acid 264, which affected the metal binding of the enzyme when modified (Hansson et al., 2007).

Iron soy proteinate (Fe-SP) is characterized as a chelated ferrous group within soy proteinate as a supramolecular environment. Supplements containing Fe-SP can significantly improve eggshell color in brown-egg layers (Seo et al., 2010), while vanadium adversely affects pigmentation of the shell (Sutly et al., 2001). Vanadium is a first-row transition metal often found in small quantities in poultry feeds. Indeed, it appears that a vitamin C dosage proportional to the vanadium intake neutralizes the effects of the metal on shell color (Odabasi et al., 2006). There have been suggestions that vitamin D could be responsible for more dilute shell coloring; however, a recent study showed no significant correlation between the 2 factors (Roberts et al., 2013).

Fe-SP supplementation to the order of 100 ppm was found to significantly increase the intensity of brown pigment in eggshells from a study of 800 26-week-old Hy-line Brown hens (Seo et al., 2010). This is in good agreement with previous work by Park et al. (2004)
and Paik et al. (2009), who suggested that increased levels of Fe led to a proportional increase in erythrocyte formation and breakdown. As erythrocytes synthesize porphyrin, this accompanied an increased intensity in pigmentation of the eggshells.

The effect of nutrition on alternative pigments also has been considered. Indeed, the Araucana breed of poultry originates from South America and lays a blue-green egg, with the color permeating throughout the shell, as opposed to being deposited mainly throughout the cuticle. Araucana chickens were treated with high levels of dietary antioxidants, namely, vitamins A retinol and E acetate, and the reflectance of the shells measured (Dearborn et al., 2012). Differences in eggshell coloration between females of the same breed were also considered on the basis of the sexual signaling hypothesis (SSH). The SSH suggests that the quality of females from a particular species or breed could be deduced from the intensity of eggshell pigmentation (Moreno and Osorno, 2003). Dearborn et al. (2012) reared birds to maturity and administered one of 2 diets, based on high and low antioxidant concentrations, respectively. These diets were reversed after 8 wk to ensure that birds were subjected to both extremes of antioxidant content. Antioxidant content of the feed was found to have little effect on the color of the eggshells produced by the Araucana in this study. Reflectance measurements, however, identified significant differences in pigmentation of eggs laid among different members of the study. It was suggested that the sensitivity of the reflectance detector did not allow for meaningful determination of the effect of antioxidant content in feed on blue-green pigmentation in eggshells of Araucana chickens.

It is clear that feed has some effect on eggshell characteristics, with individual components contributing more or less; however, the color and physical features of eggshells are also rooted in the genetic factors of the species, breeds, and individuals.

**IMPACT OF GENETIC FACTORS ON EGGSHELL COLOR AND QUALITY**

The control of eggshell color by several genes that code for proteins and enzymes regulating the production and deposition of pigments was initially documented in the late 20th century (Vanbrummelen and Bissbort, 1993). Specific brown-egg genes, however, remain elusive, although the higher activity of certain key enzymes in brown-egg layers suggests that the brown egg trait is purely of genetic origin (Schwartz et al., 1980).

Wardecka et al. (2002) established that the same region on chromosomes 2, 4, 5, 6, and 11 influences eggshell color. Phenotypic observations showed that crosses of white with brown-egg layers resulted in intermediate colors—indicating a codominance effect (Hall, 1944). The same study found higher concentrations of pigment in crosses of males from brown-egg-laying breeds with females from white-egg-laying breeds than the reverse, suggesting a degree of sex-linkage associated with this pigmentation.

The expression of 2 genes, SLC01A2 and SLC01C1, in the shell gland was found to be associated with the coloring of the eggs (Zheng et al., 2014). It was suggested by Dunn (2011) that it may in fact be more productive in the short term to explore the genetic basis of pigmentation by means of selection and breeding rather than molecular methods, until such a time as these become less practically and computationally expensive.

A recent advance in genetic and proteomic studies has been the use of novel isobaric tag technology (iTRAQ); this is used to identify specific proteins regulated by functional genes and measure their expression (Ross et al., 2004). Li et al. (2016) employed this technique to identify regulatory proteins responsible for the brown pigmentation and suggest mechanisms for their action. Two-hundred-eighty-one hens laying both light and dark brown eggs were subjected to shell gland epithelial cell sampling, with 147 differently expressed proteins between the 2 groups being identified as affecting eggshell color. Hens laying light-shelled eggs were found to express proteins that decreased the synthesis of protoporphyrin IX, whereas dark-shelled egg layers possessed expression of proteins regulating the formation of hemoglobin. The identification of these proteins by Li et al. (2016) highlights the feasibility of the iTRAQ methodology and provides a basis for protein-based studies on the mechanisms behind eggshell pigmentation.

