

Mechanisms of antimicrobial defense in avian eggs

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Abstract One of the greatest threats to the survival of avian eggs is the risk of infection by microbes; as such, a large number of parental defense mechanisms have evolved in response to the decreased fitness imposed by microbial infection. The existing literature on this topic has focused largely on the mechanisms of microbial invasion through eggshells and the identification of molecules with antimicrobial properties in eggs of commercial species. However, little is still known about antimicrobial mechanisms in wild birds or how they vary with environmental pressures. This review concentrates on recent findings that shed new light on the role of parental behaviors (including incubation and placement of vegetation with antifungal activity in the nest) and the physical properties of eggshells (including nanometer-scale spheres that prevent microbial attachment) that protect eggs from contamination in high-risk environments. In addition to presenting a summary of current information, we identify evident gaps in knowledge and highlight research avenues for the future.

Keywords Antimicrobial defense · Eggshell · Infection · Incubation

Introduction

Embryos from oviparous vertebrates are frequently exposed to environmental challenges that may reduce their viability and survival. Predation has been recognized as the major cause of egg mortality (Ricklefs 1969; Martin 1995). In a less traditional sense, predation could also be considered as the consumption of egg components by heterotrophic bacteria (Board and Hornsey 1978). Indeed, the intricate structure of avian eggshells and albumen likely evolved from the ancestral amniotic egg at least partly as a result of intense predation by microbes (Packard and Packard 1980).

Numerous studies over the past 30 years have examined the susceptibility of eggs to contamination by bacteria. However, most have focused on domestic species (primarily chickens), with the goal of improving egg hygiene and thereby reducing loss due to contamination (Board and Fuller 1994; Board and Tranter 1995; Hincke et al. 2011; Baron et al. 2011). Researchers have recently begun to expand upon the lessons learned in these studies and applying them to natural bird populations. Broadly speaking, this research has shown that microbial infection affects eggs in the wild (e.g., Pinowski et al. 1994; D’Alba et al. 2011), that certain environmental conditions promote egg contamination (e.g., Berrang et al. 1999; Cook et al. 2005a), and that birds have evolved numerous behavioral, chemical and physiological adaptations to combat infection (e.g., Gwinner and Berger 2005; Martín-Vivaldi et al. 2014; D’Alba et al. 2014).

The goal of this review is to summarize what is currently known about the factors that influence the risk of infection in avian nests and the defense mechanisms against it. Ultimately, we hope to stimulate more research on this important but understudied topic.

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Risk factors for microbial contamination of eggs

What makes some eggs, both within and between species, more susceptible to infection than others? The first step in the course of microbial contamination of eggs is the attachment to the egg surface and the proliferation of microorganisms on the shell (Board et al. 1979). Once established, the abundance of bacteria determines the probability of their penetration of the shell (Cook et al. 2003, 2005b; Shawkey et al. 2009). In tropical environments, infection risk is highest before the onset of incubation, when eggs are exposed to ambient conditions and microbial growth is highest (Cook et al. 2005a). In temperate habitats, the specific functions of environmental components in promoting egg contamination are less understood (Wang et al. 2011; Lee et al. 2014). Overall, however, ambient conditions, parental physiology and behavior, and their interactions are known to regulate microbial diversity and abundance in the immediate environment of the eggs, and are therefore expected to strongly affect the egg's susceptibility to infection.

Moisture

Water, in both liquid and vapor states, is essential for fungal and bacterial growth on nests and eggshells and for microbial penetration of eggshell pores (Board et al. 1979; Bruce and Drysdale 1991, 1994). Several influential studies have established that the risk of trans-shell infection is highest in cool, wet and humid environments (Graves and MacLaury 1962; Board et al. 1979; Cook et al. 2003, 2005b). Under these conditions, contaminated water can be drawn into the pores by capillary attraction, and fungi can grow and digest the cuticle and shell membranes, thus facilitating the passage of bacteria throughout the pores and into the albumen (Board 1966; Bruce and Drysdale 1994).

Although Cook et al. (2003, 2005a, b) emphasized the influence of ambient humidity on trans-shell infection, more recent studies have suggested that the shelter from environmental conditions provided to eggs by incubating parents mitigates the significance of large-scale climatic conditions (e.g., precipitation, Ruiz-de-Castañeda et al. 2011; temperature, Walls et al. 2011; but see Peralta-Sánchez et al. 2012). Rather, the nest microclimate—for example, the humidity inside the nest—should strongly affect the rate of microbial invasion (Horrocks et al. 2014).

