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Antimicrobial Defences of the Avian Egg

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Antimicrobial Defences of the Avian Egg

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Summary:

The successful proliferation of future avian generations is dependent on the ability of eggs to remain uncontaminated during incubation. Antimicrobial constituents are incorporated into the egg during its formation within the reproductive system of the hen. Traditionally, avian albumen is considered as the only defensive component of the egg while other egg structures (shell and membranes) are considered simply as physical barriers. In this thesis, the antimicrobial proteins identified in mature avian species and the evidence suggesting their incorporation in eggs is presented. Albumen of both domestic and wild avian species is investigated for its protein content and antimicrobial properties in order to investigate the effects of phylogeny and environmental pressures on the antimicrobial defences of the egg. Cuticle and outer eggshell protein extracts from both domestic and wild avian species as well as lipophilic outer eggshell components of domestic avian species are investigated in order to demonstrate the role of the eggshell in the antimicrobial defence of the avian egg. The role of selected eggshell proteins in the antimicrobial defence of the avian egg is investigated and discussed. The results presented demonstrate that antimicrobial defence in the avian egg is a much more complex system than previously believed. Egg white as well as eggshell specific proteins appear to enhance the antimicrobial defences of the egg. In addition, species-specific antimicrobial defensive strategies appear to be present in the eggs of avian species in response to unique environmental pressures.

Résumé:

La prolifération des futures générations d'oiseaux est dépendante de la capacité de l'oeuf de demeurer sans contamination bactérienne durant la période d'incubation. Des éléments antimicrobiens sont incorporés dans l'oeuf au cours de sa formation dans le système reproducteur de la poule. Traditionnellement, le blanc d'oeuf est considèré comme la seule défense de l'oeuf tandis que les autres structures de l'oeuf (membranes et coquille) sont perçues simplement comme étant des barrières physiques. Au cours de cette thèse, les protéines antimicrobiennes identifiées chez les oiseaux matures ainsi que les données suggérant leur incorporation dans l'oeuf seront présentées. Le contenu en protéines et les propriétés antimicrobiennes du blanc d'oeuf d'oiseaux domestiques et sauvages sont étudiées pour observer l'effet de la phylogénie et des pressions environnementales sur les défenses antimicrobiennes de l'oeuf. Les protéines extraites de la cuticule et de l'exterieur de la coquille des oeufs d'oiseaux domestiques et sauvages, ainsi que des extraites lipophyliques de l'exterieur de la coquille des oeufs d'oiseaux domestiques, sont étudiées pour déterminer le rôle de la coquille dans la défense antimicrobienne de l'oeuf. Le rôle de certaines des protéines de la coquille dans la défense antimicrobienne de l'oeuf est étudié et discuté. Les résultats obtenus démontrent que la défense antimicrobienne de l'oeuf est un système complexe. Le blanc d'oeuf ainsi que les protéines de la coquille de l'oeuf semblent accroître les défenses antimicrobiennes de l'oeuf. De plus, des défenses antimicrobiennes uniques à certaines espèces d'oiseaux semblent être présentes dans les oeufs en réponse aux pressions environnementales.

Introduction:

With approximately 10 000 species, the Aves are the most diverse class of terrestrial vertebrates with representatives in environmental niches spanning from desert to arctic. Avian eggs are exposed to the harsh conditions of the environment. The materials from which nests are constructed often provide a suitable environment for the growth of fungi (Baggott and Graeme-Cook, 2002). Many micro-organisms, such as *Escherichia coli* and *Staphylococcus* spp., have been known to penetrate the shell and cause embryonic mortality in chickens (Baggott and Graeme-Cook, 2002). Pathogens of avian embryos include protozoa and neoplasm, in addition to viruses, bacteria and fungi (Romanoff, 1972). Cook et al., (2003) noted that bacterial infection of chicken eggs is most prevalent under conditions of high relative humidity and demonstrated that the probability and magnitude of infection was positively related to exposure period. The successful propagation of future bird generations is dependent on the ability of eggs to remain uncontaminated during incubation.

In order to overcome the problem of bacterial contamination, antimicrobial strategies are undoubtedly present in avian eggs. The egg, not being a complete organism, must therefore rely on antimicrobial products from the parent hen or on other defensive strategies. Multiple antimicrobial proteins have been identified within the egg white of the domestic chicken, *Gallus gallus*. Ovotransferrin, a 78 kDa glycoprotein with two iron binding sites, has been found to inhibit bacterial growth by limiting free iron in albumen (Valenti et al., 1981a). Lysozyme, a 14 kDa enzyme which breaks down the peptidoglycan layer of Gram-positive bacteria is found in high levels in egg white (Bera et al., 2005; Burley and Vadehra, 1989). These two proteins are present in high levels within chicken albumen and are homologous to the major components of the innate immune systems of mature vertebrates.

Over a dozen antimicrobial proteins have been identified within the tissues and secretions of mature birds. Although limited, the available literature suggests that antimicrobial proteins are expressed within the reproductive tract of the domestic hen, *Gallus gallus*, and may be incorporated within the egg. Traditionally, the avian eggshell is considered solely as a physical barrier and the egg white has been regarded as the sole component responsible for the astonishing antimicrobial properties of eggs (Seviour and Board,

1972a; Ahlborn et al., 2006; Gautron et al., 2007). Studies investigating the presence of antimicrobial proteins within the avian eggshell are scarce. While multiple antimicrobial proteins have been identified within albumen, few studies have compared the antimicrobial activity of these across species and none have investigated the bacterial toxicity of albumen from wild avian species.

Thesis Objectives and Rational of Approach:

This thesis study was conducted in order to elucidate the mechanisms involved in the antimicrobial defence of the avian egg as well as to provide a significant contribution to the literature currently available on antimicrobial proteins of the avian egg. We hypothesize that proteins within the various compartments of the avian egg provide antimicrobial defence. The eggshell cuticle, the relatively thin protein-rich outer layer of the eggshell directly in contact with the environment, appears particularly promising. This outer eggshell layer is reminiscent of many physical barriers, including vertebrate skin, which have also been shown to provide chemical defence through the incorporation of antimicrobial proteins. Additionally, we hypothesize that environmental pressures, mainly the greater microbial threat present during nesting under humid conditions, can influence antimicrobial properties of the egg. We propose that the eggs of species nesting under humid conditions are at greater risk of microbial contamination and may therefore show more potent antimicrobial defences. Proteins enhancing antimicrobial defences may be localized within specific parts of the egg or be present in many egg compartments.

This thesis study is composed of four main sections each representing one or more chapters that independently contribute significantly to the scientific field and to the elucidation of the mechanisms involved in the antimicrobial defence of the avian egg. Section one, which consists of chapter one, is a literature review which describes the antimicrobial proteins identified in mature birds and their probable incorporation in the egg as well as highlighting the need for future research into avian antimicrobial proteins and the role of the egg as a potential source of bioactive molecules.

Section two, consisting of chapters two and three, compares the antimicrobial activity of avian albumen across species. Chapter two represents one of the few studies comparing

the antimicrobial activity of albumen across domestic avian species while chapter three is the first study to investigate this subject in wild avian species. As a unit, chapters two and three, contribute to the understanding of the effects of phylogeny and environmental pressures (nest humidity and associated microbial load) on antimicrobial activity of avian albumen.

Section three, which consists of chapters four, five and six, investigates the presence of antimicrobial components within the avian eggshell. Chapters four and five represent the first investigations of the antimicrobial activity of eggshell cuticle and outer eggshell protein in domestic and wild avian species. As a unit, chapters four and five contribute to the understanding of the effects of phylogeny and environmental pressures on the antimicrobial activity of the avian eggshell. Chapter six represents the first study to investigate the antimicrobial activity of lipophilic components from avian eggshell. Thus, section three is a significant contribution describing the involvement of the eggshell, an egg compartment other than egg white, in the antimicrobial defence of the avian egg.

Section four, consisting of chapter seven, is the first investigation to demonstrate that purified avian eggshell specific proteins posses antimicrobial activity. Chapter seven is an investigation of the antimicrobial activity of avian C-type lectin-like proteins, a group of major eggshell specific proteins present in the eggshells of many avian species. Finally, the major contributions of this thesis study as well as the future direction of research into antimicrobial defences of the avian egg will de discussed in a concluding section.

Section I: Chapter 1

With the emergence of antibiotic resistant bacterial strains, antimicrobial proteins present in many organisms represent an appealing alternative to conventional antibiotic agents. Much research has been conducted on insect, amphibian and mammalian antimicrobial proteins. In many cases, extraction of these highly important molecules is complicated and few viable sources of antimicrobial proteins have been identified. The avian egg, a widely available structure of high protein content, is already the source of many biomolecules and may also represent a viable source of antimicrobial proteins.

Currently, over a dozen antimicrobial proteins, including defensins, cathelicidins, lysozymes and transferrins, have been identified in mature avian species. Bioinformatics and proteomic investigations into avian antimicrobial proteins are demonstrating expression of these molecules in the reproductive tract of the hen and suggesting the incorporation of antimicrobial proteins within the egg.

In this section, the available literature on antimicrobial proteins identified in mature avian species is presented. The evidence suggesting the presence of antimicrobial proteins within the avian egg as well as the potential of the egg as an economical source of antimicrobial proteins is discussed. This section represents the first review to document the milestones which have been achieved in the field of avian antimicrobial protein research and sets the direction of future work. This review was entirely written by myself and is published in World's Poultry Science Journal (2007) 63: 421-438.

CHP: chicken heterophil peptide; **THP**: turkey heterophil peptide; **Gal**: gallinacin; **GVP**: gallopavin; **Osp**: ostricacin; **Sphe**: sphenicin; **LEAP**: liver-expressed antimicrobial peptide; **CMAP**: chicken myeloid antimicrobial peptide; **c-type**: chicken-type; **g-type**: goose-type; **HEWL**: hen egg white lysozyme; **AvBD**: avian beta-defensin

Chapter 1: Avian Antimicrobial Proteins: Structure, Distribution and Activity

Olivier Wellman-Labadie, Jaroslav Picman and Maxwell T. Hincke

Abstract:

Antimicrobial proteins are active against protozoans, fungi, viruses as well as Gram-positive and Gram-negative bacteria. In many cases, antimicrobial proteins are present as components of innate immunity and are capable of evading bacterial resistance mechanisms. Due to these characteristics, these proteins represent an appealing alternative to conventional antibiotic drugs. Considerable research has been conducted on antimicrobial proteins from invertebrate and mammalian sources. Within the last decade, over 20 novel antimicrobial proteins have been isolated from avian systems. The majority of these proteins has been isolated from the domestic chicken and therefore represents a minuscule fraction of the avian antimicrobial proteins that are potentially awaiting discovery. In this review, we elaborate on these discoveries and on the future of avian antimicrobial protein research.

Introduction:

Proteins with antimicrobial properties are produced by bacteria, fungi, plants and animals (Sugiarto and Yu, 2004; Bulet et al., 2004; Hancock and Chapple, 1999). In unicellular organisms, antimicrobial proteins are produced in order to provide a competitive edge while leaving the host unaffected (Shelburne et al., 2007; Hancock and Chapple, 1999). In multi-cellular organisms, antimicrobial proteins are vital components of innate immunity and provide protection against foreign particles while activating a multitude of immune functions (Bulet et al., 2004; Ganz, 2003). Structural features including linear helical conformations, richness in a particular amino acid residue and the presence of one or several internal disulfide bonds are seen in antimicrobial proteins (Bulet et al., 2004; Andreu and Rivas, 1998). In general there are at least two types of mechanisms by which antimicrobial proteins affect micro-organisms. Bacteriostatic antimicrobial proteins inhibit bacterial growth by limiting the availability of essential elements or inhibiting metabolism while bactericidal antimicrobial proteins lead to cell death through digestion of targeted cell walls or through the disruption of membrane potential.

Although antimicrobial proteins show great variation in origin, form and mode of action, they possess qualities which are of great interest in an age of antibiotic resistance. Antimicrobial proteins may show activity against both drug-resistant and sensitive pathogens since target components are largely conserved (Kamysz, 2005; Matsuzaki, 2001). Many contribute to rapid bacterial death and resistance is made difficult since activity is not prevented by enzymatic degradation and export processes (Matsuzaki, 2001). Antimicrobial proteins are selective; some target negatively charged bacterial membranes while others bind specific metals and vitamins, inhibit proteases or hydrolyse specific chemical bounds while leaving host and non-target cells intact (Supuran et al., 2002).