Francesch et al. (1997) estimated the genetic parameters associated with egg color, using a subset of native Catalan poultry breeds. Penedesencas Negra, Prat Lleonada, and Empordanes Roja populations were obtained from the Catalan Institute for Research, Technology, and Agriculture (IRTA) Poultry Genetic Program. Feed was uniformly administered across the breeds for each period of growth and lay. Eggs were collected from 3 consecutive d each wk, from 18 to 39 wk of age, and color was estimated by reflectometer. The complete pedigree for each hen had been recorded, and average egg weights and color measurements were obtained. The Empordanesa breed was found to possess different genetic characteristics and additional links to egg color and heritability than the other breeds in the study. Indeed, the Empordanesa breed showed a negative correlation among breeding birds selected for egg number, and intensity of pigmentation of the shells of eggs from these strains.

The impact of breeding for intensive laying on the color of eggshells has recently been revisited (Mulder et al., 2016). Environmental and genetic variance in purebred and crossbred laying hens was investigated at different points in the laying period. More than 167,000 eggshells from purebred and more than 79,000 eggshells from crossbred laying hens were analyzed for color. It was concluded that genetic selection could be used.
to judiciously select for uniformity of eggshell color, a trait often sought out by consumers. Indeed, Mulder et al. (2016) suggest future crosses between purebred and crossbred strains in order to maximize selection for uniformity of eggshell pigmentation.

Recent genome sequencing of the Araucana breed of chicken has identified genes responsible for the blue coloration of eggs from this breed. Indeed, work by Wang et al. (2013) and Wragg et al. (2013) had identified expression of the SLC01B3 gene in the uterus of the oviduct in Araucana as resulting in formation of blue eggshells. Jeong et al. (2016) extended investigations in the expression of this gene to alternative blue-egg layers, noticing that the SLC01B3 gene was expressed similarly across the breeds. Moreover, the Araucana derivative breed considered by Jeong et al. (2016) displayed characteristics clearly removed from numerous popular breeds, physical size, flight ability, and egg production being a subset of these. Indeed, the origin of this breed suggests little crossing with other breeds within the investigation, such as Dongxiang from China. It is therefore likely that expression of the SLC01B3 gene is responsible for the coloration of the eggs in breeds known to possess blue-shelled characteristics.

Genetic factors pertaining to various shell characteristics have been studied for a number of years. Indeed, although shell color is of significant commercial interest, the ability of eggshells to endure physical stress is of concern relative to the handling and packing of eggs.

**GENETIC FACTORS ASSOCIATED WITH SHELL STRENGTH**

Sun et al. (2016) published data on candidate genes expressed in the uterus during calcification and their relationship with eggshell mechanical properties. They considered the mechanism for variable eggshell strength to verify if calcification-related genes were involved in the determination of shell strength, as had previously been postulated (Ahmed et al., 2005). Quantitative polymerase chain reaction (qPCR) was used to detect the expression of the selected genes in 2 groups of White Leghorns laying strong and weak-shelled eggs. The eggs were collected at 60 wk of age to obtain samples from the optimum laying period (Zita et al., 2009).

The study found that expression of the CALB1 gene increased in the strong-shelled group compared to the weak-shelled, confirming previous work in the field (Bar et al., 1992). This gene is associated with calcium transport in the intestine and uterus; it is therefore unsurprising that CALB1 expression also affects the strength of eggshell. More intriguing is the overexpression of the DMP4 gene is the weak-shelled egg group. This gene codes for a protein used to bind calcium to the shell, a function different from the transporting behavior coded for by CALB1 (Hao et al., 2007). Sun et al. (2016) therefore suggested additional work on the behavior of DMP4 to gauge the advantageous effects of expression over strength of shell.

Recent work by Sun et al. (2016) employed genome-wide association analysis to understand the genetic basis of eggshell structure and mechanical properties. Sixty-six-week-old hens (n = 927) were subject to analysis, with eggshell thickness, effective layer thickness, mammillary density, and mammillary layer thickness being measured. Four key genes coding for ion transport, ABCC9, ITPR2, KCNJ8, and WNK1, were found to contribute significantly to the regulation of eggshell thickness. The ITM2C and KNDC1 genes also were identified as affecting mammillary thickness and mammillary density, with chromosome lengths found to be proportional to eggshell thickness variation per chromosome. ITM2C conventionally controls the assembly of protein signaling complexes, and the KNDC1 gene is best known for regulating neuronal dendrite growth (Huang et al., 2007). These robust identifications of gene expression relating to mechanics properties provide a meaningful contribution to the understanding of shell ultrastructure.

Although nutritional and genetic factors contribute significantly to numerous eggshell properties, environmental influences have been demonstrated to affect shell characteristics and, in particular, color.