With this in mind, we can predict that species using mud or platforms of vegetation on the water surface should be at greater risk than birds breeding in sites with elevated ambient humidity (e.g., rainforest species). In the former group, therefore, adaptations (e.g., a specialized eggshell cuticle, discussed below) may have evolved to waterproof

the egg, preventing flooding of the shell pores (Board 1982) and microbial penetration. Moreover, if nest humidity affects microbial processes independently of climate, we could expect to see these adaptations in a wide range of climatic conditions.

Temperature

Ambient temperature has a strong influence on microbial growth, as many mesophilic bacteria grow well at a temperature that is also favorable for incubation (37 °C; Madigan et al. 2005). Apart from the obvious effect of temperature on the rate of bacterial proliferation and consequent level of contamination (Board and Ayres 1965), there is no strong indication that temperature alone is the major determinant of trans-shell infection. For example, Cook et al. (2005a) found that bacterial growth on eggshells was enhanced in sites in which relative humidity rather than temperature was elevated. Instead, temperature differentials between the egg and its environment may be important (Lorenz et al. 1952; Padron 1990; Bruce and Drysdale 1994; Berrang et al. 1999). Such temperature gradients occur, for example, immediately after oviposition, when the egg's temperature is close to the female's body temperature of 42 °C, and then cools to the ambient temperature. In this case, negative pressure is created downward through the pores and can result in contaminated material passing through the pores and into the egg contents (Bruce and Drysdale 1994). Certain patterns of nest attentiveness can also lead to sudden changes in temperature, particularly when coupled with low ambient temperatures, rain, or the lack of cover by nest materials (Afton and Paulus 1992). These possibilities have yet to be explored.

Type of contaminant microorganisms

The eggshell microbiota is largely derived from soil, feathers and faeces, and for many years was thought to be dominated by Gram-positive bacteria (Board et al. 1994). These findings were the result of culture-based approaches that likely detect only about 1 % of microbes present (Amann et al. 1995). More recent studies have used DNA-based identification techniques that detect much higher proportions of the true diversity. These methods have shown greater representation of Gram-negative bacteria than those in previous studies (Shawkey et al. 2009; Grizard et al. 2014). However, such techniques have their own biases (e.g., PCR preferentially amplifying some sequences over others), and as such, also cannot be considered definitive. Based on evidence relying solely on culture-based methods at this point, the most common

microorganisms detected in the interior of infected eggs are Gram-negative bacteria (i.e., family *Enterobacteriaceae*), which are more resistant to the chemical protection of egg proteins like lysozyme (Board 1966). Comprehensively cataloging the microbial communities on and in eggs using both culture-based and molecular techniques will be critical going forward. In particular, comparison of communities on the egg and in the different layers (membranes, albumen, yolk) will elucidate the types of bacteria that are most likely to infect eggs. Interactions between bacteria on the shell surface can also begin to be understood in this way. For example, some bacteria (e.g., *Enterococcus*), often present on the digestive tract, skin and uropygial gland of some birds (Soler et al. 2010) are known to produce antibiotics, and the shape of communities with these bacteria may differ from those without them. Detailed understanding of the composition of eggshell communities is needed to address these fascinating possibilities.

Type of nest and parental behavior

Birds can nest either in cavities or in the open. The walls of natural cavity nests are frequently wet (McComb and Noble 1981), and are therefore damper (albeit less exposed to the elements) than open nests. As such, we can predict that cavity nests will have higher levels of microbial infection. Indeed, in a comparative study across 24 species in Mediterranean Spain, Peralta-Sánchez et al. (2012) found that the probability of microbial colonization of the eggshell surface was higher in cavity than open nests. This study was performed in active nests and investigated the effects of nest material, reuse of nest holes, and incubation patterns on eggshell bacterial loads. However, different results were demonstrated in a study by Godard et al. (2007), which exclusively tested the effect of humidity and temperature on rates of microbial infection in chicken eggs placed in artificial open nests or nest cavities. In this case, the authors found that eggs from open nests were more often colonized by bacteria. These two examples serve to illustrate the fact that estimating the risk of infection is a complex task requiring rigorous sampling of a broad diversity of nest types and designs, their microclimates, and climatic conditions.