Antimicrobial proteins have a variety of uses and are currently being used as food additives and as therapeutic agents. Chicken egg white lysozyme is used as a food preservative in cheese, wine and tofu while nisin, an antimicrobial peptide produced by some strains of *Lactococcus lactis*, is a common additive of cheese products (Mine et al., 2004; Davies et al., 1997). Clinical trials with topical creams containing the synthetic peptide pexiganan, a derivative of the magainin peptide isolated from the skin of the African clawed frog (*Xenopus laevis*), have been found to reduce viral skin infections (Sugiarto and Yu, 2004). Many ongoing clinical trials are being conducted using antimicrobials such as α-defensin, cecropin (first identified from the giant silk moth *Hyalophora cecropia*), and magainins to treat various infectious diseases (Andreu and Rivas, 1998). Antimicrobials, such as nisin and magainins, are being considered as potential contraceptives due to spermicidal and antiseptic activity (Reddy et al., 2004; Andreu and Rivas, 1998).

Due to their astonishing properties, considerable interest is being invested in antimicrobials and the search for new antimicrobial sources. Although most research is conducted in mammalian systems, over a dozen novel antimicrobial agents have been identified in avian tissues and secretions. Within the last decade, gallinacins, heterophil peptides, ostricacin, sphenicins, gallopavin and fowlicidins have all been isolated from mature birds. Although the antimicrobial properties of avian egg components have been recognized since the early 20th century, few antimicrobial agents, other than lysozyme and ovotransferrin have been identified in this medium. In this review, we elaborate on these discoveries and assess the potential of egg proteins in avian antimicrobial defence.

Since yolk is capable of supporting large bacterial populations (Humphrey and Whitehead, 1993; Humphrey et al., 1991), focus will be placed particularly on the antimicrobial proteins present in avian albumen and eggshell.

Avian Antimicrobial Peptides:

A variety of low molecular weight cationic membrane binding proteins have been identified in invertebrates and vertebrates. These fast acting proteins are routinely defined as antimicrobial peptides, due to their low molecular weight, and are effective against a wide spectrum of targets. Such antimicrobial peptides are present as components of innate immunity and provide protection against invading pathogens. In the class Aves, antimicrobial peptides are present as β-defensins and cathelicidins. The mature peptides are produced by proteolytic cleavage from a precursor form containing a signal peptide and a propiece. This mechanism of activation appears to be a conserved strategy in host defence (Tennessen, 2005).

Defensins:

Defensins are low molecular weight, non-glycosylated, cysteine-rich, cationic peptides with a triple-stranded β-sheet structure connected with a loop of β-hairpin turn (Sugiarto and Yu. 2004, Evans et al., 1994). Six-eight cysteine residues that form three-four pairs of disulfide bridges with 2-3 anti-parallel β-sheets with or without α-helix is the main characteristic of these peptides (Sugiarto and Yu, 2004; Xiao et al., 2004). Defensins contribute to the antimicrobial properties inherent in mammalian granulocytes, epithelial cells and certain secretions (Zhao et al., 2001). These antimicrobial peptides are active against Gram-positive bacteria, Gram-negative bacteria, protozoans as well as some fungi and enveloped viruses including herpes species (Sugiarto and Yu, 2004). Defensins are chemotactic for T-cells, monocytes and immature dendritic cells while having toxic effects on a wide range of normal and malignant cells including cells resistant to tumor necrosis factor-α (Bulet et al., 2004). Defensin activity is observed at micromolar concentrations but can be reduced under high ionic strength conditions (Evans et al., 1995; Bulet et al., 2004). Defensins can be classified into plant, invertebrate and vertebrate α-, β- and θ-defensins (Xiao et al., 2004).

Classical defensins, or α-defensins, are short cationic peptides composed of three pairs of disulfide-linked cysteines (Brockus et al., 1998). The slightly larger β-defensins differ in spacing and connectivity of the conserved cysteine residues while having a similar three-dimensional structure (Brockus et al., 1998). In α-defensins, the disulfide pairing is represented by Cys1-Cys6, Cys2-Cys4, and Cys3-Cys5 while pairing at Cys1-Cys5, Cys2-Cys4, and Cys3-Cys6 occurs in β-defensins (Landon et al., 2004). α-defensins and β-defensins are characteristic of vertebrates while θ-defensin is only present in primates (Xiao et al., 2004). β-defensin, the only form present in the Aves, is believed to have given rise to classical defensin from which the cyclic θ-defensin arose by posttranslational ligation of two α-defensin-like sequences (Xiao et al., 2004).

The mechanism of action of β-defensins is not fully understood. It is believed that the exposed cationic sites of β-defensin interact with the negatively charged bacterial membrane pulling the peptide toward the membrane (Sugiarto and Yu, 2004). A dimer is then formed creating a lethal voltage gated ion channel between the amino-terminal β-strands of the two β-defensin monomers resulting in changes in trans-membrane potential, membrane disruption and cell death (Sugiarto and Yu, 2004; Harmon, 1998).

Avian β-defensins can be classified according to their source. Avian heterophil peptides include two chicken heterophil peptides (CHP-1 and CHP-2), three gallinacins from chicken (Gal-1, Gal-2 and Gal-1α), three turkey heterophil peptides (THP-1, THP-2 and THP-3) and Ostricacin (Osp-1) from ostrich (Sugiarto and Yu, 2004). The non-heterophil peptides include Gal-3 and gallopavin-1 (GVP-1), expressed and characterized from the epithelial cells of chicken and turkey, as well as sphenicins (Sphe-1 and Sphe-2) from the stomach content of king penguin (Sugiarto and Yu, 2004). The antimicrobial spectrum and minimum inhibitory concentrations of selected heterophil and non-heterophil avian β-defensins are summarized in Table 1. The sequences for Gal-1 and CHP-1 as well as Gal-1α and CHP-2 are almost homologous and could be due to gene polymorphism (Sugiarto and Yu, 2004). Brockus et al., (1998) proposed to name these peptides as Gal-1/CHP-1 and Gal-1α/CHP-2. Various avian β-defensins have been identified by differing research groups which each developed their own nomenclature system. Although this original nomenclature can at times be a source of confusion, it was adopted for this

review for consistency with the literature. However, during the submission of this manuscript, Lynn et al., (2007) proposed a new standardized nomenclature system where the terms "gallinacin, heterophil peptide, ostricacin and spheniscin" will be replaced by "avian β-defensin" (AvBD) and where gene and peptide numbers correspond to each other in a systematic manner.

Heterophil Peptides:

CHP-1, CHP-2 and THP-1 are very similar in primary structure and in spectrum of microcidal activity (Evans et al., 1995). CHP-1 consisted of 39 amino acids and contains 6 cysteines and 6 arginines with a molecular mass of 5138 ± 2 daltons (Evans et al., 1994). THP-3 shares less primary structural homology with the other peptides and appeared less potent against particular microbes (Evans et al., 1995). THP-2 and THP-3, which are homologous, form a unique cysteine motif and demonstrate a similar antibacterial spectrum (Evan et al., 1994). THP-1 and THP-2 as well as Gal-1 and Gal-2 proregions are similar to storage granule-free β-defensin proregions and lack the long negatively charged propiece reported in classical defensins that is thought to stabilize, inactivate and target the propeptide for storage granules (Brockus et al., 1998).

Gal-1, Gal-1α and Gal-2 were expressed strongly in healthy bone marrow and to a lesser extent in lung (Zhao et al., 2001). Ohashi et al., (2005) reported strong Gal-1 and Gal-2 expression in the chicken infundibulum while weaker expression was detected in the magnum, isthmus, uterus and vagina. Harwig et al., (1994) reported a calculated molecular weight for Gal-1α, Gal-1 and Gal-2 of 4582, 4504 and 3916 Da respectively. Molecular weight determined by ESI-MS of the three peptides was 4582, 4505 and 3916 Da respectively (Harwig et al., 1994). Expression of Gal-1 and Gal-2 as well as Gal-3, a non-heterophil peptide, has also been demonstrated in the vaginal mucosa of the domestic chicken (Yoshimura et al., 2006). Yoshimura et al., (2006) noted that the expression was greater in older birds and increased with *S.* Enteritidis or lipopolysaccharide stimulation. Expression of the three gallinacins decreased in the vaginal mucosa of the regressed oviduct of non-laying birds when compared with laying birds (Yoshimura et al., 2006).

Heterophil peptides have been identified in the chicken and turkey but are also present in non-Galliform species. Ostricacin-1 (Osp-1) was characterised from ostrich leukocytes by Yu et al., (2001). This 36 residue peptide has a molecular mass of 4011 Da and is active against *E. coli* and *S. aureus* (Yu et al., 2001). Sugiarto and Yu (2006) purified and characterised three additional β-defensins from ostrich heterophils. The peptides are composed of 36-42 amino acids with molecular weights ranging from 4.70-4.98 kDa (Sugiarto and Yu, 2006), and represent the active, mature form of these defensins. Osp-2, Osp-3 and Osp-4 were active against *E. coli* and *S. aureus* while only Osp-2 showed activity against *C. albicans* (Sugiarto and Yu, 2006).

Non-Heterophil Peptides:

In chicken, Gal-3 was especially prominent in the tongue, bursa of Fabricius and trachea but also occurred in the skin, esophagus, air sacs, large intestine and kidney (Zhao et al., 2001). Strong expression of Gal-3 was detected in chicken infundibulum and vagina while weaker expression was reported in magnum, isthmus and uterus (Ohashi et al., 2005). Weaker expression of Gal-3 was seen in the large intestine, bone marrow, kidney and ovary (Zhao et al., 2001). Gal-3 is also present in epithelial tissues and colon (Townes et al., 2004). Tracheal expression of Gal-3 was increased significantly after experimental infection whereas expression in tongue, esophagus and bursa of Fabricus was unaffected (Zhao et al., 2001). The deduced Gal-3 prepropeptide contained 80 amino acids and had a mass of 8,723 Da and a pI of 9.42 while the propeptide contained 38 residues with a mass of 4,234 Da and pI of 9.49 (Zhao et al., 2001). An epithelial β-defensin homologous to Gal-3 was found in turkey tracheal tissue and named gallopavin-1 (GVP-1) (Zhao et al, 2001). The cationic prepropeptide of GVP-1 has a calculated mass of 6,598 Da and a pI of 9.49 (Zhao et al., 2001).

Sphe-1 (synonym pBD-1) and Sphe-2 (synonym pBD-2) have 38 amino acid residues and molecular weights of 4482 and 4501 Da respectively (Thouzeau et al., 2003). The two peptides were isolated from the stomach contents of the king penguin, *Aptenodytes patagonica.* It is believed that the two peptides aid in the preservation of stomach contents during the extended incubation period where king penguins are unable to leave the nest and forage. Spheniscins were found to be active against both Gram-positive and Gram-negative bacteria as well as fungi (Thouzeau et al., 2003). The synthetic version of

Sphe-2 shows bactericidal activity against Gram-positive *S. aureus* while being bacteriostatic against Gram-negative *E. coli* (Landon et al., 2004). Residue 14 of Sphe-1 is a histidine while an arginine is present in Sphe-2 (Thouzeau et al., 2003). A large positive net charge is believed to explain the high activity of Sphe-2 in the salt-rich environment of the penguin stomach (Landon et al., 2004).

Bioinformatically Identified Defensins:

Two additional chicken β-defensins, Gal-11 and Gal-12, were identified by hidden Markov model profile searching of the chicken genome (Higgs et al., 2005). Gal-11 was approximately 4,500 Da and expressed highly in the small intestine, the liver, gall bladder and spleen while the tongue, proventriculus, bursa of Fabricius, trachea, lungs, air sacs, kidney and pancreas showed moderate expression (Higgs et al., 2005). Gal-11 was active against *L. monocytogenes*, *S. enteritidis* serovar Typhimurium, *E. coli* and *Streptococcus pyogenes* but not against *S. aureus* (Higgs et al, 2005). Gal-12 has a more restricted expression pattern and is highly expressed in the liver and gall bladder (Higgs et al., 2005).

