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Maxwell Hincke, Mylène da Silva, Nicolas Guyot, Joël Gautron, Marc Mckee, et al.. Dynamics of structural barriers and innate immune components during incubation of the avian egg: critical interplay between autonomous embryonic development and maternal anticipation. *Journal of Innate Immunity*, 2019, 11 (2), pp.111-124. 10.1159/000493719 . hal-02622940

HAL Id: hal-02622940

<https://hal.inrae.fr/hal-02622940>

Submitted on 26 May 2020

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Dynamics of Structural Barriers and Innate Immune Components during Incubation of the Avian Egg: Critical Interplay between Autonomous Embryonic Development and Maternal Anticipation

Maxwell T. Hincke^{a–d} Mylène Da Silva^d Nicolas Guyot^d Joël Gautron^d
Marc D. McKee^e Rodrigo Guabiraba-Brito^f Sophie Réhault-Godbert^d

^aDepartment of Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, Canada; ^bDepartment of Innovation in Medical Education, University of Ottawa, Ottawa, ON, Canada; ^cLE STUDIUM Research Consortium, Loire Valley Institute for Advanced Studies, Orléans-Tours, Nouzilly, France; ^dBOA, INRA, Val de Loire Centre, Université de Tours, Nouzilly, France; ^eDepartment of Anatomy and Cell Biology and Faculty of Dentistry, McGill University, Montreal, QC, Canada; ^fISP, INRA, Université de Tours, Nouzilly, France

Keywords

Chorioallantoic membrane · Chick embryo · Innate immunity · Antimicrobial peptides · Avian β -defensins · Toll-like receptors · Eggshell

course of egg incubation, and also discusses the critical interplay between autonomous development and maternal anticipation.

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Abstract

The integrated innate immune features of the calcareous egg and its contents are a critical underpinning of the remarkable evolutionary success of the Aves clade. Beginning at the time of laying, the initial protective structures of the egg, i.e., the biomineralized eggshell, egg-white antimicrobial peptides, and vitelline membrane, are rapidly and dramatically altered during embryonic development. The embryo-generated extra-embryonic tissues (chorioallantoic/amniotic membranes, yolk sac, and associated chambers) are all critical to counteract degradation of primary egg defenses during development. With a focus on the chick embryo (*Gallus gallus domesticus*), this review describes the progressive transformation of egg innate immunity by embryo-generated structures and mechanisms over the 21-day

Introduction

Avian eggs are continuously exposed to microbes. They are challenged with high numbers of potentially pathogenic agents from the laying hen during oviposition, through the air and litter, and during natural incubation. Despite this exposure, most eggs remain viable up to hatching. The foremost reason for this is the highly efficient early defense response of the egg's innate immune system. In birds, there are 2 main, complementary types of immune defense: (1) nonspecific mechanisms, which act on pathogens in a nontargeted manner (physical and

Dr. Maxwell T. Hincke
Faculty of Medicine, University of Ottawa
451 Smyth Road
Ottawa, ON K1H 8M5 (Canada)
E-Mail mhincke@uottawa.ca

Dr. Sophie Réhault-Godbert
INRA-Centre Val de Loire
UMR0083 Biologie des Oiseaux et Aviculture (BOA)
FR-37380 Nouzilly (France)
E-Mail sophie.rehault-godbert@inra.fr

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chemical barriers, and components of innate immunity including antimicrobial molecules and cellular mechanisms, i.e., heterophils and macrophages), and (2) adaptive mechanisms, which target specific pathogens (antibodies and lymphocytes). The innate immune responses can directly control the replication or spread of pathogens by induction of phagocytosis or antimicrobial products. This review covers studies on the well-documented chick embryo (*Gallus gallus domesticus*), from which most of our knowledge on avian embryogenesis is derived [1].

The first line of defense against pathogenic microorganisms is formed by physical barriers such as the skin or, in the egg, the eggshell (ES), as well as by chemical innate immune protective mechanisms; together, these resist pathogen invasion from a contaminated environment. Because the development of an avian embryo occurs in an egg chamber that is physically separated from the hen, the egg contains all the required elements to nourish and protect the developing embryo during the entire cycle of its development prior to hatching. However, the innate defenses initially present within the egg disappear gradually during incubation; therefore, to prevent the penetration and growth of bacteria in the egg during embryonic development, new defense systems (not yet fully characterized) are required [2, 3]. Defensive responses also involve the recognition of pathogens by Toll-like receptors (TLRs) present in blood vessels, and by leukocytes that develop within the embryo (heterophils and macrophages) [4]. While there are good indicators that an innate immune response can be triggered, it is not yet clear how an inflammatory response in the embryonated egg would be controlled, as the cellular and molecular checkpoints in such a process are completely unknown.

The developing chicken embryo is able to trigger an immune response to a pathogen just prior to hatching, a characteristic that is routinely exploited in modern, large-scale poultry production with the administration of in ovo vaccination for multiple pathogens, including Marek's disease (MD) and infectious bursal disease (IBD). Embryonic development takes 21 days, and the first signs of a developing immune system are observed by the 10th day (ED10). On ED11 and ED12, T cells and B cells are developed, respectively, with B cell differentiation occurring after ED15. By ED18, the chicken embryo is immunocompetent and capable of producing both an innate and an adaptive response to pathogens [5, 6]. During incubation and after hatching, the yolk sac (YS) membrane transfers nutrients and immunoglobulin (Ig)Y from the

egg yolk to the developing embryo or the newly hatched chick. Therefore, during the first week after hatching, before the immune system is mature enough to produce its own B lymphocytes, a chick's humoral immunity depends on maternal antibodies (IgY) received from the egg yolk.