The selection of a suitable nest site and construction materials (discussed below) is likely to influence the risk of infection. For example, birds that reuse nest cavities across seasons might experience increased risk of egg infection, as viruses, fungi and bacteria can remain quiescent in nest materials for long periods and can withstand freezing temperatures (Davies et al. 1971; Hubalek 1978). Indeed, several strains of bacteria, including fecal-borne pathogenic strains (*Pseudomonas*, *Bacillus* and *Staphylococcus*), have been found in old nests of house wrens *Troglodytes*

aedon (Singleton and Harper 1998), great tits *Parus major*, and blue tits *Cyanistes caeruleus* (Goodenough and Stallwood 2010). Passerines that tend to reuse cavities, however, are also more likely to add fresh vegetation to their nests (Harrison 1975 in Clark and Mason 1985) that could serve as nest sanitizers (see below).

Certain parental behaviors may enhance some aspects of fitness while putting the eggs at higher risk of contamination. For example, some species (e.g., ducks, gannets, cormorants, kittiwakes) defecate in their nests, likely as anti-predatory behavior (McDougall and Milne 1978) or as part of their nest architecture (Nelson 1978; Cooper 1986). Fecal matter is known to contain microorganisms, including several pathogens such as salmonellae and campylobacter, or antibiotic-producing bacteria (e.g., *Enterococcus* spp.; Brandl et al. 2014), which can be horizontally transmitted to eggs (Cox et al. 2000, 2002) and can change the composition of bacterial communities on shells.

In other cases, birds such as vultures that feed almost entirely on carcasses of dead animals incorporate these materials into their nests (del Hoyo et al. 1994), providing a breeding ground for pathogenic bacteria. The incidence of particular behaviors like these and their role in shaping the evolution of antimicrobial defenses in eggs should be an extremely interesting topic for future studies.

Mechanisms of antimicrobial defense

Behavioral

Keeping eggs dry during incubation

Successful embryo development requires a relatively narrow range of temperature and humidity (Drent 1975; Webb 1987). Therefore, during incubation, parents make dramatic changes to the nest microclimate to promote optimal embryonic growth, respiration and hydration. Parental regulation of hydric conditions inside the nest has been thoroughly investigated with regard to water vapor gradients between eggs and the environment (Chattock 1925; Lomholt 1976; Rahn et al. 1977; Walsberg 1980), and it is recognized that parents help maintain a nesting environment that is generally above ambient humidity (i.e., water vapor). Of more direct importance to egg microbial infection, however, is the accumulation of liquid water on the egg surface. Two studies thus far have investigated whether parental incubation inhibits bacterial growth through the drying of eggshells. D'Alba et al. (2010a) found that microbial growth was highest on un-incubated, experimentally moistened eggs, and that incubation nullified these effects by removing water from the eggs' surface.

Similarly, in a correlative study, Ruiz-de-Castañeda et al. (2011) found that incubation reduced relative humidity in the nest and bacterial loads on shells. These findings suggest that this non-specific defense mechanism may be common across birds.

Use of plants as nest sanitizers

Birds of several species use fresh vegetation in their nests, including leaves, sprigs or small branches, that are not integral elements of the nest architecture. These green materials have been proposed to function as nest decorations for mate attraction (Gwinner 1997; Brouwer and Komdeur 2004; Veiga et al. 2006), to boost nestling development by activating their immune system (Gwinner et al. 2000), and to protect eggs and nestlings from insect pathogens (Lafuma et al. (2001) or bacterial contamination (Clark 1991). Birds often exhibit non-random selection of plants from the pool of available vegetation. Preferred plants contain high concentrations of aromatic compounds (e.g., monoterpenes and sesquiterpenes) produced as herbivore toxins and fungal growth inhibitors (Gwinner 1997; Mennerat et al. 2009b; Pires et al. 2012), suggesting that antimicrobial defense may be an additional or alternative function.