**EFFECTS OF AGE, STRESS, AND MEDICATION ON LAYING AND PIGMENTATION**

It is well known that uniform, dark-brown eggs are the goal of many commercial breeders. It has been noted that there are significant, quantifiable shell color differences among breeds laying brown eggs, and among individuals within those breeds (Grover et al., 1980). Although seemingly evident, the fact that blue and brown pigment concentrations are higher within the glands of each respective egg layer is an indication of the pigments being breed specific, as opposed to being unilaterally produced in all breeds (Liu et al., 2010).

Odabasi et al. (2007) followed a flock of commercial hybrids at different ages, noting the differences in degree of pigmentation of the shell. They found that color remained relatively constant with the bird, most specimens having lighter eggs at the start of the laying period and correspondingly light eggs at the end.

A general trend of decreased pigmentation with age often has been observed (Samiullah et al., 2016). A number of hypotheses suggest that the increase of egg size with age results in a dilution of the pigment on the shell, a symptom of pigment concentration cover over the entirety of the egg.

Samiullah and Roberts (2013) conducted a longitudinal study on a flock of commercial hybrids. Longitudinal studies differ from horizontal studies in that factors from the same flock are considered at different ages, as opposed to different flocks at different ages.
They found no significant difference in eggshell color between 35 and 75 wk of age, noting, however, that the color at 25 wk was quantifiably darker than for other age groups. The color of shells became lighter as age increased; the amount of protoporphyrin IX in one gram of whole eggshell from 33-, 50-, and 67-week-old Hyline Browns did not differ significantly. Conversely, the concentration of the pigment in the cuticle layer itself was found to be significantly higher in 50-week-old birds than that of 33 and 67 weeks. The study therefore concluded that following the initial optimum laying period, eggshell color became paler, suggesting that age was proportional to paleness of shell in brown-egg layers.

The effect of stress from such stimuli as handling and relocation on the laying trends of brown-egg layers has been of particular commercial interest (Reynard and Savory, 1999). Indeed, for 4.5 h prior to oviposition, stress was found to cause a delay in laying of up to 3 hours. A stress threshold was observed whereby hens became unable to lay if the levels and duration of the stress reached or surpassed this.

Molting stress is an example of a condition significantly affecting the variations observed in eggshell color once laying resumes. Aygun (2013) showed that the response to molting with respect to eggshell color varied significantly among individuals in a flock. For caged flocks, high cage densities were proportional to the level of stress exhibited by the birds. Cage design, fear, and frequency of disturbance were also identified as contributing to changes in egg color through the effects of stress.

It has been noted that abnormalities such as paleness, calciferous deposits, and some structural defects were related to environmental influences (Mills et al., 1987). This was explained by the retention of the egg in the shell gland, where additional calcium deposition onto the formed egg results in masking of the ground color. Physical stress such as feather removal and extremes of temperature also were confirmed to adversely affect pigment deposition on the shell.

Drugs have been identified to have a profound effect on both laying and the color of the shell. Prostaglandins are active lipid compounds with biological effects similar to those of hormones. The effects of intrauterine injections of prostaglandin F2α (PGF2α) on pigmentation of brown eggs laid in the time following administration has been determined (Soh and Koga, 1994). The PGF2α caused oviposition to occur on a reduced timescale as compared to the control group, but resulted in the production of paler eggs—likely due to the time reduction of the egg residing in the shell gland. Indomethacin also was independently administered and was found to entirely inhibit pigment secretion.

Nicarbazin, an anti-coccidial medication, has been noted to result in production of unpigmented eggs for up to a wk after first dose (Hughes et al., 1991). The concentration and duration of treatment with the medication affected pigmentation, but not synthesis of the pigments themselves. Indeed, the effect was reversed within a wk of completion of the treatment, the birds returning to producing eggs of the same coloration as previously. It has been concluded that although pigment is produced under treatment, it is the medication that affects deposition onto the shell and cuticle, as opposed to its synthesis (Schwartz et al., 1975).

**IMPORTANCE OF COLOR AND QUALITY OF SHELLS**

The weight of the egg itself, as well as that of the shell, its strength, abnormalities in structure, yolk color, and properties of the albumen, are indicators for uniformity of the shell physical properties, and internal standard of the egg.

A significant correlation between brown-shelled eggs and the strength of the shell has been previously reported (Yang et al., 2009), with a similar study (Joseph et al., 1999) noting that the specific gravity of the eggshell was higher in brown eggs than blues and whites.

There was no correlation between internal quality of the egg, and the color of the shell (Sekeroglu and Duman, 2011); however, shell structure strength and thickness were found to be related to pigmentation (Schreiweis et al., 2006).

Mroz et al. (2007) reported on the relationship between surface ultrastructure and hatchability of turkey eggs. The hatchability of the eggs from Broad-breasted White Turkeys was considered as a function of shell surface character. In this report, eggs (n = 17,590) were split into 3 groups. Group 1 contained regular eggs with no visible calciferous deposits or deformities, group 2 consisted of rough eggs with some calcium deposition and surface nodules, and group 3 contained eggs with significant pigment-spotting.