Innate Immune Receptors and Antimicrobial Peptides

TLRs recognize microbes by binding to pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), lipoteichoic acid, bacterial flagellin, lipoproteins, peptidoglycans, glycosylphosphatidylinositol, and bacterial DNA, in addition to single- and double-stranded viral RNA. At this time, 10 TLRs have been identified in chickens; most of them still require better characterization on ligand-receptor interactions and the associated downstream signaling pathways involved in their immune effector functions [7]. However, it is clear that chick embryonic tissues express TLRs from ED3 onwards, recognizing viral ligands and responding to them, thereby exhibiting an innate preparedness [4, 8, 9]. Avian β -defensins (AvBDs) and cathelicidins (CTHLs) are major classes of antimicrobial peptides with distinctive expression patterns during early embryonic development [9]. There are 14 AvBD genes (*AvBD1–14*) and 4 members of the CTHL gene family (*CTHL1*, *CTHLB1*, *CTHL2*, and *CTHL3*) [9]. Antimicrobial peptides such as AvBDs have a broad spectrum of activity against Gram-negative and Gram-positive bacteria, as well as fungi [10]. The ovodefensin OvoDA1/gallin is a novel β -defensin-related antimicrobial peptide which appears to be expressed specifically in the avian oviduct and possesses anti-*Escherichia coli* activity [11–13]. Much less is known about the antibacterial peptide natural killer (NK)-lysin (the chicken ortholog of human granulysin), which is a novel effector of cytotoxic T cells and NK cells [14, 15]. The chicken ortholog of liver-expressed antimicrobial peptide-2 (cLEAP-2) is a cationic antimicrobial peptide (CAMP) that is expressed in chicken epithelial tissues and upregulated in response to *Salmonella enterica* serovar Enteritidis infection [16, 17]. NK-lysin and cLEAP-2 have not been detected in the egg or within extra-embryonic structures to date, but are expressed in the chick embryo [14, 16]. Maternal stimulation with TLR ligands was observed to modulate oviduct expression of components of innate immunity such as proinflammatory cytokines, AvBDs, and CTHLs [18, 19].

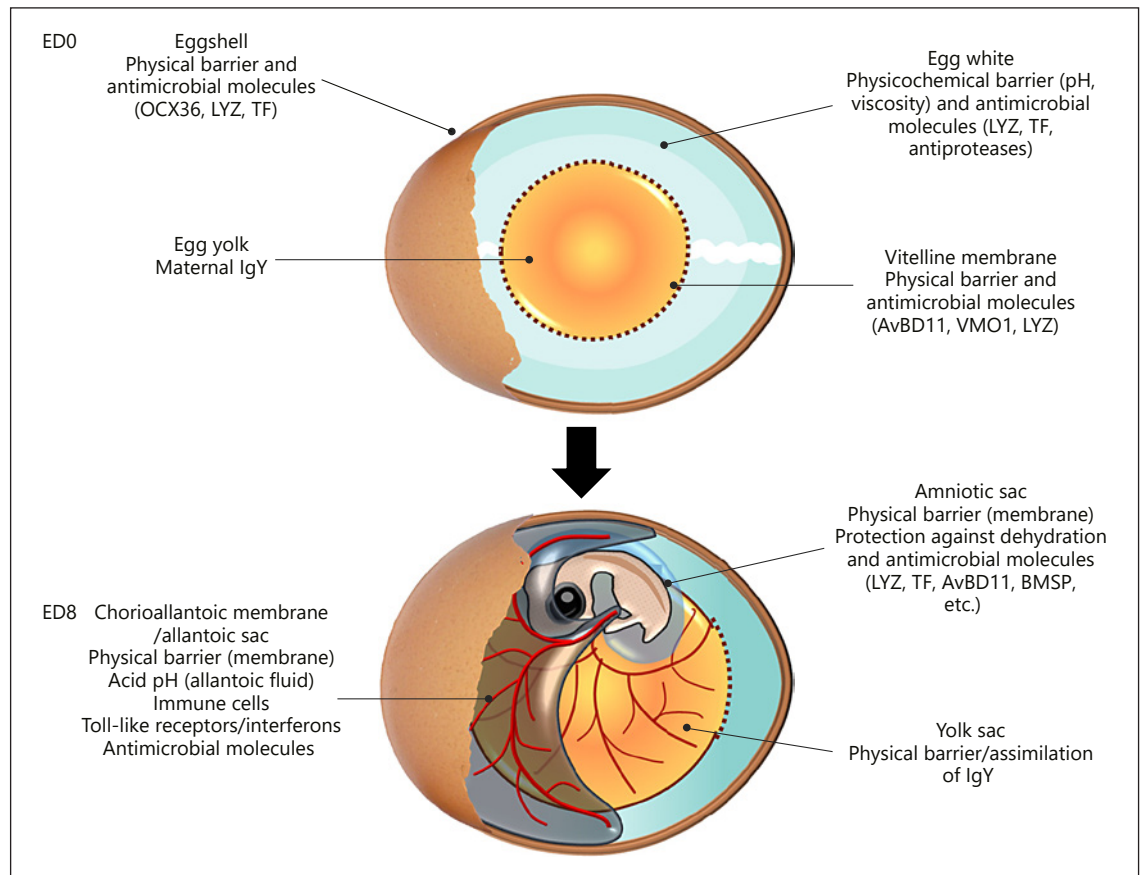


Fig. 1. Schema contrasting the basal innate defenses of the egg between ED0 (or unfertilized) and ED8 (developed extra-embryonic membranes).