Despite the high use of fresh vegetation in nests among bird taxa, functional and experimental studies of this behavior are limited to three passerine species (Dubiec et al. 2013). Moreover, most of those studies have focused on deterrence of nest ectoparasites, and only two have tested the specific effect of green plant deposition on nest microbiota. The first of these showed that the experimental addition of herbs, including *Achillea millefolia*, *Mentha suaveolens*, *Heracleum sphondylium*, and *Salix alba* leaves, on the nests of European starlings resulted in a reduction in bacterial abundance (Gwinner and Berger 2005). Later, Mennerat et al. (2009a) demonstrated that the presence of *Lavandula stoechas* and *Helichrysum italicum* in nests reduced bacterial richness on nestling skin.

Experimental investigations demonstrating the effect of fresh plants on egg microbiota are still lacking. More importantly, it is still not known whether fresh plants affect pathogenic microorganisms and, ultimately, embryo survival.

Inoculation of eggs with uropygial secretions

A third mechanism of antimicrobial defense in eggs involves oils secreted from the uropygial gland, which are then spread onto feathers through preening. The uropygial gland in birds produces a mixture of chiefly monoester and diester waxes whose main functions include waterproofing of the plumage and maintaining the flexibility and physical

integrity of the feathers (Elder 1954; Jacob and Zisweiler 1982). Another important function is the promotion of plumage hygiene (Jacob and Zisweiler 1982). The inhibitory effects of uropygial oils on bacterial and fungal infection of feathers have been well documented in vitro (Baxter and Trotter 1969; Jacob and Zisweiler 1982; Jacob et al. 1997; Shawkey et al. 2003; Ruiz-Rodríguez et al. 2009, 2014). However, in vivo studies have been restricted to one species, the mallard (*Anas platyrhynchos*), in which it was shown that, contrary to predictions, covering eggs with feathers (Javůrková et al. 2014) or directly with preen oil (Giraudeau et al. 2014) did not affect infection rates or bacterial loads on eggs.

In addition to its chemical properties, uropygial secretion of certain species also harbors antibiotic-producing bacteria (e.g., the green wood hoopoe [formerly red-billed hoopoe] *Phoeniculus purpureus* and the European hoopoe *Upupa epops*; Law-Brown and Meyers 2003 and Soler et al. 2008, respectively). These bacteria, from the genus *Enterococcus*, are known to produce bacteriocins (Martín-Platero et al. 2006; Franz et al. 2007a) and several antimicrobial volatile substances (Martín-Vivaldi et al. 2010) that are active against a broad range of bacteria.

For this type of defense to be effective, the egg surface must come in direct contact with the uropygial oils, thus requiring that parents either actively add waxed feathers to their nests or directly coat the eggs with these secretions. Birds frequently use feathers to line their nests (Cramp 1998; Hansell 2000), but their effect on either abundance or diversity of eggshell microbiota has been tested in only two studies to date. Peralta-Sanchez et al. (2010) showed that the number of feathers in the nests of barn swallows (*Hirundo rustica*) negatively correlated with bacterial load on eggshells. Although this effect could result from inhibition of eggshell bacteria by uropygial oil, Peralta-Sánchez et al. (2014) showed that bacteria living on feathers (some of which produce antibiotics) had an inhibitory effect.

The suggestion that parents may directly inoculate eggshells with antimicrobial compounds from the uropygial gland has been repeatedly proposed (Menon and Menon 2000; Cook et al. 2005a; Shawkey et al. 2009) but not experimentally tested until recently. In a study of European hoopoes by Martín-Vivaldi et al. (2014), the authors experimentally prevented female access to the gland and recorded the behavior of females during incubation, demonstrating that female European hoopoes smeared their eggs with preen oil and that their eggs contained specialized shell structures that enhanced the adhesion of oil and symbiotic bacteria onto the egg surface.

Similar observations of parental application of uropygial oil have not been reported for any other species, perhaps

simply due to a lack of research effort. However, it is worth noting that this behavior could impair the gas exchange between egg and atmosphere, leading to potentially lethal effects on the embryo. This is because the shell pores—the structures that mediate gas exchange—would become blocked and rendered nonfunctional (Board 1982). Our knowledge of the role of uropygial secretions in preventing microbial infection of eggs is still incomplete and taxonomically limited. At this time, it appears that its effects may vary during the avian annual cycle (Jacob and Balthazart 1979) and among species with different ecological and life history traits (Vincze et al. 2013).