Xiao et al., (2004) reported that the chicken β-defensin genes, designated Gallinacin 1 to 13, are clustered densely within an 86 kb distance on chromosome 3q3.5-q3.7 and code for peptides that are 63 - 104 amino acids in length. The Gallinacin 1 to 7 genes showed expression in bone marrow and respiratory tract while the Gallinacin 8 to 13 genes were expressed in liver and urogenital tracts (Xiao et al., 2004). Simultaneously, Lynn et al., (2004) reported seven novel gallinacins (Gal-4 to Gal-10), a novel cathelicidin and a novel liver-expressed antimicrobial peptide (LEAP-2) by bioinformatic search of chicken expressed sequence tags. Chicken liver-expressed antimicrobial peptide (LEAP-2) was expressed in the small intestine, liver, lung and kidney (Townes et al., 2004). The predicted molecular masses of the pro- and mature peptides were 6,074 and 4,593 Da (Townes et al., 2004). A significant increase in the expression of LEAP-2 in the intestine and liver of chickens infected with *Salmonella* was detected (Townes et al., 2004). The gene coding for LEAP-2 is located within chromosome 13 and is expressed in epithelial tissues, liver, lung, kidney and small intestine (Townes et al., 2004). LEAP-2 is induced in the liver and small intestine by *Salmonella* infection and the response of *S.* Enteritidis strains to LEAP-2 peptides varies for the various strains (Townes et al., 2004).

Cathelicidins:

Cathelicidins are highly variable cationic antimicrobial proteins encoded by prepropeptides containing a highly conserved cathelin pro-sequence at the N-terminus (Lynn et al., 2004: Xiao et al., 2006a). The prepropeptide is activated by proteolytic cleavage to a peptide of 12 to 97 amino acids in length (van Dijk et al., 2005). The mature peptide demonstrates broad spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria as well as fungi, protozoa and enveloped viruses (van Dijk et al., 2005). Mature cathelicidin possesses the ability to bind and neutralize endotoxin and induce chemotaxis of neutrophils, T-cells and monocytes (van Dijk et al., 2005). Cathelicidins are chemo-attractant, activate a variety of immune cells, inhibit NADPH oxidase, interact with lipopolysaccharide, kill activated lymphocytes as well as promoting angiogenesis, cytolysis and wound healing (Xiao et al., 2006a; Ramanathan et al., 2002).

The mechanism of cathelicidins is believed to be similar to that of defensins in that the peptides bind lipopolysaccharide membranes by physical interaction with negatively charged microbial membrane phospholipids (Xiao et al., 2006a). Bacterial cell death through membrane permeabilization and disruption is the final result.

Cathelicidins are well represented in mammals and have recently been identified in the Atlantic hagfish and the chicken *Gallus gallus* (van Dijk et al., 2005). Cathelicidins are differentially expressed in a variety of tissues. Phagocytic cells, such as neutrophils, are a rich cathelicidin source in many species while mucosal epithelial cells and skin keratinocytes show lower expression (Xiao et al., 2006a: van Dijk et al., 2005).

Xiao et al., (2006a) identified 3 novel cathelicidins (fowlicidin-1, fowlicidin-2, and fowlicidin-3) densely clustered within 7.5kb on the p-arm of chromosome 2 less than 3.5Mb from the proximal end. The genes coding for the three fowlicidins consists of the 4 exon 3 intron structure typical of mammalian cathelicidin (Xiao et al., 2006a). Fowlicidin-2 and fowlicidin-3 are placed in a head-to-head orientation separated only by 736bp from the stop codon of both genes while fowlicidin-1 and fowlicidin-2 are separated by 2.4kb (Xiao et al., 2006a). The three peptides are positively charged at the

C-terminus due to an excess of arginine and lysine. Fowlicidin-1 is composed of 26 amino acid residues with a net charge of +8, fowlicidin-2 is composed of 32 amino acid residues with a net charge of +10 while fowlicidin-3 is composed of 29 amino acid residues with a net charge of +7 and has high sequence homology to fowlicidin-1 interpreted to have arisen as a result of gene duplication (Xiao et al., 2006a).

Xiao et al., (2006a) reported that synthetic fowlicidin-1 and fowlicidin-2 had potent salt-independent antimicrobial activity against both Gram-negative and Gram-positive bacteria. The synthetic proteins retained their activity in salt concentrations as high as 100mM NaCl. The MIC of the peptides was found to vary between 0.4-2.0 μM although both fowlicidin-1 and fowlicidin-2 were found to be cytotoxic against mammalian erythrocytes and epithelial cells at 6-40μM (Xiao et al., 2006a). Fowlicidin-1 was found to be slightly more active than fowlicidin-2. Fowlicidin-1 and fowlicidin-2 are among the most potent cathelicidins known and represent promising theurapeutical agents due to their salt-independent activity against both drug-resistant and sensitive bacteria at sub-cytotoxic levels.

Sequences identical to fowlicidin-1 and fowlicidin-2 were independently reported by Lynn et al., (2004) and van Dijk et al., (2005). Lynn et al., (2004) noted that a cathelicidin (fowlicidin-1) was expressed in chicken bursa, testis, gizzard and bone marrow. Van Dijk et al., (2005) reported an avian cathelicidin, chicken myeloid antimicrobial peptide 27 (CMAP27 synonym fowlicidin-2), which is composed of 27 residues and is predicted to form an α-helical configuration with a hydrophobic tail. CMAP27 is expressed in bone marrow, bursa of Fabricius, testis, spleen, kidney, liver, thymus, caecal tonsil and lymphoid tissue at the base of the caeca whereas lower expression is found throughout the rest of the intestinal tract (van Dijk et al., 2005). CMAP-27 expression is low in skin tissue while much higher in the uropygial gland tissue (van Dijk et al., 2005). This sebaceous gland is located at the base of the tail of birds and secrets a holocrine solution of waxes and oils possessing antimicrobial properties and serves to maintain feather condition (van Dijk et al., 2005). It is probable that these secretions become in contact with the surface of the egg during incubation and may provide some protective value.

Higher Molecular Weight Avian Proteins with Antimicrobial Activity:

Although proteins with antimicrobial properties are widely recognized as low molecular weight proteins, we have chosen to incorporate larger antimicrobials in this review due to their potent activity and prevalence in avian systems. Lysozyme and transferrin are such higher molecular weight antimicrobial proteins present in the class Aves that share properties with antimicrobial peptides.

Lysozyme:

Lysozyme, or N-acetylmuramideglycanohydrolase, splits the bond between the glycosidic beta-1, 4-linked residues of N-acetylneuramic acid (NAM) and N-acetylglucosamine (NAG) in the peptidoglycan structure of Gram-positive bacteria and to a lesser extent in some Gram-negative bacteria (Bera et al., 2005; Burley and Vadehra, 1989). Lysozyme is widely distributed in eukaryotes and prokaryotes and has been identified in viruses, bacteria, plants and animals. Lysozymes are self-defense enzymes found mainly in egg whites, tears and various secretions of eukaryotic cells (Pooart et al., 2004).

There are three main types of lysozyme; phage type, chicken-type (c-type) and goose-type (g-type). Phage type, or T4-type, is a viral lysozyme while c-type and g-type were first isolated from avian egg white. The different classes of lysozymes have overall similarities in tertiary structure although their amino acid sequences are entirely different (Pooart et al., 2005) and are products of different genes (Irwin and Gong, 2003). As can be seen on the NCBI database, both the c-type lysozyme gene (GeneID: 396218) and g-type lysozyme gene (GeneID: 418700) of *Gallus gallus* are located on chromosome 1 but are separated by a large distance. In the domestic chicken, g-type lysozyme is expressed in bone marrow and lung tissue while c-type lysozyme is expressed in several tissues, including red isthmus and uterus, and is incorporated in the avian eggshell, mammillary layer and eggshell membranes (Prager and Jolles, 1996; Nakano and Graf, 1991; Hincke et al., 2000). G-type lysozyme has been identified in various tissues of the goose and in goose egg white (Irwin and Gong, 2003; Hindenburg et al., 1974). The egg white of chicken and Peking duck have been found to contain only c-type lysozyme while black

swan and Canada goose egg white both contain c-type and g-type lysozyme (Hindenburg et al., 1974).

C-type Lysozyme:

Chicken-type or c-type lysozyme, also known as hen egg white lysozyme (HEWL), was first isolated from the egg white of the chicken, *Gallus gallus*, at the beginning of the 20th century by Sir Alexander Flemming and has since been identified in various eukaryotic secretions. This 14.4kDa protein constitutes 3.5% of chicken egg white protein and is highly active against *Micrococcus* and *Bacillus* species while demonstrating lower activity against Gram-negative bacteria (Bera et al., 2005; Burley and Vadehra, 1989). In addition to direct bacteriolytic action, other biological functions have been reported for lysozyme such as antiviral, antibiotic, anti-inflammatory, antihistaminic, anti-heparinic, anti-tumor and fusogenic activity as well as agglutination and immune cell activation (Mine et al., 2004). Lysozyme plays a role in antitumor activity, inactivation of certain viruses, induction of phospholipid vesicles, enhancement of phagocytic activity as well as stimulating monocytes and influencing surveillance of mammalian cell membranes (Ibrahim et al., 2001b). This protein has a broad pH optimum of 5.5-7.0 and is present mainly in Galliformes and Anseriformes. Three c-type lysozymes, some showing greater activity than hen egg white lysozyme have been isolated from egg white of the Peking duck (Kondo et al., 1982). Hermann et al., (1971) also reported two c-type lysozymes in the albumen of the Kaki duck. These species represent the first examples of c-type lysozyme polymorphism which has been attributed to multiple alleles at a single locus (Prager and Jolles, 1996).

Chicken egg white lysozyme is a medically and commercially important protein. It has been estimated that more than 100 tons of lysozyme is used annually for pharmacological and therapeutic applications as well as in the preservation of food products such as vegetables, fish, meat, sea food, butter, cheese and wine (Mine et al., 2004). Austria, Australia, Belgium, Denmark, Finland, France, Germany, Italy, Japan, Spain and the United Kingdom have approved the use of lysozyme for pharmacological and therapeutic use (Mine et al., 2004). Oral and topical application of lysozyme has been found to inhibit viral skin infections such as herpes simplex and chicken pox (Sava, 1996). Lysozyme has even been reported by Lee-Huang et al., (1999) to be active against HIV

type I. With its broad working range, hen egg white lysozyme is the most effective lysozyme and is the only lysozyme that is currently used in commercial applications (Nakimbugwe et al., 2006).

Lysozyme is an enzyme which acts by extensive hydrolysis of peptidoglycan and also demonstrates a non-lytic bactericidal mechanism involving damage without hydrolysis. Thermal and chemical-thermal modification of lysozyme leads to the formation of polymers and thereby leads to the loss of enzymatic activity while extending the range of lysozyme activity to include Gram-negative bacteria such as *E. coli* (Lesnierowski et al., 2004; Ibrahim et al., 1996). In a site-directed mutagenesis experiment conducted by Ibrahim et al., (2001a), peptidoglycan hydrolysis was inactivated by substituting one of the catalytic residues in chicken egg white lysozyme. The mutant protein retained bactericidal activity against Gram-positive bacteria, indicating that antimicrobial activity could be independent from catalytic activity (Ibrahim et al., 2001a). Clostripan digestion of lysozyme produced an antimicrobial peptide composed of residues 98-112 with broad spectrum activity (Ibrahim et al., 2001b). This sequence was found to be part of the helix-loop-helix domain (residues 87-114) located at the upper lip of the active site cleft of lysozyme and had potent activity against both Gram-positive and Gram-negative bacteria as well as the fungus *C. albicans* (Ibrahim et al., 2001b).

G-type Lysozyme:

Goose-type or g-type lysozyme was first isolated from the egg white of the domestic goose (Canfield and McMurray, 1967; Dianoux and Jolles, 1967). Although absent from chicken egg white, g-type lysozyme is widely distributed and has been identified in 9 orders including the Anseriformes (Florkin et al., 1978; Hemmen et al., 1992; Prager and Wilson, 1974; Nakano and Graf, 1991). This 20kDa muridase has a 3 to 6 fold higher specific activity but lacks the chitinase activity that is characteristic of c-type lysozyme (Hindenburg et al., 1974). Canfield and McMurry (1967) reported that the enzymatic activity of goose egg white lysozyme is three times greater than an equimolar amount of hen egg white lysozyme at pH 6.2. A partial common domain structure for both types of lysozyme has been determined but only slight amino acid sequence homology is found between g-type and c-type lysozyme (Hemmen et al., 1992). Antibodies directed against c-type hen egg white lysozyme do not cross-react with the g-type lysozyme and *vice*

versa (Hemmen et al., 1992). The pH optimum of g-type lysozyme of goose and ostrich was found to be at pH 5.5-6.0 while that of rhea was at pH 6.0 and was found to show more activity than the other g-type lysozymes (Pooart et al., 2005). Rhea, goose, ostrich and cassowary g-type lysozymes were all found to be inactive at a pH below 3.0 or greater than 8.0 (Pooart et al., 2005). Cassowary g-type lysozyme was found to have a narrow and acidic pH optimum at pH 5 which contrasted with HEWL which had a broad maximum pH range of 5.5 to 7.5 and was about 3 times more active than HEWL at pH 5.5 (Thammasirirak et al., 2001). Cassowary g-type lysozyme had its optimum temperature at around 30°C while HEWL had a broad optimum temperature from 40 to 60°C (Thammasirirak et al., 2001).