Egg Basic Structures and Innate Immunity

The egg is formed as it traverses the oviduct of the sexually mature hen, and it consists of 4 basic structures: yolk, vitelline membrane (VM), egg white (EW), and ES (Fig. 1). These acellular structures serve as a source of nutrients and energy as well as physical, chemical, and molecular defenses to protect the chicken embryo against physical shock and microbial infection in the course of its 21-day development [20–22] (Fig. 2). The freshly laid egg is therefore an enclosure that must remain free of any microorganisms in spite of the surface microbiota of the ES, where a variety of bacterial species coexist [23, 24]. The microbiome is essential for development, health, and homeostasis throughout an animal's life. However, the origin and transmission processes governing animal microbiomes remain elusive for nonhuman vertebrates, and oviparous vertebrates in particular. Eggs may function as transgenerational carriers of the maternal microbiome, thus warranting characteriza-

tion of the egg microbiome assembly and a link with the developing immune system after they hatch. Bacteria can infect the egg in 2 possible ways: by vertical transmission, directly from hen reproductive tissues to the egg during its formation; or horizontally, by contact with the environment once the egg has been laid, through a defective shell or incomplete cuticle [25, 26]. These protective systems are very effective against most pathogens except *S. enterica* serovar Enteritidis, a Gram-negative bacterium responsible for food-borne illness, that is able to survive and grow in the EW because it can evade most egg antimicrobial mechanisms [25, 27, 28]. However, bacteria that reach the yolk can easily proliferate, thanks to the abundance and necessarily complete diversity of all the yolk nutrients required for chick development and growth in the absence of a maternal blood supply. This remarkable yolk is maintained in the center of the egg by 2 EW-derived “suspensory ligaments” (chalazae) and is surrounded by various protective layers (the VM, EW, and ES).

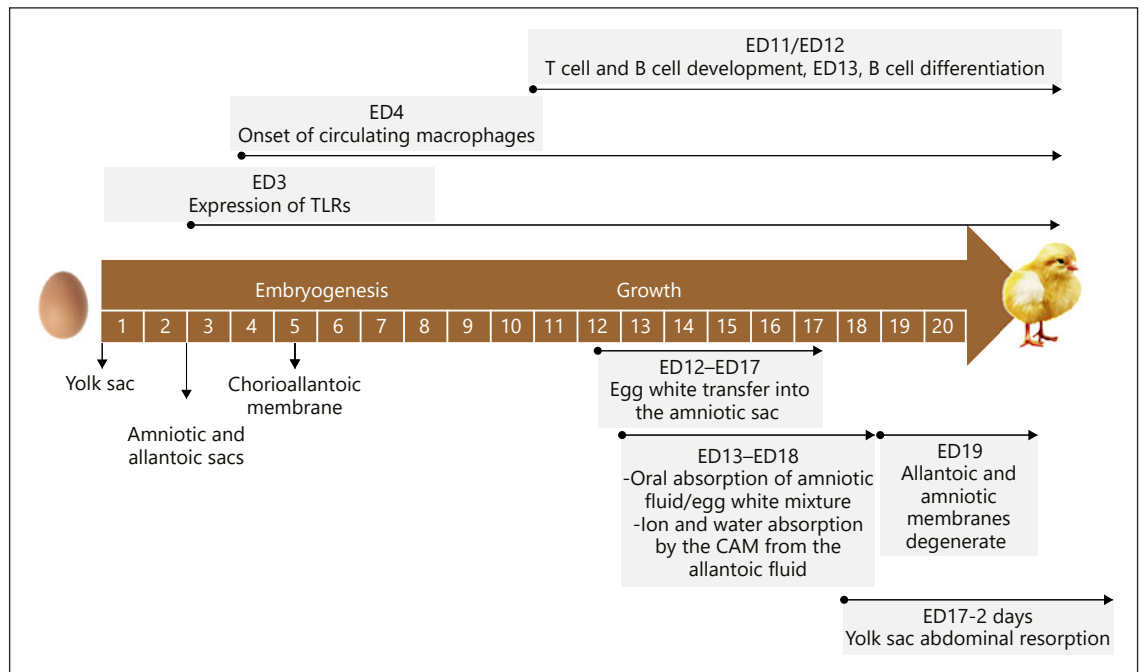


Fig. 2. Timeline contrasting the evolution of innate and adaptive immune systems between ED0 and ED21, over the course of embryogenesis and growth (ED0 to ED21). TLR, Toll-like receptor; CAM, chorioallantoic membrane.

Egg Yolk

The yolk accumulates during the process of vitellogenesis in the ovary of the hen [29]. With the exception of maternal immunoglobulins, yolk compounds are largely secreted by the liver and transported to the ovary via the blood, mainly in the form of very low-density lipoproteins [30]. Yolk proteomic studies have identified over 200 proteins [31–34], with the most abundant including IgY, avidin (AVD), ovotransferrin (TF), transthyretin (TTR), cystatin (CST3), α -2 macroglobulin (A2M), apolipoprotein A1 (ApoA1) and a protein predicted to be a β -microseminoprotein (BMSP). Some antimicrobial molecules, e.g., lysozyme (LYZ) or TF, are found in the yolk but, overall, this fluid is rich in nutrients and provides a favorable environment for bacterial growth. The maternal immunoglobulins concentrated in this compartment are mainly used by the embryo at the time of hatching, and within the following few days. In parallel, the B lymphocytes first emigrate from the bursa to seed secondary lymphoid organs (tonsils, etc.) about 3 days before hatching (ED18) whereas the first population of T cells leaves the thymus around ED6, with the second and third waves of migration taking place on about ED12 and around the time of hatching. Therefore, the embryo can-

not produce antibodies or mount an effective adaptive response [35].

The immunoglobulins in the blood of the hen at the time of egg formation are transferred to the yolk by endocytosis via the Fc γ receptor (Fc γ R). Their specificity therefore represents a snapshot at a given time of the maternal immune system, reflecting the microbiota to which the hen has been exposed [33, 34]. In the yolk, IgY is the dominant immunoglobulin, with a singular structure that is similar to that of mammalian IgG. However, the hinge region of IgG is larger, which makes it more flexible than IgY [36]. Indeed, the limited flexibility of avian IgY may account for some unique biochemical properties, such as the inability to precipitate antigens at physiological salt concentrations seen in chickens and ducks. For example, the 2 arms may be so closely aligned that they preclude crosslinking of epitopes on large antigens, and IgY recognition is biased towards short sequences compared to the mammalian antibody [37, 38].