Chemical and physical egg attributes

In the absence of a fully developed immune system, the avian embryo and neonate require a non-specific defense mechanism to prevent contamination by microorganisms. Seminal papers by Board and Fuller (1974) and Board (1980, 1982) have shown that this mechanism exists and is composed of a complex network of physical and chemical properties integrated across all egg compartments (Fig. 1).

Physical defense

The passage of microbes from the external environment to the nutritious yolk and embryonic tissues is physically hindered by the shell, its membranes and the albumen (Board and Hornsey 1978). The eggshell provides physical protection from the external environment, functions as the main source of calcium for the growing embryo, prevents water loss, and mediates gas diffusion between the external and internal egg environment. It is also the first barrier encountered by microorganisms and, as such, plays an important role in preventing microbial contamination of the egg contents. Although most eggs do not contain microorganisms at the time of oviposition (Brooks and Taylor 1955), they rapidly become exposed to infection through contact with contaminated nest materials or parental tissues (Board and Fuller 1974; Berger et al. 2003; Mills et al. 1999; Singleton and Harper 1998).

The eggshell comprises four morphologically distinct regions that are formed sequentially, starting with the innermost mammillary zone, followed by the palisade, the vertical crystal layer (these three layers constitute the “true shell”), and the outermost cuticle (Fig. 1). Thus the eggshell can be considered as a series of resistance barriers that have evolved as a result of pressures from specific nesting and environmental conditions, physiological demands by the embryo, and the nature of microorganisms (Board and Fuller 1974). For example, Stein and Badyaev (2011) recently demonstrated that the structure of the eggshell in house finch (*Carpodacus mexicanus*) populations

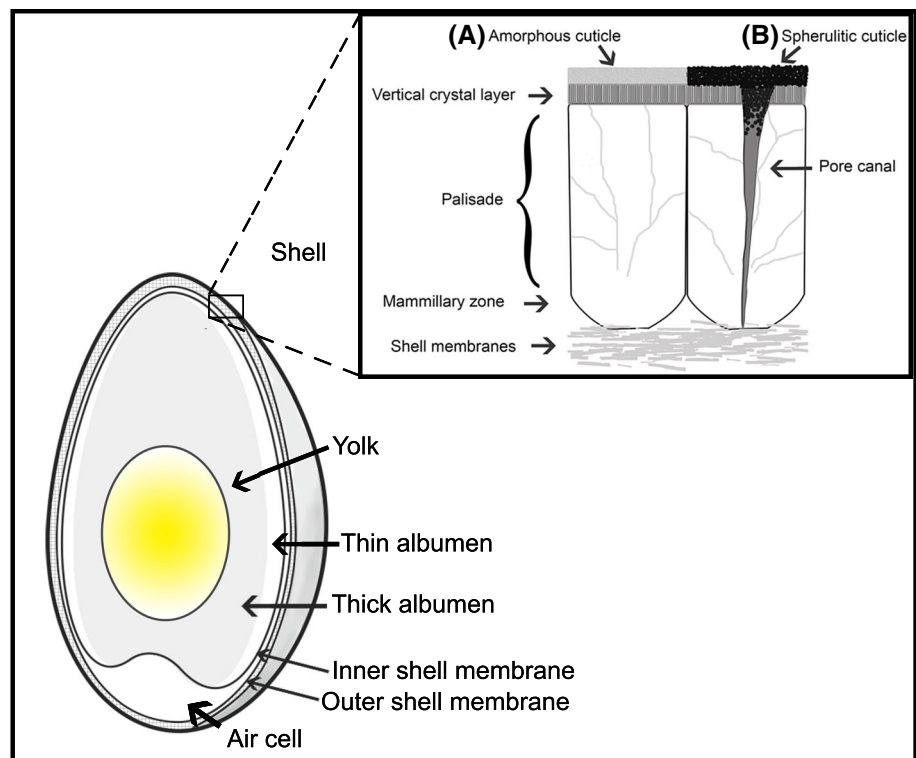
rapidly evolved in response to pressures from the environment (e.g., ambient humidity). A similar evolutionary response to the pressure of invasion by microorganisms has been proposed but not yet investigated.