Transferrins:

Transferrins are 80kDa glycoproteins composed of two domains that each reversibly bind one molecule of iron (Valenti et al., 1981a). These proteins are present in various concentrations in the cells and biological fluids of mammals and birds. Transferrins play a role in iron transport and inhibit the growth of micro-organisms at pH 6 to 10 by limiting the amount of free iron available (Phelps and Antonini, 1975). Transferrins bind a number of other transition metals but the affinity of the protein is always higher for iron than the various other metals (Valenti et al., 1987). The amino acid composition has been found to be similar across transferrins while the number and composition of carbohydrate groups attached to different transferrins has been found to be variable (Graham and Williams, 1975). Transferrins are present in mammals as serum transferrin and lactoferrin while ovotransferrin and serum transferrin are the forms present in birds. Serum transferrin, also known as siderophilin, has been found to play a role in iron transport in the plasma of vertebrates including birds (Rawas et al., 1989). In the chicken, the protein moieties of serum transferrin and ovotransferrin appear to be identical while the carbohydrate groups differ (Graham and Williams, 1975).

Ovotransferrin, also known as conalbumin, is a major egg white protein and represents 10% of chicken albumen protein (Burley and Vadehra, 1989). The unavailability of iron through chelation by ovotransferrin is the principle impediment to the growth of bacteria in the albumen of the hen's egg (Seviour and Board, 1972a). In *E. coli* and other Gram-negative micro-organisms which possess an iron transport system mediated by citrate, it

was demonstrated that bicarbonate enhanced the antimicrobial action of ovotransferrin while citrate had an antagonizing effect (Valenti et al., 1981a; Valenti et al., 1985). The addition of citrate and bicarbonate to ovotransferrin inhibits the growth of *S. aureus* by 50% (Valenti et al., 1981a). *Pseudomonas sp.*, *E. coli* and *Streptococcus mutans* were most sensitive while *S. aureus*, *Proteus sp.* and *Klebsiella sp.* were most resistant to ovotransferrin (Valenti et al., 1983). In a study by von Hunolstein et al., (1992), iron-binding proteins, such as ovotransferrin and lactoferrin, failed to show any activity towards Streptococcal species such as *S. bovis*, *S. mutans* and *E. faecalis*. Valenti et al., (1981b) noted that almost 100% of the strains tested from the genus *Proteus* were capable of multiplying in the presence of 5mg/ml of ovotransferrin.

Ovotransferrin has also been found to show antiviral and antifungal activity. Giansanti et al., (2002) reported antiviral activity against infection by Marek's Disease Virus. Valenti et al., (1985) reported antifungal activity of ovotransferrin towards *C. albicans* and noted that the activity did not appear to depend only on iron chelation but involved a more complex mechanism such as an interaction of protein and fungal cells. Of the 100 strains within the *Candida* genus tested by Valenti et al., (1985), only *C. krusei* showed noticeable resistance to ovotransferrin. Fungus was found to be more sensitive to ovotransferrin than bacteria and retained activity even during iron saturation suggesting that there is a direct interaction of ovotransferrin and fungal cells (Valenti et al., 1985).

Recently, an antimicrobial region termed ovotransferrin antimicrobial peptide 92 (OTAP-92) has been identified within the ovotransferrin protein sequence which appears to be the analog of lactoferricin, an antimicrobial sequence isolated from bovine lactoferrin. The cationic antimicrobial sequence is composed of 92 residues within the 109-200 sequence of the N2-domain of the N-lobe of ovotransferrin and contains three intrachain disulfide bridges of which two are surface exposed cringle bridges showing similarity to insect antimicrobial peptides (Ibrahim et al., 1998; Ibrahim et al., 2000). The 9.9kDa sequence was isolated by trypsin-nicking and was active against *S. aureus* and *E. coli* K12 (Ibrahim et al., 1998; Ibrahim et al., 2000). It is believed that the mode of action of this peptide involves bacterial membrane penetration and dissipation of membrane potential (Aguilera et al., 2003).

Other Proteins:

Recently, a 3.5 kDa peptide was purified from the mucosa and epithelial cells of the chicken intestine and demonstrated antimicrobial activity against *Salmonella* (Nile et al., 2006). The peptide, AWAP IV, was found to correspond to the C-terminal WAP domain of the 81 kDa AWAK [Avian WAP (whey acidic protein) domain- and Kunitz domain-containing] protein (Nile et al., 2006). AWAP IV expression was detected in small intestine and kidney tissues of 5-day-old *Salmonella*-infected chicks (Townes et al., 2006). Recombinant AWAP IV cell lysate was found to demonstrate inhibition of subtilisin and proteinase K as well as antimicrobial activity against *Salmonella*, *Streptococcus* and *Staphylococcus* spp (Townes et al., 2006).

The immunoglobulins present in birds are likely to play a role in immunity and potentially in egg defence. Both IgA and IgM have been identified in chicken egg white while avian IgG, also termed IgY, is present in the yolk in large concentrations and may be present in trace levels in albumen (Rose et al., 1974; Rose and Orlans, 1981). The transfer of immunity to tetanus toxin from hen to chick has been demonstrated (Rose and Orlans, 1981). In addition, the presence of antibodies to Newcastle disease virus, the protozoan *Leucocytozoon* as well as other viruses and bacteria have been found in chicken egg white and yolk (Rose and Orlans, 1981). Kassaify and Mine (2004) reported that non-immunized egg yolk powder supplemented into diets of laying hens can eliminate and prevent intestinal *S.* Enteritidis colonization suggesting that egg yolk contains novel immunomodulatory or anti-adhesive components.

Phosvitin is a mineral binding protein which forms important storage sites within the granule subfraction of avian yolk (Richards, 1997). Due to a high proportion of phosphorylated serine residues, phosvitin avidly binds iron (Taborsky, 1980; Greengard et al., 1964). Chicken egg yolk phosvitin has been reported to be antibacterial against thermally stressed *E. coli* (Sattar Khan et al., 2000). Riboflavin-binding protein and avidin are two vitamin-binding proteins of avian albumen (Muniyappa and Adiga, 1979). The substrate of riboflavin-binding protein, vitamin B12, also known as cobalamin, is synthesized only in prokaryotic organisms and is an essential cofactor for several important bacterial enzymes that catalyze a variety of transmethylation and rearrangement reactions (Martens et al., 2002). Biotin, the substrate of avidin, is required for fixation of carbon dioxide in aspartic acid and is essential for the growth of bacteria

(Broquist and Snell, 1951). Phosvitin, riboflavin-binding protein and avidin may therefore be involved in the antimicrobial defence of the avian egg by limiting the availability of essential elements.

Avian albumen is known to contain protease inhibitors which may also play a role in antimicrobial defence. Chicken egg white ovomacroglobulin, a broad spectrum protease inhibitor, inhibits the bacterial growth and bacterial proteases of *Serratia marcescens* and *Pseudomonas aeruginosa* (Molla et al., 1987; Miyagawa et al., 1991a). Ovomacroglobulin has also been reported to reduce corneal destruction and accelerate wound healing in experimental keratitis in rabbits (Miyagawa et al., 1991b). The chicken egg white cysteine protease inhibitor, cystatin, alters intracellular proteolytic processing of poliovirus proteins and reduces virus yield (Korant et al., 1985). The third domain of the Kazal-type turkey egg white ovomucoid forms a complex with protease B, a serine protease of *Streptomyces griseus* (Read et al., 1983). Chicken egg white ovoinhibitor contains seven Kazal-type domains and has been reported to inhibit protease F from bacterial fibrinolysin (Birk et al., 1983). Chicken egg white ovomucin binds bovine rotavirus, hen new castle disease virus and human influenza virus (Tsuge et al., 1996a).

Some egg white proteins have been found to incorporate antimicrobial peptides within the mature sequence as previously discussed for lysozyme and ovotransferrin. Ovalbumin is the major protein of egg white and comprises 50% of total egg white protein in the chicken (Burley and Vadehra, 1989). This 45kDa glycoprotein has an isoelectric point of 4.7 and is a member of the serpin family; however, it lacks any protease inhibitor activity. Pellegrini et al., (2004) noted that proteolytic digestion of ovalbumin by tryptic and chymotryptic digestion produced fragments with strong antimicrobial activity against *B. subtilis* and weak activity against *Candida albicans*. Ovomucin, the egg white protein which is responsible for the characteristic viscosity of albumen, is another such protein. Ovomucin-derived peptides have been found to show activity against Newcastle disease virus, bovine rotavirus and human influenza virus in vitro (Tsuge et al., 1996a; 1996b; 1997a; 1997b; Watanabe et al., 1998).

Potential of Antimicrobial Proteins in the Avian Egg and Eggshell:

The avian egg provides the developing embryo with essential nutrients and protection from physical and microbial damage while allowing water and gas exchanges. Egg white is well known for its antimicrobial properties and Guerin-Dubiard et al., (2006) recently demonstrated using two-dimensional electrophoresis associated with mass spectrometry that this medium consisted of up to 69 proteins suggesting a much greater complexity than previously believed and hinting to novel and potentially antimicrobial proteins. The albumen and central yolk are surrounded by the eggshell membranes and eggshell. The mineral phase (95%) of the calcified eggshell is predominantly composed of calcium carbonate while the organic phase (3%) is composed of protein and lipids. In direct contact with the environment, the eggshell cuticle is a thin layer composed of proteins, lipids and hydroxyl apatite crystals residing on the surface of the eggshell. This layer is believed to regulate the entry of water in the eggshell pores and may also be involved in antimicrobial defence. There is reason to believe that antimicrobial proteins other than lysozyme and ovotransferrin are present in avian eggs and especially outer eggshell. Mann et al., (2006) recently identified 520 proteins within the acid-soluble organic matrix of the chicken eggshell using MS-based technology further reinforcing the concept that the avian egg is a relatively unexplored source of novel and potentially antimicrobial proteins.

Mine et al., (2003) noted that protein extracted from the eggshell of the domestic chicken demonstrated activity against *P. aeruginosa, Bacillus cereus, S. aureus, E. coli, S. enteritidis* at concentrations suggesting the activity of proteins other than lysozyme. Mann et al., (2006) identified low levels of gallinacin-8 and β-defensin 11 as components of the chicken eggshell matrix using MS-based technology. The recent identification by Silphaduang et al., (2006) of two antimicrobial histone proteins (H1 and H2B), expressed by the chicken ovary and oviduct, suggests that these may be incorporated in the egg during formation. Xiao et al., (2004) reported the expression of the Gallinacin 10 to 13 genes in the chicken reproductive system. In addition, Yoshimura et al., (2006) reported expression of Gal-1, Gal-2 and Gal-3 in the vaginal mucosa of the chicken. Gal-1, Gal-2 and Gal-3 may therefore come into contact with the egg during oviposition and be present on the eggshell surface.

A novel chicken eggshell matrix protein, ovocalyxin-36, shows homology to lipopolysaccharide-binding proteins, bactericidal permeability-increasing proteins, Plunc

family proteins and is believed to play a role in antimicrobial defence (Gautron et al., 2007). Ovocalyxin-32, a chicken eggshell matrix protein found in the cuticle and outer calcified zone of the shell, has been shown to share homology with TIG1, a skin protein and latexin, a carboxypeptidase inhibitor of the rat (Hincke et al., 2003; Gautron et al., 2001a). Recently, recombinant *Gallus gallus* ovocalyxin-32 has been demonstrated to inhibit the growth of *B. subtilis* and to possess carboxypeptidase inhibitory activity (Xing et al. 2007). While recombinant OCX-32 protein may show activity varying from that of native OCX-32, this experimental evidence suggests that it is involved in antimicrobial defence. Chicken egg white ovotransferrin immobilized on Sepharose 4B maintains its iron-binding capacity and bacteriostatic activity (Valenti et al., 1982). In addition, immobilized chicken egg white c-type lysozyme, as occurs naturally within the eggshell matrix, retains its enzymatic activity (unpublished data) suggesting that eggshell matrix antimicrobial proteins may be active *in situ*.