The selective transport of IgY from the yolk to the embryonic circulation through the YS begins slowly on ED7 (<100 μ g/day) [39] and involves YS receptors whose IgY-binding is pH-dependent [40]. IgY is then detected in other compartments such as the EW, and the amniotic

and allantoic sacs [41–43]. Transport accelerates 3 days before hatching (>600 µg/day), to strengthen the defenses of the future chick at the time of hatching, but also during the following weeks, pending the activation of B lymphocytes and antibody synthesis by the embryo [44]. Only 10% of total yolk IgY will be absorbed by the embryo, corresponding to YS resorption in the gut of the embryo [22, 30]. It is likely that residual IgY from the YS provides local enteric protection [45, 46]. Overall, the contribution of the yolk to the protection of the embryo is minimal before hatching.

Vitelline Membrane

The VM forms an extracellular protein matrix around the oocyte and yolk and provides a physical separation from the EW. It consists mainly of fibrous structural proteins and antibacterial polypeptides. Its inner layer is equivalent to the mammalian zona pellucida, and its constituents are secreted by the ovarian granulosa cells and the liver [47, 48]. The inner membrane contains mainly zona pellucida proteins, which are critical for the adherence of spermatozoa to the oocyte during fertilization [47–49]. Conversely, the outer membrane is formed after ovulation in the infundibulum, the first segment of the oviduct and the site of fertilization, which also provides both physical and molecular protection to prevent pathogens from reaching the yolk [50]. A proteomic study of total VM identified 137 proteins, including 4 major ones: ovalbumin (OVAL, approx. 75%), LYZ (approx. 21%), VM outer membrane 1 (VMO1, approx. 1%), and AvBD11 (approx. 1%) [50, 51]. The disulfide bridges formed between ovomucins are essential to maintain the integrity of the fibrous VM network, even after the solubilization of the 2 major outer membrane proteins LYZ and VMO1 [52, 53]. At least 35 of the proteins identified in the VM have confirmed or predicted antibacterial activity [51]. Three of the major proteins, LYZ, VMO1, and AvBD11, can act directly against *S. enterica* serovar Enteritidis [54]. The total concentrations of ovomucin and LYZ are 17 times higher in the outer membrane than in the EW, indicating the strong antibacterial potential, both bacteriostatic and bacteriolytic, of the VM. However, the integrity of the membrane is severely impacted by the age of the hen, and also affected by the duration and temperature of egg storage [55]. This deterioration is partially due to the solubilization of membrane proteins such as VMO1 and AvBD11 [56].

Egg White

The EW is deposited around the VM during the passage of the forming egg through the magnum. In addition

to its role in nutrition of the embryo, its strategic position around the embryo and the yolk as well as its unique physicochemical properties (pH and viscosity) and complement of antibacterial proteins make it an effective barrier against pathogens. More than 300 proteins have been identified in the EW, 8 of which account for approximately 90% of the protein content: OVAL (54%), TF (12%), ovomucoid (SPINK7, 11%), ovoglobulin G₂ (BPIFB2/G2/TENP, 4%), ovomucin (3.5%), LYZ (3.4%), ovoinhibitor (SPINK5, 1.5%), and ovoglycoprotein (ORM1, 1%) [55, 57–62]. OVAL likely serves as a nutritional source of amino acids for the embryo [63]. Ovoda1/gallin, AvBD11, TF, LYZ, ovomucin, SPINK5, and SPINK7 participate in the antimicrobial activity of EW [64–67]. In addition, SPINK5 and SPINK7 are major EW protease inhibitors [68], which could protect EW proteins from proteolysis until their assimilation by the embryo [69, 70].

The antimicrobial nature of unfertilized EW and its proteins encompass at least 4 distinct mechanisms: (1) the chelation of compounds essential for survival and bacterial growth, (2) direct interaction with the bacterial wall, (3) inhibition of invasive proteases, and (4) limiting bacterial adherence [54, 64, 69, 70]. Although maternal exposure to environmental microbes can selectively increase EW antibacterial activity, the molecular details of this phenomenon have not yet been deciphered [71]. IgA and IgM are found in EW [59, 60], but their role and mechanism of action during embryonic development remain unclear.

During incubation, water from the EW is transferred to egg yolk (76% by ED10) [72, 73]. On ED12, EW is transferred to the amniotic sac. The mixture of amniotic fluid (AmF) and EW is then orally absorbed by the embryo from ED13 onwards, to accompany the intensive phase of growth of the embryo body and organs during the second half of incubation [22, 74, 75]. Part of the EW protein is absorbed across the gut epithelium and redirected to the organs of the embryo [76, 77], and OVAL can be detected in the brain, spinal cord, and muscle tissues [69, 70]. Other proteins are transported to the YS [74, 78, 79], to be digested with the other yolk compounds before transfer to the embryo. The amino acids, peptides, or proteins resulting from embryonic metabolism are secreted into the allantoic sac [80, 81], from where they can be reabsorbed by the chorioallantoic membrane (CAM) [82]. Direct transfer of gut proteins to the allantoic sac is also likely to occur [83]. EW anti-*Listeria monocytogenes* and anti-*Streptococcus uberis* activities are maintained during embryonic development up to ED12 [84], and EW

antibacterial proteins have been shown to be active even after their transfer into the amniotic sac [20].

In addition to the activity of antibacterial proteins and peptides, the physicochemical properties of the EW also play a major role in preventing the proliferation of invading pathogens. In a freshly laid egg, whether fertilized or not, the EW pH increases rapidly from 7.6 to 9.0, attributable to the diffusion of CO₂ through the ES [84]. This phenomenon not only affects the survival and growth of bacteria, but also their flagellar mobility and the oxidative stress experienced by bacteria [85]. It also modulates the activity of certain antibacterial proteins, such as TF, which chelates iron better, an element essential to certain bacteria such as *S. enterica* serovar Enteritidis [86], or LYZ which loses its N-acetylmuramide glycanhydrolase activity at an alkaline pH [87]. Overall, the bactericidal and bacteriostatic activities of EW are enhanced at elevated pH values [88]. The pH of the fertilized EW then gradually decreases to 7.5 by ED12. Conversely, the pH of EW in incubated, unfertilized eggs continues to increase, reaching 9.7 on the ED12 [84]. This difference is explained by the respiratory metabolism of the embryo, which causes a decrease in the EW pH in embryonated eggs and modifies the antibacterial properties (increased hydrolase activity of LYZ and decreased iron chelation by TF).