Each of the eggs was individually marked and all were obtained at the peak of the laying season. Scanning electron microscopy (SEM) was used to observe the shell microstructure, and hatchability was estimated based on fertility rate and number of dead embryos—specifically of healthy poults. The main differences between groups 1 and 2 included varying cuticle size, size of crystal, palisade and mammillary layers, curved mammillary walls, and thicker inner shell membrane fibers. Group 3 was found to contain more eggs with thicker crystal layers and curved fibers of inner membranes, in particular, compared to group 1.

Of all eggs included in the study, those in group 1 had the highest hatch rate, at 77%. Groups 2 and 3 had gradually higher embryo mortalities at just under and just over 5%, respectively. The findings therefore suggested that, certainly in Broad-breasted White Turkeys, smoother eggs with less pigmentation tend to have higher hatchability than their rougher, more pigmented counterparts.
EGGS AS A BARRIER TO INFECTION

It is generally thought that the eggshell itself serves as both a barrier to physical impact, as well as to pathogens (Sá e Silva et al., 2016). There has therefore been a great deal of research carried out to understand the factors influencing the ability of the shell to act as a preventative medium to infection, as well as methods of treatment to avoid consumer infection (Berrang et al., 1999; Messens et al., 2005).

The penetration of 7 common pathogenic agents, Staphylococcus warneri, Acinetobacter baumannii, Alcaligenes sp., Serratia marcescens, Carnobacterium sp., Pseudomonas sp., and Salmonella enteritidis, through the eggshell has previously been reviewed (De Reu et al., 2006). This research correlated bacterial penetration, with structural features of the eggshell and particular strains of the bacteria.

Shells were inoculated with selected strains and stored at 20°C and 60% humidity for a period of 3 wk, with eggshell penetration being regularly monitored by the Berrang agar method, and whole egg contamination sampled after 3 weeks. The method consists of draining the egg contents and filling the shell with treated agar and sealing it with commercial silicone. Among the constituents of the treated agar was triphenyl tetrazolium chloride (TTC). As bacterial colonies grew, the TTC was reduced to formazan, which has a distinctive red coloration. Through candling the eggs daily during the first wk and 3 times the following wk, it was possible to record bacterial penetration through the shell (Berrang et al., 1998).

It was found that shell thickness and number of pores did not influence penetration. For each bacterial strain, the average cuticle deposition was lower for penetrated than unpenetrated shells, confirming that some strains digest the cuticle itself before penetrating. Non-clustered strains and those capable of movement were found to penetrate more frequently than others, with Pseudomonas sp. and Alcaligenes sp. showing highest contamination through the shells, most samples being penetrated after 4 to 5 days.

Shell thickness and the influence of age on penetration were not statistically significant; however, eggs with high specific gravities (good quality) were less penetrated than inferior examples. The number of pores did not correlate with penetration, but pore sizes were proportional to inner contamination of the shell, suggesting that perhaps a threshold diameter is necessary for additional penetration.

The cuticle on the shell serves as the first line of defense against pathogens, and acts as a waterproofing agent. Removal of the cuticle increased penetration by 20 to 60%. Eggs with poor-quality cuticles were found to have 40% more contamination than examples with more intact cuticles (26%). Finally, as a useful and non-invasive measure, a statistically significant correlation was found between the level of contamination of the outside of the shell and degree of penetration, suggesting that eggs can be at least partially judged in terms of contamination from an outer inspection of the eggshell itself.

CONCLUSIONS AND FUTURE RESEARCH

Recent scientific developments in the area of avian egg research have been discussed. This review has covered a broad spectrum of applications and practice related to the science of eggs, and in particular those of laying hens. Numerous factors have been shown to affect eggshell structure and color, implicating fields from enzyme reactivity to calcite formation (Wilson and Williams, 2015; Wilson et al., 2015; Leitch et al., 2016; Wilson and Williams, 2016). It has, however, been noted that a substantial number of questions remain in fully understanding the avian egg; the precise chemical formation of the crystalline structure of the eggshell, the distribution of pores, and processes behind termination of calcification all remain relatively unknown. Indeed, in an era in which hybrid research is becoming the norm, the greatest discoveries are likely to be made by incorporating powerful theoretical methodologies, such as computer simulations (Williams and Wilson, 2017), into biological research, as has been the subject of this review. Our understanding of the eggs and eggshells is continuously developing; however, the avian egg has not yet yielded all of its secrets.

ACKNOWLEDGMENTS

The author would like to thank Brogan Wilson for his influence. Andrew Sheppy (Cobthorn Trust, Bristol, UK) is greatly acknowledged for his assistance and advice.

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