Holocrine secretions of the avian uropygial gland are believed to contain antimicrobials and may come in contact with outer egg surfaces during incubation. Deeming (1987) reported that Muscovy ducks, as is case with most ducks, often smear eggs with preening oil. CMAP-27 has been reported to be expressed in the uropygial gland of the chicken (van Dijk et al., 2005) and may be present in preening oil. Martin-Platero et al., (2006) reported the secretion of antimicrobial peptides from bacteria isolated within uropygial gland secretions of the hoopoe, *Upupa epops*. Bandyopadhyay and Bhattacharyya (1996; 1999) demonstrated that uropygial secretions of the chicken inhibited fungal and bacterial growth while removal of uropygial gland in hens promoted bacterial and fungal skin infections. Shawkey et al., (2003) reported that the uropygial oil of house finches, *Carpodacus mexicanus*, inhibited bacterial growth.

These findings demonstrate the expression of antimicrobial proteins in the female reproductive tract and suggest that antimicrobial proteins are incorporated into eggs and especially eggshell and outer eggshell. Further studies to demonstrate the incorporation of antimicrobial proteins in the egg and eggshell as well as studies investigating the biological activity of individual egg and eggshell proteins are needed. Such studies would demonstrate that the eggshell, in addition to the egg white, plays an important role in the antimicrobial defence of the avian embryo and food safety of the table egg. Moreover, several studies have demonstrated that certain peptides derived from egg white proteins

possess antimicrobial activity (Pellegrini et al., 2004; Tsuge et al., 1996a; 1996b; 1997a; 1997b; Ibrahim et al., 2001b; Watanabe et al., 1998) and such peptides may exist *in vivo* or could easily be produced *in vivo* following enzymatic digestion by bacterial proteases. The development of proteomics, transcriptomics and bioinformatics will facilitate the identification of potentially new antimicrobial agents. Antimicrobial proteins of the egg, such as lysozyme and ovotransferrin, represent ideal conservation agents which are already in use in the food industry. Similar antimicrobial egg components are likely to be identified in the near future and represent molecules of economic and medical importance.

Table 1: Activity spectrum of avian antimicrobial peptides described in literature. The minimum inhibitory concentration of peptides is indicated in μg/ml. The presence of inhibition is denoted by "+". The lack of inhibition is denoted by "-". Conversion of MIC data to μg/ml from μM is denoted by "Ψ".

	Pasteurella multocida	*Mycoplasma gallisepticum*	*Candida albicans*	*Salmonella enteriditis*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*	*Campylobacter jejuni*	*Bordetella avium*	*Escherichia coli*	*Salmonella typhimurium*	*Staphylococcus aureus*	*Listeria monocytogens*	*Streptococcus pyogenes*	
Protein Name														**Reference**
CHP-1/ Gal-1	>16	16	8	16			2	16	16	4	+	+		Evans et al., 1994; 1995; Harwig et al., 1994
CHP-2/ Gal-1α	>16	16	16	8			4	16	16	8	+	+		Evans et al., 1994; 1995; Harwig et al., 1994
Gal-2			-						+			+		Harwig et al., 1994
Gal-11									+	+	-	+	+	Higgs et al., 2005
THP-1	>16	8	8	8			2	8	8	8	+			Evans et al., 1994; 1995
THP-2									-		+			Evans et al., 1994
THP-3	>16	8	16	8			16	-	-	-	+			Evans et al., 1994; 1995
Osp-1			-						1.29		1.17			Sugiarto & Yu, 2006
Osp-2			6.2						0.96		1.25			Sugiarto & Yu, 2006
Osp-3			-						2.41		2.78			Sugiarto & Yu, 2006
Osp-4			-						12.03		11.48			Sugiarto & Yu, 2006
Sphe-2			225-450$^{\Psi}$		27-54$^{\Psi}$	225-450$^{\Psi}$			3.6-13.5$^{\Psi}$	27-54$^{\Psi}$	6.75-13.5$^{\Psi}$	27-54$^{\Psi}$		Thouzeau et al. 2003
Fowlicidin-1					10$^{\Psi}$	1.3-2.5$^{\Psi}$			2.5-12.6$^{\Psi}$	1.3-6.3$^{\Psi}$	1.3-2.5$^{\Psi}$	2.5-6.3$^{\Psi}$		Xiao et al., 2006a; b
Fowlicidin-2					10-20$^{\Psi}$	2.5-5$^{\Psi}$			2.5-10$^{\Psi}$	2.5-5$^{\Psi}$	1.2-10$^{\Psi}$	5$^{\Psi}$		Xiao et al., 2006a; b
LEAP-2				+						+				Townes et al., 2004

Section II: Chapter 2

A literature review of the currently known avian antimicrobial proteins was presented in the first section (Chapter 1) of this thesis. This work emphasized that many of the antimicrobial proteins which have been identified in mature birds may also be present within the avian egg. In addition, a wide range of the known and suspected antimicrobial proteins which are present within avian albumen were presented. The literature review also demonstrated that, while many avian albumen proteins have been investigated in the chicken, few have been investigated in other domestic avian species and even fewer have been investigated in wild avian species. In addition, the current literature accounts for few studies that evaluate the antimicrobial activity of albumen itself and even less have been conducted in a comparative approach investigating the albumens of domestic or wild avian species.

Section two of this thesis was designed in order to remedy some of the deficiencies identified within the literature on the antimicrobial proteins of the avian egg. For this reason, the antimicrobial activity of albumen from both domestic and wild avian species will be investigated in a comparative approach during the following two chapters. We therefore begin with an investigation of the antimicrobial activity of albumen from domestic chicken, turkey, duck and goose in the first part of this section (Chapter 2). This experimental work was performed entirely by myself and is accepted for publication in the British Poultry Science Journal.

Chapter 2: Comparative Antibacterial Activity of Avian Egg White Protein Extracts

Olivier Wellman-Labadie, Jaroslav Picman and Maxwell T. Hincke

Abstract:

Egg white proteins from the eggs of domestic chicken (*Gallus gallus*), turkey (*Meleagris gallopavo*), duck (*Anas platyrhynchos*) and goose (*Anser anser*) were analysed in order to compare the antimicrobial activity of these products. Albumen from each species was sampled and analysed by SDS-PAGE and Western blotting. Antimicrobial activity and lysozyme activity were measured. Ovotransferrin and ovalbumin were identified in all species while c-type lysozyme was present in chicken, turkey and duck egg white samples, but not in goose. Galliformes appear to possess albumens with greater antimicrobial activity than those of the Anseriformes. This can be attributed to higher concentrations of ovotransferrin and the broad acting c-type lysozyme.

Introducton:

The avian albumen is a complex multifunctional medium promoting the growth and development of the embryo. In the domestic chicken (*Gallus gallus*), the albumen is composed of 88 % water and 10 % protein (Burley and Vadehra, 1989). In addition to providing water and nutrients to the developing embryo, albumen prevents the growth of micro-organisms. The alkaline pH of egg white and the presence of antimicrobial proteins significantly reduce the growth of micro-organisms (Baggott and Graeme-Cook, 2002).

Many albumen proteins, including cystatin (Wesierska et al., 2005; Saxena and Tayyab, 1997), ovomacroglobulin (Miyagawa et al., 1991a) and avidin (Board and Fuller, 1974), have been implicated in the antimicrobial defences of *Gallus gallus* egg white. Ovotransferrin, a major egg white protein, is considered to be the principal impediment to the growth of bacteria in the albumen of the hen's egg (Seviour and Board, 1972a). Ovotransferrin is composed of two domains that each reversibly bind iron, limiting the amount of this essential element and thereby inhibiting the growth of micro-organisms (Phelps and Antonini, 1975; Valenti et al., 1981a). Large microbial populations form in

albumen when supplemented with iron (Seviour and Board, 1972a; Schade and Caroline, 1944). Lysozyme, or N-acetylmuramideglycanohydrolase, is another major egg white component (3.5% of chicken egg white protein). It is an enzyme that splits the bond between the glycosidic beta-1,4-linked residues of N-acetylneuramic acid (NAM) and N-acetylglucosamine (NAG) in the peptidoglycan structure of Gram-positive bacteria and to a lesser extent in some Gram-negative bacteria (Bera et al., 2005; Burley and Vadehra, 1989).

Many studies have investigated the antimicrobial properties of albumen from the domestic chicken. Raw hen egg white has been found to inhibit the growth of *Staphylococcus aureus*, *Shigella dysenteriae*, *Escherichia coli* and *Saccharomyces cerevisiae* (Schade and Caroline, 1944). Wang and Shelef (1991) reported that *Listeria monocytogenes* strain Scott A and Brie-1 are highly sensitive to raw chicken albumen. Sahin et al., (2003) reported that the viability of inoculated *Campylobacter jejuni* was dramatically reduced in albumen while bacteria were able to survive up to 14 days in chicken egg yolk. Nonetheless, literature describing the antimicrobial properties of albumen or albumen components in species other than the chicken, *Gallus gallus,* is scarce. In this study, we investigate and compare the antimicrobial properties of albumen from four domestic avian species: chicken (*Gallus gallus*), turkey (*Meleagris gallopavo*), duck (*Anas platyrhynchos*) and goose (*Anser anser*).

Materials and Methods:

Egg White Protein Sampling: Fresh unfertilized eggs of domestic chicken (*Gallus gallus*), turkey (*Meleagris gallopavo*), duck (*Anas platyrhynchos*) and goose (*Anser anser*) were obtained from a local farm in Perth (Ontario, Canada). After cleaning of eggshell surface with running deionised water, eggs were cracked open and egg whites sampled. Pooled egg whites were dialyzed 6 times, using cellulose dialysis membranes (MWCO 3500 Da, Fisher Scientific, Ottawa, ON), against a 100 fold greater volume of deionised water at 4 °C for 24 h periods, lyophilized and stored at -20 °C.

Analysis of Proteins: Protein concentration of samples used for denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as well as those used for lysozyme and antimicrobial assays was determined by bicinchoninic acid (BCA)

protein assay (Pierce, Rockford, IL), using bovine serum albumin (Bioshop, Burlington, ON) as a standard. For SDS-PAGE analysis, samples were dissolved in 4 % sodium dodecyl sulphate 25 % glycerol 1.5 % Tris-HCl pH 6.8. Bromophenol blue (0.125 mg/ml) and 1,4-dithiothreitol (7.7 mg/ml) was added to the samples before heating (5 min at 90 °C) and loading. SDS-PAGE was carried out on 12 % polyacrylamide gels and visualized by Coomassie Blue staining. Molecular weight markers (MBI Fermentas, Burlington, ON) were included in gels during SDS-PAGE while prestained protein standards (Bio-Rad, Mississauga, ON) were used for blotting.

Western blotting was performed for selected chicken proteins with rabbit antiserum (1: 5000) in 0.01 M phosphate buffer 0.0027 M potassium chloride 0.137 M sodium chloride pH 7.4 Tween-20 (0.1 %) as described by Hincke et al., (1999). Polyclonal antibody to chicken egg white c-type lysozyme was a kind gift from Dr. J. Gautron (SRA, INRA, Nouzilly, France). Polyclonal antibody to chicken egg white ovotransferrin was a kind gift from Dr. A. B. Mason (University of Vermont, U.S.A.). Polyclonal antibody against chicken egg white ovalbumin was obtained from Chemicon (Temecula, CA). Bovine serum albumin (3 % solution) was used during blocking of nitrocellulose membranes and 1 : 5000 anti-rabbit IgG horse radish peroxidase linked whole antibody (GE Healthcare Bio-Sciences, Montreal, QC) was used to reveal immunoreactive bands by enhanced chemiluminescence method (PerkinElmer BioSignal, Montreal, QC).

Determination of Lysozyme Concentration by Enzymatic Activity: Lysozyme concentration of samples was determined using a bioassay developed by Liao et al., (2001). Protein samples as well as serially-diluted chicken egg white lysozyme (Sigma-Aldrich, Oakville, ON), used as standards, were suspended in 0.01 % acetic acid. Samples and standards were added to a 1 mg/ml dry cell wall suspension of *Micrococcus lysodeikticus* ATCC 4698 (Sigma-Aldrich, Oakville, ON) in 10 mM sodium phosphate buffer (pH 7.3 and 5.3) and absorbance (600 nm) at room temperature was recorded over time. A standard curve was constructed by plotting the log reciprocal of the time required for a 0.05 unit change in absorbance *vs.* log concentration of chicken egg white lysozyme (mg/ml) standards. Lysozyme activity was expressed as a percentage of total protein concentration within samples.