The mobility of pathogens is modified by fluid viscosity [89], and EW will therefore slow pathogens and limit their access to yolk nutrients. The gelled structure of EW is directly related to the presence of ovomucin, a highly glycosylated protein composed of 2 subunits: an α subunit (MUC5B), low in carbohydrates, and a β subunit, with a higher carbohydrate content (MUC6) [55]. The formation of complexes with LYZ reinforces this structure. However, the viscosity of EW is affected by many parameters, such as storage conditions and time [90]. Indeed, the increase in the pH of the EW during storage causes dissociation of the complex formed between ovomucin and LYZ, which liquefies the EW and promotes the mobility of invasive bacteria. However, this liquefaction may solubilize some proteins that were initially entrapped in the gel structure, resulting in the release of their intrinsic antimicrobial activity.

Eggshell

The final step in egg formation is shell deposition, a process that lasts about 18 h. The ES surrounds all the other structures of the egg and forms the first physical barrier protecting the egg against physical and microbial aggression. The ES has different layers, including noncal-

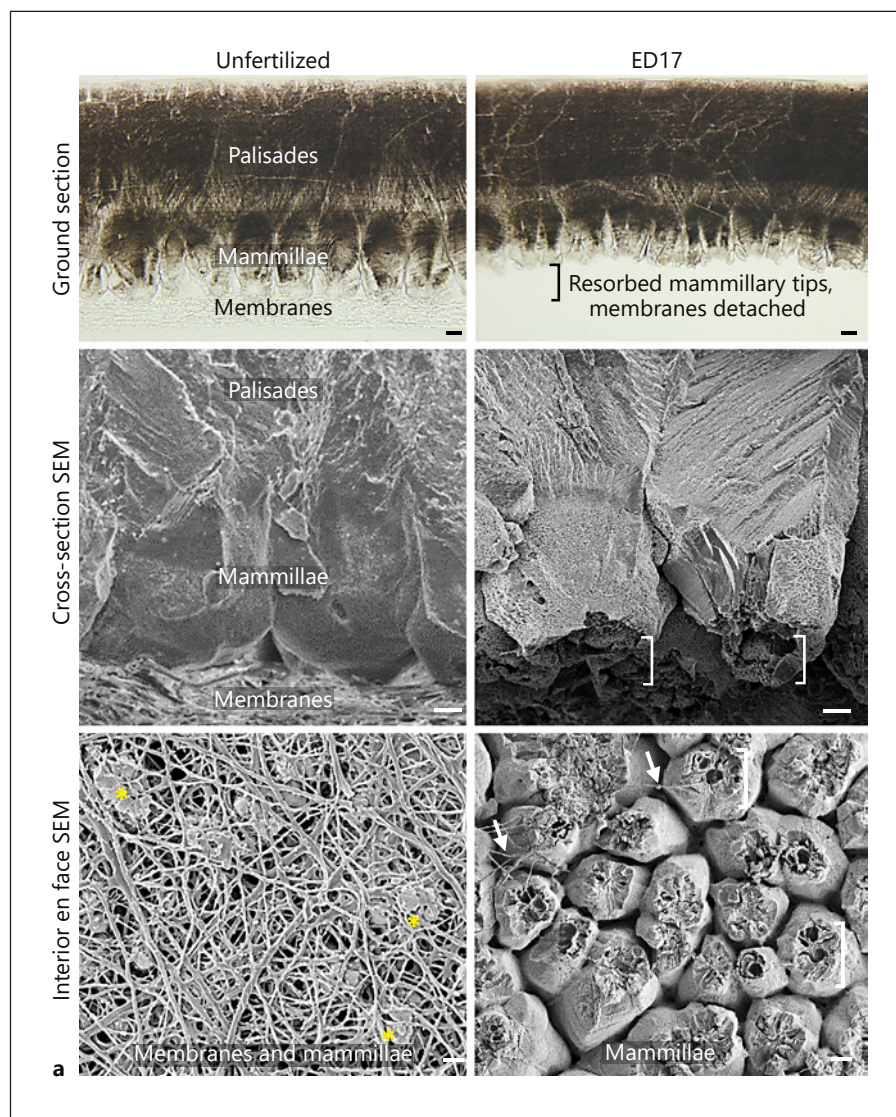
cified membranes that enclose the EW and provide a scaffold for the nucleation and growth of the calcitic mammillary cones and palisade layer (Fig. 3). Respiratory pores traverse the ES to regulate the exchange of water and respiratory gases, and partial dissolution of its inner portion with its calcium-rich composition provides an essential component for skeleton mineralization during the latter half of development [22, 91].

Regulation of ES mineralization within the acellular uterine fluid is a poorly understood process; however, the organic matrix plays a key role [92–94]. More than 700 proteins have been identified in this organic matrix [93, 95]. Some are also associated with other egg structures (OVAL, LYZ, and TF), while others are relatively ES-specific (ovocleidin [OC]17 and OC116; ovocalyxin [OCX]21, OCX25, OCX32, and OCX36) and involved in the biomineralization of the ES [92, 96]. The ES protein osteopontin has recently been shown to control ES nanostructure, where the dimensions of the calcite mineral structure correlate with ES hardness [97]. The outermost layer of protein-rich cuticle contains antimicrobial proteins such as LYZ [98, 99].

The ES protects the contents of the egg and the embryo from physical assaults and is an impervious barrier to penetration by microorganisms. Its strength is related not only to its thickness (300–400 μ m), but also to its ultrastructure [92, 100]. Indeed, the morphology, size, number, and orientation of calcite crystals provide the ES with remarkable and unique mechanical properties. The final layer of vertical crystals (perpendicular to the ES) as well as the cuticle that covers the entire shell, prevent not only water loss but also bacterial penetration. Cuticle quality (thickness and completeness of coverage) is highly heritable and strongly linked to resistance to bacterial penetration [27, 101].