The cuticle is a layer with highly variable thickness, structure and composition among bird taxa, and that is absent in entire groups such as parrots, pigeons, and petrels (Mikhailov 1997). One of the main functions of the cuticle is thought to be the waterproofing of eggs by capping the shell pores, while allowing the diffusion of respiratory gasses (Board 1980). A more specific role of the cuticle in the prevention of microbial egg invasion has been hypothesized in a few studies (Board et al. 1982; Sparks and Board 1984; Sparks 1994), but experimental evidence supporting this function is very limited, and no studies have addressed this issue since the early work performed by Kim and Slavik (1996). Those authors found that treating eggshells with various acidic solutions produced various changes in the microstructure of cuticles, and that increased deterioration of the eggshell cuticle allowed greater rates of bacterial penetration. Very recently, however, renewed interest in this topic has begun to shed light on the mechanisms behind the proposed effect of the cuticle against microorganisms. D’Alba et al. (2014) conducted an experimental study on the Australian brush-turkey (*Alectura lathami*). This mound-builder from the family Megapodiidae relies on heat produced by bacterial decomposition of plants for incubation of their eggs (Jones 1988). This, and the fact that humidity inside the mounds is continuously near saturation (Booth and Thompson 1991), leads to a high risk of microbial infection for the clutches of these birds. The study by D’Alba et al. (2014) demonstrated that the eggshells of this species were covered with an inorganic layer of nanometer-scale spheres of hydroxypapatite, rendering them superhydrophobic and preventing attachment by bacteria, thus creating a very effective physical defense against microbial penetration. A large number of species have cuticles with similar layers of spheres of varying size and chemical composition (Sparks 1994; Mikhailov 1997 L. D’Alba, unpublished), yet their functional significance and relationships with specific nesting ecologies are completely unknown. Investigation of the ecological basis and mechanisms of immunity against microbes by these structures will be a fertile area of research.

Chemical defense

In 2006, fewer than ten chicken egg proteins had been identified. With the development of high-throughput methods used in combination with genomic databases, this number has since increased to over 520 in eggshells (Mann et al. 2006) and 148 in albumen (Mann 2007). Not all of

Fig. 1 Schematic representation of the components involved in the antimicrobial defense of avian eggs. Chemical (e.g., antimicrobial proteins) and physical (e.g., barriers, albumen viscosity, cuticle topography) defenses are integrated across egg compartments. The cuticle of eggs can be **a** amorphous or **b** formed by nanometer-scale spheres of various chemical composition (see text)



these proteins are involved in antimicrobial defense, however; in fact, the function of 95 % of those identified proteins has not been tested directly or at all (Rehault-Godbert et al. 2011). Nevertheless, proteins with antimicrobial properties are highly effective, as they cover a broad spectrum of inhibitory and bactericidal activities. The mechanism of action in antimicrobial egg proteins can be grouped into three main categories: (1) chelation of vitamins or minerals essential for microbial growth, (2) direct degradation of microbial components, and (3) inhibition of bacterial proteases involved in pathogen invasion (Table 1).

In chicken eggshells, these proteins are deposited to varying degrees (ovotransferrin 12, ovomucoid 11, lysozyme 3.4, ovoinhibitor 1.4, and avidin 0.05 %) in the albumen (Li-Chan and Kim 2008) and, to a lesser extent, in the eggshell matrix and cuticle.

The true eggshell is composed primarily of calcium carbonate in the form of calcite, which is embedded in an organic matrix of diverse elements (Rose and Hincke 2009) that includes proteins, glycoproteins and proteoglycans. Most of these matrix proteins are calcium-binding constitutive proteins that regulate the mineralization process during eggshell formation (Eckert et al. 1986; Hincke et al. 2011), and some are also involved in the antimicrobial defense of eggs (Mine et al. 2003; Wellman-Labadie et al. 2008). For example, lysozyme and ovotransferrin have been detected in the eggshell matrix

of chicken eggs (Gautron et al. 1997). Ovotransferrin is also present in turkey (*Meleagris gallopavo*) and quail (*Coturnix japonica*) eggshells (Panheleux et al. 1999). Matrix proteins extracted from true eggshell and cuticle have shown antimicrobial activity in vitro against *Pseudomonas aeruginosa*, *Bacillus cereus*, *Staphylococcus aureus* (Mine et al. 2003), *Bacillus subtilis*, and *Escherichia coli* (Wellman-Labadie et al. 2008). Interestingly, all data on chemical defense features of eggshells are from species with organic cuticles, which are mostly (87 %) composed of protein (Wedral et al. 1974). In contrast, nothing is known about the chemical defense of inorganic cuticles occurring in some groups of birds (e.g., Pelecaniformes, flamingos, megapodes, grebes). Inorganic cuticles are largely composed of either calcium carbonate crystals (vaterite) or amorphous calcium phosphate (Sparks 1994).