Bacteriostatic Activity: Bacteriostatic activity of protein samples was evaluated using an antimicrobial assay adapted from Valenti et al., (1981a). A sample of an overnight bacterial culture was grown to log phase in Luria-Bertani (LB) broth (Bioshop, Burlington, ON) or LB broth supplemented with salt. Gram-negative bacteria were grown in LB broth supplemented with 50 mM sodium bicarbonate while Gram-positive bacteria were grown in LB broth supplemented with 10 mM sodium citrate 50 mM sodium bicarbonate. Valenti et al., (1981a) demonstrated that these salts promote the binding of iron by ovotransferrin, thereby inhibiting the growth of micro-organisms. In Gram-negative micro-organisms, bicarbonate enhanced the antimicrobial action of ovotransferrin while citrate had an antagonizing effect since such bacteria possess an iron transport system mediated by citrate (Valenti et al., 1981a; 1985). Bacterial inoculum was concentrated by centrifugation at 3000 g, 4 °C for 10 minutes and adjusted to ~ 1 x 10^5 CFU's/ml. After determination of protein concentration by BCA assay, protein samples in 0.01 % acetic acid were added to bacterial inoculum. Growth at 37 °C, 250 rpm (Multitron HT Infors incubator, Rose Scientific, Mississauga, ON) was monitored by spectrophotometric reading at 600 nm after 24 hours incubation. 0.01 % acetic acid was used as a control and 1 mg/ml of bovine lactoferrin (Sigma-Aldrich, Oakville, ON) was used as a positive control. Lactoferrin is an iron binding glycoprotein with similar bacteriostatic activity as ovotransferrin and other transferrins (Ward et al., 2002). Through the use of a low initial bacterial population, the bacteriostatic assay was designed for sensitivity to detect the inhibition of bacterial growth. Antimicrobial activity was evaluated against two Gram-positive (*Bacillus subtilis* ATCC 19659 and *S. aureus* ATCC 6538) and two Gram-negative (*Pseudomonas aeruginosa* ATCC 15442 and *E. coli* D31) bacteria.

Bactericidal Activity: Bactericidal activity of protein samples was evaluated by an antimicrobial assay adapted from the micro-broth dilution assay developed by Steinberg and Lehrer (1997). A sample of overnight bacterial culture was grown to log phase in LB broth, centrifuged (3000 g, 4 °C, 10 min) washed and resuspended in 10 mM sodium phosphate buffer (pH 7.3). Protein samples were dissolved in 0.01 % acetic acid and concentration determined by BCA protein assay. Protein samples were further diluted with 0.01 % acetic acid 0.1 % BSA and incubated 1 hour at 37 °C, 250 rpm with a ten fold greater volume of bacterial suspension. After incubation, a serial dilution of sample was plated on LB agar, incubated over night at 37 °C and CFU/ml determined. 0.01 %

acetic acid 0.1 % BSA was used as a control and 100 µg/ml bovine lactoferricin B (Sigma-Aldrich, Oakville, ON) was used as a positive control. Lactoferricin B is an antimicrobial peptide sequence found within the N-terminus of lactoferrin (Bellamy et al., 1992). The bactericidal assay was designed for sensitivity to detect reductions in bacterial populations due to cell death. Antimicrobial activity was evaluated against two Gram-positive (*B. subtilis* ATCC 19659 and *S. aureus* ATCC 6538) and two Gram-negative (*P. aeruginosa* ATCC 15442 and *E. coli* D31) bacteria.

Statistical Analysis: Data was analysed using SYSTAT Version 8.0 (SPSS, Chicago, IL). An analysis of variance (ANOVA) followed by pair-wise analysis was conducted to identify any significant differences between the growth of bacteria in the presence and absence of protein samples. If the data did not meet the assumptions of the statistical model, the Kruskal-Wallis test followed by a Kolmogorov-Smirnov test was conducted to identify reductions in bacterial populations.

Results:

Egg White Composition: SDS-PAGE (Figure 1a) revealed two major bands, at 45 kDa and 78 kDa, that appeared to be shared across species and were identified as being ovotransferrin and ovalbumin by western blotting (Figure 1b). These bands showed mild differences in intensity across species during SDS-PAGE analysis (Figure 1a) and slight differences in reaction intensity across species during western blotting (Figure 1b). A 14 kDa band present in chicken, turkey and duck egg white was immunoreactive with antibody to chicken c-type lysozyme during western blotting (Figure 1b). This band was absent from goose egg white which also failed to react during western blotting for c-type lysozyme (Figure 1b). A 20 kDa Coomassie Blue stained protein band, possibly g-type lysozyme, was detected during SDS-PAGE analysis in goose egg white (Figure 1a).

Lysozyme activity of egg white samples was determined by enzymatic activity. Chicken egg white had the highest lysozyme activity, followed by turkey, duck and goose egg white, when tested at pH 7.3 (Table 1). This activity correlates with the intensity of the 14 kDa band visualized by SDS-PAGE (Figure 1a) and western blotting (Figure 1b). When tested at pH 5.3, the lysozyme activity of chicken, turkey and duck egg white did not show significant change, in contrast to goose egg white which showed an almost 9

fold increase (Table 1). Canfield and McMurry (1967) reported that the enzymatic activity of goose egg white lysozyme is three times greater than an equimolar amount of hen egg white lysozyme at pH 6.2. The pH optimum of g-type lysozyme of goose and ostrich was reported to be at pH 5.5-6.0 (Pooart et al., 2005) while chicken c-type lysozyme possesses a broad pH range of 5.5 to 7.5 (Thammasirirak et al., 2001). This is visualized in Figure 2 where chicken egg white demonstrates uniformly high lysozyme activity between pH 5-8 while goose egg white demonstrates maximal lysozyme activity around pH 5.3. Increased lysozyme activity at pH 5.3 can therefore be used as an indication of the presence of g-type lysozyme.

Antimicrobial Activity of Egg White Samples: The antimicrobial activity of albumen is a combination of bacteriostatic activity, whereby bacterial growth is inhibited, and bactericidal activity, in which cell death results in the reduction of bacterial populations. These two possible antimicrobial mechanisms were evaluated separately.

The experiment depicted in Figure 3a was conducted under conditions designed to detect ovotransferrin-mediated bacteriostasis. In the presence of 10 mM sodium citrate and 50 mM sodium bicarbonate, *B. subtilis* was completely inhibited by all egg white samples while *S. aureus* showed significant inhibition in the presence of 10 mg/ml chicken or turkey egg white (Figure 3a). In the presence of 50 mM sodium bicarbonate, all egg white samples inhibited the growth of *P. aeruginosa* while chicken, turkey and duck egg white inhibited *E. coli* D31 (Figure 3a).

On the other hand, in the absence of salts, none of the egg white samples inhibited *E. coli* D31 while only turkey egg white caused significant inhibition of *S. aureus* and *P. aeruginosa* (Figure 3b). At a concentration of 10 mg/ml, chicken, turkey, duck and goose egg white completely inhibited the growth of *B. subtilis* regardless of the presence of salts (Figures 3a and 3b) suggesting the action of a protein with an antimicrobial mechanism independent of the iron-binding activity of ovotransferrin.

The bactericidal activity of egg white was evaluated in a different assay (Figure 4) using a low egg white concentration in order to distinguish species differences. At a concentration of 300 μg/ml, avian egg white did not significantly reduce the populations of *S. aureus*, *P. aeruginosa* or *E. coli* D31 (Figure 4). As can be seen in Figure 5, *S.*

aureus, *P. aeruginosa* and *E. coli* D31 are insensitive to the action of lysozyme while *B. subtilis* is highly sensitive. Chicken egg white followed by turkey, duck and goose egg white showed significant reductions of *B. subtilis* (Figure 4). Bactericidal activity of egg white against *B. subtilis* (Figure 4) and lysozyme activity at pH 7.3 (Table 1) appear to correlate since both lysozyme activity and bactericidal activity was highest in chicken followed by turkey, duck and goose egg white samples.

Discussion:

In avian species, the albumen provides the developing embryo with nutrients and water while limiting microbial proliferation. Egg white proteins act synergistically to contribute to antimicrobial activity. Ovotransferrin is implicated in iron-dependent bacterial inhibition (Valenti et al., 1981a; von Hunolstein et al., 1992). Enhanced binding of iron to ovotransferrin is favoured in the presence of sodium citrate and/or sodium bicarbonate and leads to inhibition of bacterial growth. During the current study, we observed enhanced inhibition in the presence of these salts for albumen samples from all species; this difference corresponds to the activity of ovotransferrin.

The ovotransferrin activity of turkey albumen showed the greatest inhibition of bacterial growth during the bacteriostatic assay, followed by that of chicken egg white (Figure 3a). Within the Anseriformes, duck ovotransferrin activity was greater against *S. aureus*, *B. subtilis* and *E. coli* D31 than that of goose albumen ovotransferrin, while the opposite was observed against *P. aeruginosa*. SDS-PAGE analysis (Figure 1a) and western blotting (Figure 1b) demonstrated that ovotransferrin is present at higher levels in Galliform albumen than in Anseriform albumen.

Regardless of the presence of salts, *B. subtilis* was completely inhibited by high levels of albumen from all of the species examined using the bacteriostatic assay (Figure 3b), indicating the presence of an antimicrobial protein which acts by another mechanism. In both the bacteriostatic (Figure 3) and bactericidal (Figure 4) assays, Gram-positive bacteria were affected by egg white samples to a greater extent than Gram-negative bacteria. Avian albumen is known to be a rich source of lysozyme. Western blotting revealed that the intensity of the 14 kDa c-type lysozyme varied between species (Figure 1b). Moreover, SDS-PAGE analysis (Figure 1a) revealed that chicken albumen showed a

most intense Coomassie blue-stained band at 14 kDa, followed by that in the turkey and duck; no 14-kDa band was detected in the goose albumen sample. This pattern was confirmed by determination of lysozyme enzymatic activity (Table 1).

Lysozyme effectively breaks down the cell walls of some Gram-positive bacteria. *S. aureus*, *P. aeruginosa* and *E. coli* D31 are insensitive to lysozyme while *B. subtilis* is highly sensitive as demonstrated in Figure 5. In the bacteriostatic assay, the albumen concentration of 10 mg/ml tested corresponded with approximately 0.01 (goose), 0.05 (duck), 0.1 (turkey) and 0.24 mg/ml (chicken) lysozyme, by extrapolation using lysozyme activity determined at pH 7.3 (Table 1). This high lysozyme concentration would completely kill off *B. subtilis* populations and prevent their further growth in the bacteriostatic assay. Utilizing lower levels of the albumen samples, as tested in the bactericidal assay (sample concentration corresponds to a 300-fold dilution of natural egg white), would not have completely killed the bacilli, in contrast to the bacteriostatic assay, and allow detection of species differences in ability of albumen to kill bacteria. Chicken egg white lysozyme is highly active against *Micrococcus* and *Bacillus* species while demonstrating lower activity against Gram-negative bacteria (Bera et al., 2005; Burley and Vadehra, 1989). In our study, the bactericidal activity of egg white samples against *B. subtilis* was strongly correlated with lysozyme activity. This relationship, as well as the fact that only a bacterial species sensitive to lysozyme showed significant sensitivity, is consistent with the notion that lysozyme is a major bactericidal egg white protein although it demonstrates limited specificity.

Two types of lysozyme activity: c-type (chicken-type) and g-type (goose-type) have been described. These two lysozymes have similarities in their tertiary structures although their amino acid sequences are entirely different and antibodies directed against c-type lysozyme do not cross-react with g-type lysozyme, and *vice versa* (Hemmen et al., 1992; Irwin and Gong, 2003; Pooart et al., 2005). C-type lysozyme has been demonstrated in the albumens of Galliformes and Anseriformes, whereas the g-type is found in the albumen of Anseriformes, Struthioniformes, Rheiformes, Apterygiformes, Tinamiformes, Podicipediformes, Sphenisciformes, Casuariformes and Charadriiformes. Simultaneous expression of both c-type and g-type lysozyme is observed in the albumens of some Anseriformes (Florkin and Scheer, 1978; Hemmen et al., 1992). G-type lysozyme is up to 3 times more active at pH 6.2 than equimolar amounts of c-type

lysozyme, although it has much less activity at neutral pH (Canfield and McMurry, 1967; Hindenburg et al., 1974). Consistent with this, we observed that goose egg white exhibited a 9-fold increase in lysozyme activity at pH 5.3 (Table 1) in the absence of immunoreactive c-type lysozyme as demonstrated by Western blotting (Figure 1b). The pH of non-incubated chicken albumen rises from about 7.6 to 9.5 within a few days after oviposition (Lapao et al., 1999). Non-incubated Galliform eggs may therefore represent a safer food for human consumption, since these eggs should remain free of lysozyme-sensitive micro-organisms for longer periods during storage than g-type lysozyme - containing Anseriform eggs. Gram-positive bacilli are major contaminants on the eggshell surface (Cook et al., 2003) and are associated with food-borne illness. Other lysozyme-sensitive egg contaminants include *Micrococcus* spp., a Gram-positive cocci associated with embryonic death (Cook et al., 2003; Baggott and Graeme-Cook, 2002).