Interactions between inorganic mineral and organic matrix proteins establish the unique ES architecture that prevents most pathogens from accessing the egg interior. This organic matrix contains a number of antibacterial proteins such as LYZ, TF, OCX36, OC17, OCX32, CST3, and the AvBDs [93, 95, 99, 102]. The mechanism of action of these molecules within the ES remains unclear, but it is likely that they are solubilized during limited ES dissolution that occurs during embryonic development and could also provide local protection at the interface between the ES and extra-embryonic structures (Fig. 3). Nevertheless, some pathogens can reach the egg interior because of irregularities in the ES (a nonhomogeneous cuticle, an abnormal mammillary layer, microcracks related to physical damage, etc.) [103].

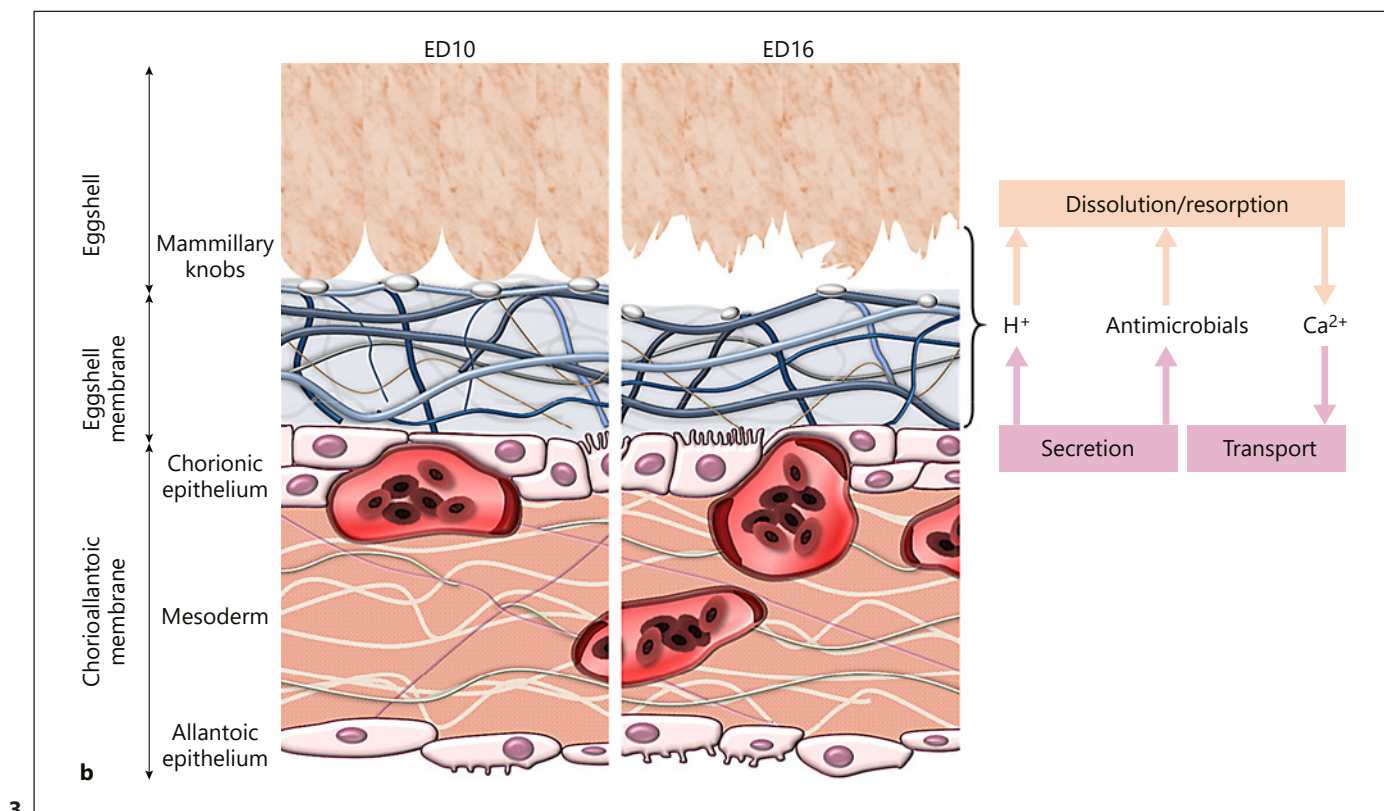
Fig. 3. a Ground and polished sections and scanning electron microscopy (SEM) of the eggshell (ES) and its membranes, showing dissolution of the innermost portion of the ES and detachment of the membranes during incubation. Brackets, dissolved mammary tips; asterisks, intact mammary tips; arrows, residual membrane fibers. Scale bars, 10 μ m. **b** Cartoon depicting the impact of the chorioallantoic membrane on ES dissolution and its predicted role in innate defense.



(Figure continued on next page.)

From the second half of incubation (ED10/ED11) to hatching (ED21), the ES is progressively degraded and used as a source of calcium by the embryo [82] (Fig. 3). While most of the solubilized calcium is transported to the embryo through the CAM for skeletal mineralization, a certain proportion is redirected to the YS for storage, to meet the nutritional needs of the chick after hatching, i.e., at the time when the immune system starts to develop. In this way, calcium may also be mobilized for biological functions that start to gain prominence, such as hematopoiesis and the development of lymphoid organs. The contribution of calcium to these processes in ovo remains unknown.

During the ES dissolution process, the tips of mammary cones are gradually dissolved, resulting in detachment of the shell membranes from the mineralized ES (Fig. 3) [104]. Solubilization and calcium transport involve a number of mechanisms, including a proton pump, calcium-binding CAM proteins, calcium ATPases, and carbonic anhydrases [105, 106]. The process releases occluded antimicrobial proteins from the organic matrix (Fig. 3b), which, we hypothesize, are liberated to act locally. By the end of incubation, the thinning of the ES facilitates the emergence of the hatchling from the egg [104]. However, this weakened ES could also more easily result in contamination of the egg and embryo in the lat-



3

er stages of development, and new developing structures are necessary to resist such threats.