While it is clear from these studies that the avian egg contains many molecules that may help protect the embryo from microbial attacks, in most cases, their function has yet to be validated experimentally, and many questions remain. For example, (1) are these molecules actively incorporated into the egg to protect the embryo, or is their presence the result of passive transference from the female's reproductive system? (2) What is the biological function of these molecules in the egg after oviposition? And (3) how does the chemical defense vary among different nesting environments and risk of infection?

Table 1 Molecules with potential antimicrobial properties identified in domestic chicken eggs, categorized by their function against microbes and their location in the egg

Function	Number of identified proteins	Examples	Location in the egg
(A) Degrade microbial components			
Hydrolases	5	Lysozyme	A, C, TS, SM
Antimicrobial peptides	19	β -Defensin	A
LBP–BPI ^a proteins	4	Ovocalyxin-36	A, TS
C-type lectin	7	Ovocleidin-17	A, TS
(B) Decrease bioavailability of iron and vitamins			
Iron chelators	6	Ovotransferrin	A, SM, TS
Vitamin chelators	5	Avidin	A
(C) Inhibit the activity of microbial proteases			
Serine protease inhibitors	>30	Ovoinhibitor	A
		Ovomucoid	
Cysteine protease inhibitors	2	Cystatin	A
Metalloprotease inhibitors	10	TIMP2	A
Broad-spectrum inhibitors	7	Ovostatin	A, TS

Data compiled from Rehaalt-Godbert et al. (2011) and Gautron et al. (2011)

A albumen, C cuticle, TS true shell, SM shell membranes, TIMP tissue inhibitor of metalloproteinase

^a LBP–BPI lipopolysaccharide-binding protein–bactericidal/permeability-increasing protein

While much has yet to be investigated with regard to antimicrobial allocation in eggs, particularly in relation to the mechanisms of protein deposition, several studies have found evidence suggesting that mothers distribute antimicrobial proteins differentially within and among clutches. In studies of the barn swallow (*Hirundo rustica*), Saino et al. (2002) found that, within a clutch, earlier-laid eggs had higher levels of lysozyme than later-laid eggs. Similar patterns have been reported for ovotransferrin in blue tits (*Cyanistes caeruleus*; D'Alba et al. 2010a, b) and avidin in yellow-legged gulls (*Larus michahellis*; Bonisoli-Alquati et al. 2010). Opposite allocation patterns or a lack of patterns have been observed in other species (e.g., Shawkey et al. 2008). One theory has proposed that females may enhance antimicrobial defense of early-laid eggs to better protect them from the increased risk of infection before the onset of incubation.

Females are also known to increase their antimicrobial allocation to clutches sired by attractive males (blue tits, D'Alba et al. 2010a, b; mallards, Giraudeau et al. 2011) or mates that perform more complex songs (Eurasian reed warbler *Acrocephalus scirpaceus*, Krištofik et al. 2014). In pied flycatchers (*Ficedula hypoleuca*), females transfer lower concentrations of lysozyme when the risk of nest predation is high (Morosinotto et al. 2013).

In summary, these studies provide some degree of evidence that the differential allocation of antimicrobial proteins to eggs may be an adaptive response evolved to increase the probability of offspring survival. In the future, addressing the effects of the maternal physiology and the environment on the allocation of these molecules will be

useful for understanding the evolution of antimicrobial defense under various risks of infection.

Conclusions

Here, we have summarized, to the best of our knowledge, the current understanding of antimicrobial defenses in avian eggs. Although the majority of this work has been conducted by the poultry industry, the past 10 years have witnessed a dramatic increase in the number of studies on wild bird populations following the groundbreaking work of Cook et al. (2003, 2005). The directions of future research are numerous, but among the most critical is the identification of micro-environmental factors (i.e., at the level of the nest) that affect the risk of infection and the evolutionary responses of birds to these elements. An integrative approach incorporating such fields as ecology, evolutionary biology, physiology, behavior, chemistry, and material science will be essential as we move forward.

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