The lower defensive capabilities of eggs against Gram-negative bacteria represent a potential hazard to human health. Gram-negative bacteria, such as *E. coli*, *Salmonella enterica* and *Pseudomonas* spp. are known to penetrate the eggshell and potentially produce embryonic mortality (Baggott and Graeme-Cook, 2002; Messens et al., 2006). For this reason, eggs destined for human consumption are sanitized and kept in low humidity conditions in order to prevent the proliferation of Gram-negative pathogens. Considering that 92 % of fowl eggshell contaminants from commercial duck hatcheries are Gram-positive cocci, mainly *Micrococcus* spp. and *Staphylococcus* spp., (Seviour and Board, 1972a), it is likely that evolution and possibly human selection, has led to more potent avian egg white antimicrobial defences against the attack of Gram-positive micro-organisms.

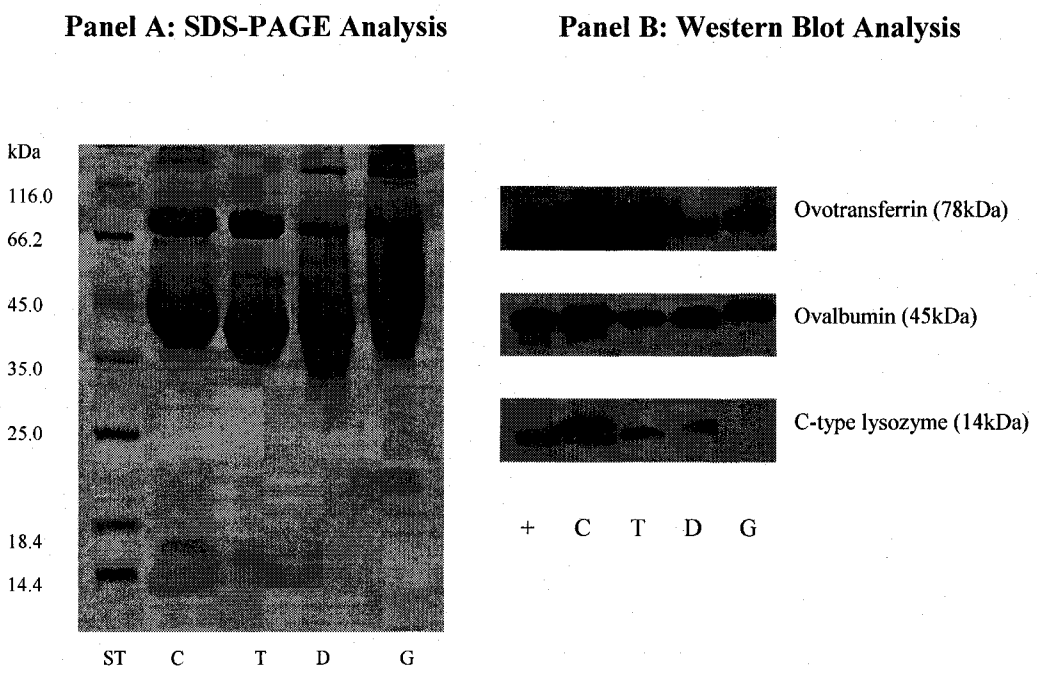

Figure 1: SDS-PAGE analysis (panel A) and Western blot analysis (panel B) of avian egg white samples. For SDS-PAGE analysis, protein samples (20 μg) were loaded into each well of a 12 % polyacrylamide gel and visualized by Coomassie Blue staining. Molecular weight of standard (ST) is indicated on the left. For western blot, protein samples (1 μg) were loaded in each well. Purified chicken egg white lysozyme, ovotransferrin or ovalbumin (0.1 μg) was used as a positive control (+). Egg white samples are labelled on the bottom (C-chicken, T-turkey, D-duck, G-

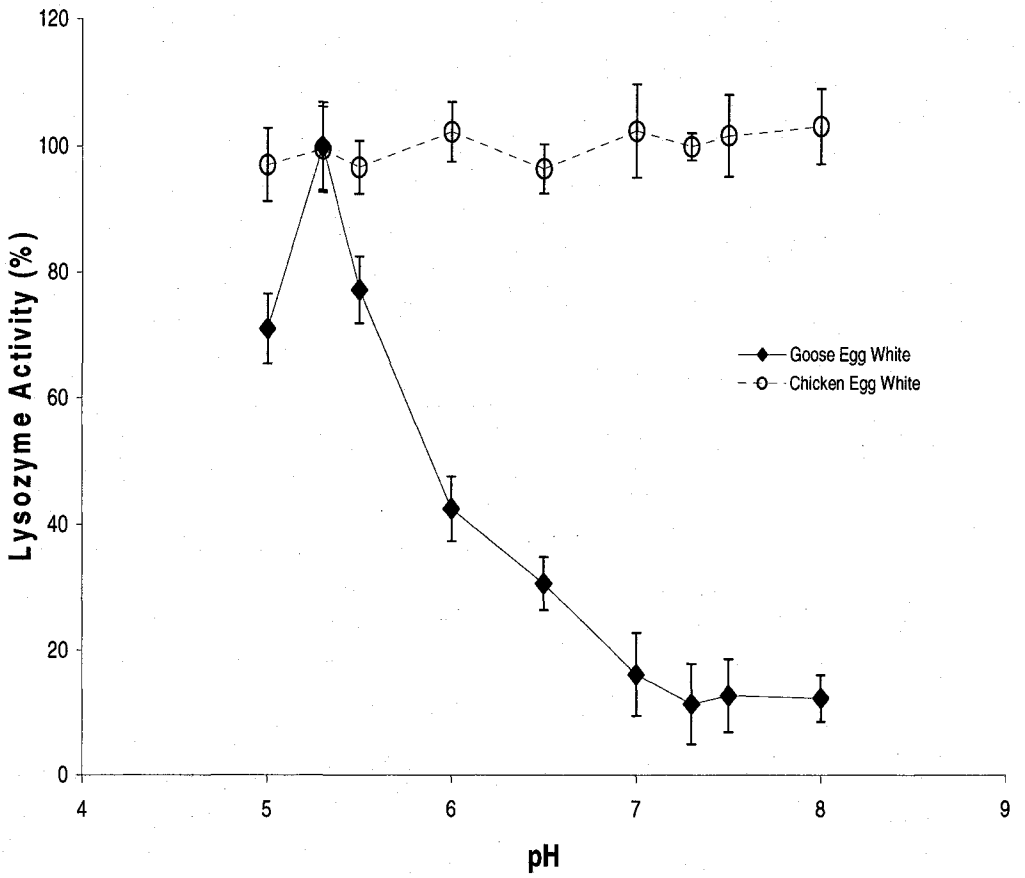

Figure 2: Lysozyme activity of chicken and goose albumen (1 mg/ml) in buffered 10 mM sodium phosphate at various pH conditions. Samples of chicken and goose albumen were added to *Micrococcus lysodeikticus* cell wall suspension in 10 mM sodium phosphate buffer (pH 5.0-8.0) and optical density at 600 nm was recorded over time. Experiment was conducted on two separate occasions with triplicate readings.

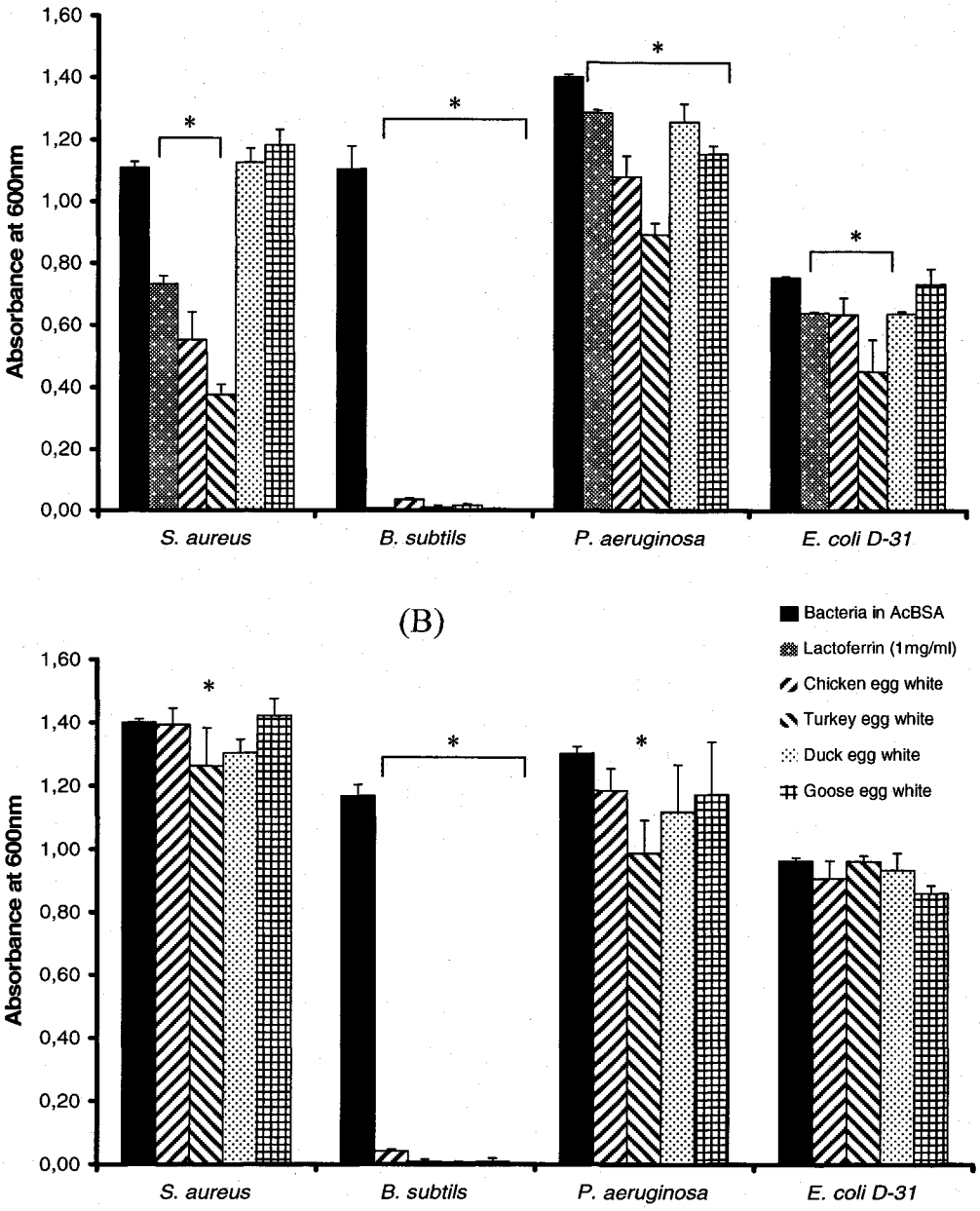

Figure 3: Bacteriostatic activity of 10 mg/ml egg white protein. Bacteria were incubated for 24 hours in LB broth with protein samples in the presence **(A)** and absence **(B)** of salts. When salt was required, 10 mM sodium citrate 50 mM sodium bicarbonate was used for Gram-positive bacteria and 50 mM sodium bicarbonate was used for Gram-negative bacteria. Bovine lactoferrin (1 mg/ml) was used as a positive control and 0.01 % acetic acid was used a negative control. Experiment was performed on two separate occasions with triplicate readings. Average absorbance ± standard deviation is indicated. An ANOVA was performed to compare protein samples to control and statistically significant differences ($p < 0.05$) are indicated by an asterix