Microorganisms in close interaction with eggs may act as a selective force on avian hatching success [107, 108]. In this earliest stage of life, they may be harmful because of their potential pathogenicity against embryos. However, only a small subset of bacterial species might actually be pathogenic to the embryo, and an increase in the number of nonpathogenic bacteria during incubation could be seen as a complementary parental approach to avoid colonization by pathogenic bacteria through direct inhibition or competitive exclusion [109]. Understanding which factors drive the microbial communities on the ES may lead to a better comprehension of the evolutionary strategies that improve embryo survival [110, 111].

Embryonic Structures and Their Role in Egg Defense

During the development of the embryo, extra-embryonic compartments and their contents are elaborated to support the vital functions of the embryo, such as circula-

tion, digestion, and respiration. These are the YS (surrounding the yolk), the amniotic sac (amniotic membrane and AmF), and the allantoic sac (CAM and allantoic fluid [AIF]). The adaptive defense mechanisms of the embryo become functional only in the first few weeks after hatching [44, 112]. Indeed, during embryonic development, the primary (bursa of Fabricius, thymus) and secondary (spleen, cecal tonsils [CT], and Peyer's patches [PP], i.e., the lymphoid organs in the gut intestine) organs of the immune system are formed, but no antibodies are secreted. The development of PP and CT starts during late embryogenesis (ED13), at the same time as the follicle of the bursa. The appearance of surface IgM-positive cells in PP and CT is independent of the development of the follicle of the bursa [113]. Mature lymphocytes capable of secreting immunoglobulins are not detected in the chick until 6 days after hatching [39]. Therefore, before hatching, the IgY, IgA, and IgM present in the egg originate solely from maternal sources. The yolk IgY may defend the embryo during and after hatching, functioning similar to colostrum and breast milk immunoglobulins in viviparous species; the IgA and IgM contained in the EW are absorbed by the embryo during incubation (trans-

ferred from the albumen to yolk at ED12) and come into contact with the intestinal mucosa, and could thus provide enteric protection [83, 114].

Concomitant with the progressive alteration/modification of basic egg structures, new defense structures essentially composed of supporting tissues are put in place quickly during incubation. Intestinal epithelial cells at ED17 have the capacity to take up and process bacteria, and they respond to bacterial products such as LPS and lipoteichoic acid with enhanced expression of proinflammatory genes (interleukin [IL]-6 and IL-18), acute-phase proteins (AVD and LYZ) and secretory components from the polymeric immunoglobulin receptor [115]. Immune cells such as macrophages enter the liver and kidneys at ED12 and ED16, respectively [116]. Already at ED4/ED5, embryonic macrophages are observed in the blood vessels and at perivascular locations; chicken embryonic macrophages are not recruited to incisional wounds, but they are able to recognize and phagocytose microbial antigens [117]. These immune cells can also be found in extra-embryonic membranes, particularly in the chorionic surface of the CAM in response to an immune challenge [118]. Moreover, macrophages are active in embryonic tissues; they can recognize and engulf microbes associated with blood vessel walls and respond to chemotactic signaling [117].

A general trend of increased expression of certain AvBDs and CTHLs, as well as cLEAP-2 and NK-lysin, during embryogenesis is observed; notably, the expression of AvBD9 and AvBD10 increases considerably by ED9 and ED12, respectively, during normal development [9]. From ED12 onwards, AvBD9 is predominantly found in enteroendocrine cells throughout the gut, while CTHL2 is exclusively found in heterophils [119]. Embryonic expression of cLEAP-2 is maximal at ED7 [16], while NK-lysin expression by thymic T cells increases continuously from ED16 [14].

A comprehensive assessment of the role of the extra-embryonic structures in the defense of the embryo is not yet available. Their strategic position around the embryo and their role in the assimilation of the compounds of the ES, yolk, and EW, however, give these compartments priority for the relocation of the molecular defenses initially present in the egg and the implementation of new defense systems. The appearance of extra-embryonic membranes at the beginning of incubation adds an additional level in the defense of the egg and the embryo: the CAM envelops all the internal structures of the egg, the amniotic membrane isolates the embryo from the other compartments, and the YS surrounds the egg yolk [1] (Fig. 1).

Yolk Sac

During incubation, the cell structure of the YS gradually replaces the VM, thus forming a new barrier. Analysis of its transcriptome has revealed that the YS synthesizes many lipid metabolism proteins, but no antibacterial effectors [120]. However, the YS serves as a support for the cells of the innate immune system, including monocytes and macrophages, from ED10 and ED12, respectively [121]. The generation of phagocytes (e.g., macrophages) in the YS and their infiltration of the embryo have been demonstrated in the chick and in the mouse. YS-derived macrophages in the chick were shown to enter the developing central nervous system independently of vascularization. Their origin was confirmed through the use of quail-chick YS chimeras. However, the interspecific chimera system used did not permit full development, so there was no evidence from the chick system as to whether the YS-derived cells were retained after hatching [121].

Amniotic Membrane

The amniotic membrane represents the last physical barrier before reaching the embryo. Initially, it contains only a few cells, but becomes more complex with the appearance of epithelial cells on its surface, as well as a fibrous matrix that seems to strengthen the structure [22]. However, from ED14, the membrane no longer grows and, therefore, to support the growth of the embryo, it stretches and becomes weaker. The plasticity of this membrane is essential to support the progressive but massive inflow of water from the beginning of development, and in EW during the second half of incubation. During the first half of incubation, the water contained in EW is redirected to the extra-embryonic compartments, passing through the yolk before accumulating in the amniotic and allantoic compartments to form the AmF and ALF. During the second half of incubation, these fluids will be reabsorbed directly or indirectly by the embryo, to support the acceleration of its metabolism during the growth phase (Fig. 2) [72, 122].

In humans, proteome analysis of AmF has identified >900 proteins, 25% of which are predicted to be involved in the immune response [123, 124]. However, unlike in mammals, chicken AmF does not collect excretory products and its protein composition strikingly changes at mid-development due to the massive inflow of EW proteins, which are thereafter swallowed by the embryo to support its growth. Proteomics has identified 91 nonredundant proteins delineating the chicken AmF proteome at ED11, before EW transfer [20]. These proteins are es-

entially associated with the metabolism of nutrients, immune response, and developmental processes. Forty-eight proteins are common to both chicken and human AmF, including serum albumin, ApoA1, and α -fetoprotein. Chicken AmF exhibits antibacterial activity on ED11, which is greatly enhanced after EW transfer, presumably due to the activity of LYZ, AvBD11, VMO1, and BMSP as the most likely antibacterial candidates. Following EW transfer, its antibacterial proteins may also contribute to protect the embryo before and after hatching, like the vernix caseosa in humans [125]. Interestingly, several proteins recovered in the AmF prior and after EW transfer are uniquely found in birds (OVAL and its related proteins OVAX and OVAY, and also AvBD11) or oviparous species (vitellogenin [VTG]1, VTG2, and riboflavin-binding protein [RBP]) [20].

Allantoic Fluid

The AIF is a product of embryonic metabolism, specifically the metabolism of the main digestive organ, the YS. The composition of the fluid is rich in nitrogen metabolites such as uric acid and ammonia, a difficult medium for the survival and growth of bacteria. As the volume of AIF decreases from ED13, these compounds are concentrated and eventually precipitate to form urate crystals [80]. From the second half of incubation, the important secretion of protons into the compartment, following the acceleration of embryonic metabolism, induces a drop in pH of about 2 units (pH 7 on ED8 to pH 5.5 on ED18) [126]. This phenomenon can induce functional changes at the protein level.

Major AIF proteins are mostly yolk proteins involved in lipid and vitamin metabolism, and metal ion transport, with few changes in this profile between ED8 and ED16. Characterization of proteolytic enzymes in these embryonic fluids has identified 12 proteases in the AIF, compared to 5 in the AmF [127]. AIF appears to concentrate digestive proteases such as aminopeptidase N (ANPEP), dipeptidyl peptidase-4 (DPP4), meprin A (MEP1A), and 72-kDa type IV collagenase preproprotein (MMP2), while proteases identified in both fluids could have a specific role in morphogenesis (hepatocyte growth factor activator [HGFA], suppressor of tumorigenicity 14 [ST14], and astacin-like metalloendopeptidase [ASTL]) and hemostasis (prothrombin [F2] and coagulation factor X [F10]). However, aside from the acidic pH that may challenge bacterial survival in AIF if contaminated, there is, to date, no clear evidence that AIF plays a significant role in antibacterial defense during incubation.

Chorioallantoic Membrane

The CAM lines the ES and covers all the structures of the egg by ED11/ED12 [105]. Because its epithelium is in direct contact with ES membranes, it is the second physical barrier after the ES during development (Fig. 2). This strategic position allows the cells of innate immunity to act locally, in case of bacterial penetration through the ES. In fact, a bacterial infection simulated by the deposition of LPS (an essential component of the outer membrane of Gram-negative bacteria) directly on the CAM induces a significant inflammatory response, resulting in heterophil and monocyte recruitment to the stimulated site [118]. The CAM was the first tissue to reveal the presence and action of interferon γ following viral infection [128], and a functioning response to TLR ligands is present by ED10–14 [4].

CAM proteomic analysis at ED19 identified 2 bacteriostatic proteins, TF and RBP [129], which are potentially expressed by the CAM cells or arrive via the bloodstream. Because of their proximity, it is likely that other proteins of the EW or ES are found in the CAM, especially since it has been demonstrated in quail that the CAM can endocytose large quantities of EW by fluid-phase endocytosis [130]. Further analysis by proteomics and transcriptomics would provide a better understanding of the development of CAM defense mechanisms that protect the egg from pathogens. Moreover, live imaging of the CAM to gain insight into leukocyte recruitment, penetration, and response to pathogens, using, for example, colony-stimulating factor 1 receptor (CSF1R) reporter chicken immune cells [117], would be an exciting research avenue to explore.

Conclusions

The calcareous egg of birds and reptiles, and previously dinosaurs, is a successful reproductive adaptation to the desiccating terrestrial environment. Embryonic development within this autonomous chamber has been shaped through evolution to resist physical and pathogenic challenges, while satisfying the metabolic and nutritional needs of the developing embryo. The evolution of the oviparous reproduction model to the viviparous model has led to important distinctions between corresponding extra-embryonic structures, particularly concerning the placenta. In humans, for example, the allantoic sac is not an independent structure as in the avian embryo, but forms part of the umbilical cord. The urine of the human embryo is therefore secreted directly into

the amniotic sac whereas the chicken embryo secretes metabolic waste into the allantoic sac, thus forming the AIF. However, the important presence of proteins and peptides in connection with the immune response and defense in human AmF is mirrored by the demonstration of such molecules in the avian AIF and AmF. In contrast, much less is known about the specific molecules that intervene in the mobilization of calcium (decalcification) from the avian ES, and the potential upregulation of innate immune genes at this critical site.

Transcriptomic and proteomic studies of the developmental changes that occur in the CAM, a structure that embraces both the embryo and all associated extra-embryonic structures, and that constitutes the first barrier against ES-penetrating pathogens, will be fruitful, particularly if augmented with functional studies of cellular and molecular changes. We believe that the CAM (that can be

likened to the mammalian placenta) has many unexplored functions and plays a major role in the development and protection of the living avian embryo.

Acknowledgements

M.T.H. is grateful to LE STUDIUM for support during the preparation of this manuscript, and is a LE STUDIUM Research Fellow, Loire Valley Institute for Advanced Studies, Orléans-Tours, and BOA, INRA, Val de Loire Centre, Nouzilly, France. M.T.H. and M.D.M. acknowledge funding from the Natural Sciences and Engineering Research Council of Canada.

Disclosure Statement

The authors declare no conflicts of interest.

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