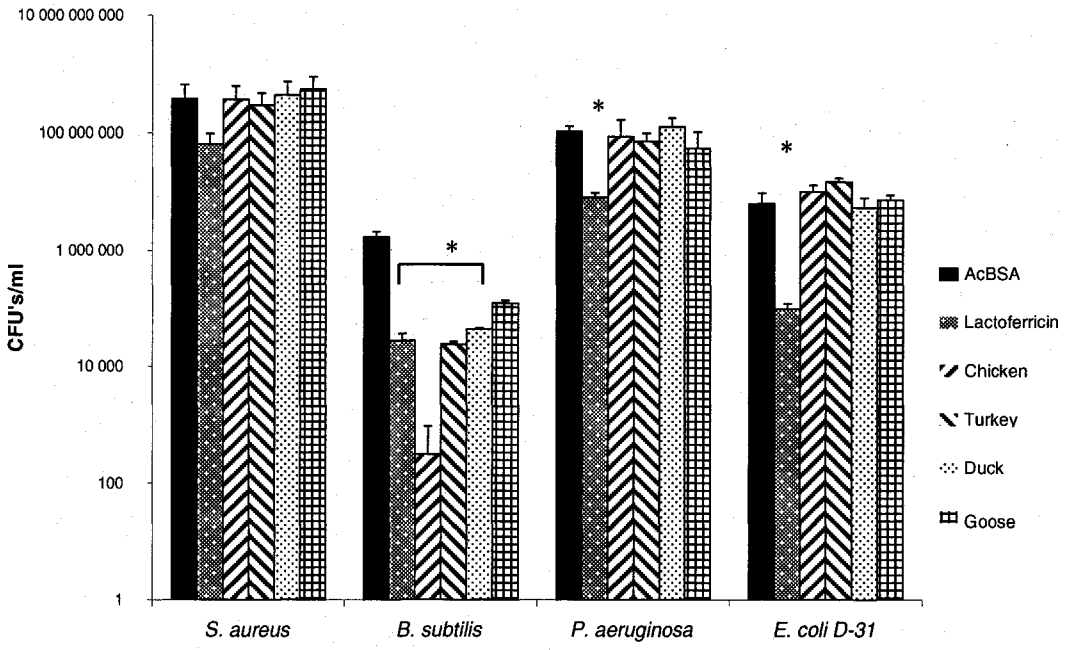

Figure 4: Bactericidal activity of 300 μg/ml egg white protein. Bacteria in 10 mM sodium phosphate buffer (pH 7.3) were incubated in the presence of protein samples. Samples were plated on LB agar and CFU's/ml determined after over night incubation at 37 °C. Bovine lactoferricin B (100 μg/ml) and bovine serum albumin (0.1 %) in 0.01 % acetic acid were used as controls. Experiment was performed on two separate occasions with triplicate readings. Average CFU's/ml ± standard deviation is indicated. An ANOVA was performed to compare protein samples to control and statistically significant differences ($p < 0.05$) are indicated by an asterix (*).

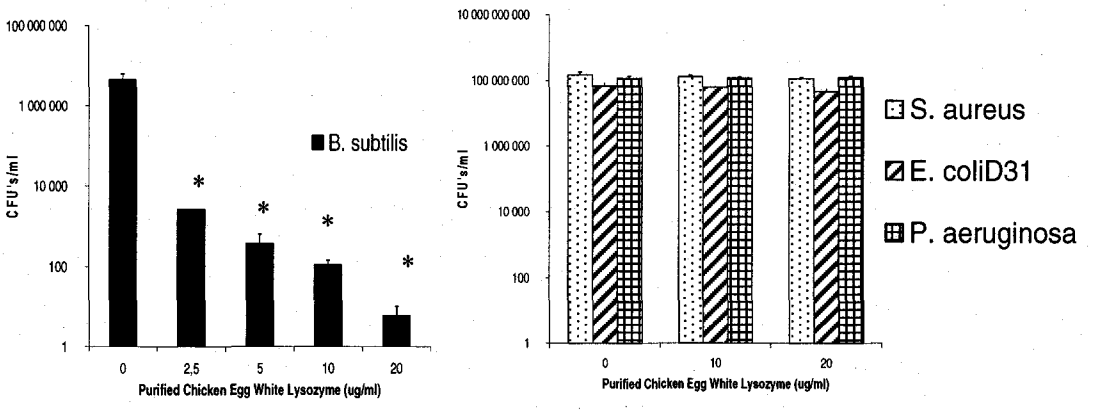

Figure 5: Bactericidal activity of purified chicken egg white lysozyme against selected Gram-positive and Gram-negative bacteria. Lysozyme dose response against *Bacillus subtilis* (**panel A**) and response of *Staphylococcus aureus*, *Escherichia coli* D31 and *Pseudomonas aeruginosa* to lysozyme (**panel B**). Bacteria in 10 mM sodium phosphate buffer (pH 7.3) were incubated in the presence of protein samples. Samples were plated on LB agar and CFU's/ml determined after over night incubation at 37 °C. Experiment was performed on two separate occasions with triplicate readings. Average CFU's/ml ± standard deviation is indicated. Statistically significant inhibition ($p < 0.05$) of growth is indicated by an asterix (*).

Table 1: Determination of lysozyme content of pooled avian egg white samples by measurement of enzymatic activity against *M. lysodeiticus* cell walls at pH 5.3 and 7.3 in 10 mM sodium phosphate buffer. Lysozyme activity is expressed as a percent by weight (± standard deviation) of total protein. Experiment was performed on two separate occasions with triplicate readings.

Sample	Lysozyme (% ± Standard Deviation)		Ratio
	pH 7.3*	pH 5.3‡	pH 5.3/pH 7.3
Chicken	2.44 ± 0.22	2.43 ± 0.21	1.0
Turkey	0.94 ± 0.06	1.20 ± 0.34	1.3
Duck	0.52 ± 0.07	0.56 ± 0.06	1.1
Goose	0.14 ± 0.03	1.22 ± 0.21	8.7

* pH optimum of c-type lysozyme (*Gallus gallus*)

‡ pH optimum of g-type lysozyme (*Anser anser*)

Section II: Chapter 3

In the previous chapter (Chapter 2), the antimicrobial properties of albumen from domestic avian species were investigated. The comparative approach which was used permits an investigation of the effects of phylogeny on the antimicrobial defences of the avian egg. In continuation with the theme of section two, chapter three is a comparative study investigating the antimicrobial activity of albumen from wild Anseriform species. This chapter represents one of the few studies investigating the properties of albumen in wild avian species. In addition, chapter three is unique as it begins to investigate the effects of environmental pressures, mainly the higher potential of egg contamination present under humid nesting conditions, on the antimicrobial defences of the avian egg. This experimental work was performed entirely by myself and is published in Physiological and Biochemical Zoology (2008) 81(2): 235-245.

Chapter 3: Enhanced C-type Lysozyme Content of Wood Duck (*Aix sponsa*) Egg White: An Adaptation to Cavity Nesting?

Olivier Wellman-Labadie, Jaroslav Picman and Maxwell T. Hincke

Abstract:

Wild waterfowl species often nest in conditions where high humidity and microbial contamination may influence egg survival and quality. Albumen is traditionally regarded as the major impediment to microbial contamination of eggs, and its composition and activity may be selected by environmental pressures. Egg white protein from the eggs of wood duck (*Aix sponsa*), hooded merganser (*Lophodytes cucullatus*), Canada goose (*Branta canadensis*), and mute swan (*Cygnus olor*) was evaluated in order to compare the antimicrobial defences of these species. Ovotransferrin and ovalbumin were identified in all species, but c-type lysozyme was present only in wood duck and hooded merganser egg white samples. Wood duck egg white showed the greatest bacterial activity as well as the highest lysozyme content. Egg white from wood duck and hooded merganser possessed greater lysozyme activity under acidic conditions, suggesting a c-type lysozyme with a pH optimum lower than that of *Gallus gallus* c-type lysozyme or the presence of g-type lysozyme. Ovotransferrin bacteriostatic activity appeared to be similar across the species investigated. The results suggest that lysozyme and ovotransferrin play a role in the antimicrobial defence of the avian egg. High levels of the broad-acting c-type lysozyme appear to have evolved in the albumen of the wood duck in order to ensure proper development of the embryo in the humid conditions of the cavity nest.

Introduction:

Duck reproductive success often depends on the inherent ability of eggs to remain fresh and contamination-free during the long incubation period common to many Anseriform species. Anseriformes often nest near water exposing the eggs and nesting material to moisture. Cook et al., (2003) demonstrated that infection of unincubated chicken eggs is dependent on the length of the exposure period and is most prevalent under conditions of high relative humidity. The materials from which nests are constructed often provide a suitable environment for the growth of pathogens and the reuse of old nests exposes eggs

to established bacterial populations (Pugh 1972; Baggott and Graeme-Cook 2002). The relative humidity of cavity nests has also been reported to be higher than that observed for open-bowl nests (Kern and Cowie 1995). Therefore, the eggs of ducks and especially cavity nesting ducks may be exposed to a greater risk of microbial contamination. In order to prevent microbial attack, the developing embryo must rely on the antimicrobial properties of the albumen and albumen components.

The albumen is acquired during the passage of the yolk through the oviduct and is composed of 88 % water and 10 % protein (Burley and Vadehra 1989). In the chicken, egg white protein is composed of 54% ovalbumin, 13% ovotransferrin, 11% ovomucoid, 8% ovoglobulins, 3.5% lysozyme, 3% beta-ovomucin, 1.5% alpha-ovomucin and trace levels of minor constituents (Burley and Vadehra 1989). Guerin-Dubiard et al., (2006) recently analysed hen egg white using two-dimensional electrophoresis associated with mass spectrometry and revealed that the protein composition of albumen is much more complex than previously believed. The avian albumen is a multifunctional medium promoting the growth and development of the embryo. Albumen is involved in many roles essential to the survival of the developing embryo. The viscous nature of the albumen may impede the movement of invading micro-organisms while providing external impact protection by dissipating mild forces (Burley and Vadehra 1989). In addition to providing water and nutrients to the developing embryo, the albumen prevents the growth of micro-organisms Burley and Vadehra 1989).

Many studies have investigated the antimicrobial properties of albumen from the domestic chicken, *Gallus gallus*. Raw hen egg white has been found to inhibit the growth of *Staphylococcus aureus*, *Shigella dysenteriae*, *Escherichia coli* and *Saccharomyces cerevisiae* (Schade and Caroline 1944). Albumen has been described to be more potent to bacteria at 39.5 °C than at 30 °C while demonstrating bactericidal activity at pH 9-10 but bacteriostatic activity at pH 6-8 (Tranter and Board 1984). The addition of iron to albumen has been found to abolish the characteristic bacteriostatic activity of egg white (Seviour and Board 1972a; Schade and Caroline 1944). Wang and Shelef (1991) reported that *Listeria monocytogenes* strain Scott A and Brie-1 are highly sensitive to raw chicken albumen. Sahin et al., (2003) reported that the viability of inoculated *Campylobacter jejuni* was dramatically reduced in albumen while bacteria were able to survive up to 14 days in chicken egg yolk.

The individual components of chicken albumen have also been extensively studied. *Acinetobacter iwoffii*, *Escherichia coli*, *Oligella* sp. and *Pseudomonas aeruginosa* have been reported to be highly sensitive to cystatin, a chicken egg white cysteine protease inhibitor (Wesierska et al., 2005; Saxena and Tayyab 1997). Ovomacroglobulin, a broad spectrum protease inhibitor, inhibits the growth of *Serratia marcescens* and *P. aeruginosa* (Miyagawa et al., 1991a). Avidin forms a stable complex with biotin and is thought to play a role in the antimicrobial defence of the egg by limiting the availability of this essential vitamin (Board and Fuller 1974). Ovotransferrin, an iron-binding glycoprotein, is well known for its bacteriostatic activity against a wide range of bacterial and fungal species (Valenti et al., 1983; 1985). Chicken egg white lysozyme, the only lysozyme currently used in commercial applications is capable of hydrolyzing the peptidoglycan cell wall of many Gram-positive bacteria (Nakimbugwe et al., 2006).

Although albumen and egg white components of the domestic chicken have been extensively studied, the currently available literature on the egg white properties of other avian species is scarce. McCabe and Deutsch (1952) compared the protein composition of avian egg white from selected Galliform, Anseriform, Passeriform, Gruiform, Charadriiform, Podicipediform and Columbiform species by moving boundary electrophoresis to demonstrate the value of this technique for taxonomic purposes. Seviour and Board (1972a) investigated bacterial metabolism in albumen of the domestic hen and waterfowl. Feeney et al., (1960) determined the egg white content of lysozyme, ovotransferrin and flavoprotein from species in 6 avian orders. Miguel et al., (2005) investigated egg white protein composition of chicken, pheasant, quail, duck and ostrich using chromatographic and electrophoretic methods. In this study, we investigate the egg white protein composition of four wild Anseriformes and report their antimicrobial properties. The major egg white proteins, mainly ovalbumin, ovotransferrin and lysozyme, were compared across species with contrasting nesting strategies.

Materials and Methods:

Egg White Protein Sampling: Wood duck (*Aix sponsa*) and hooded merganser (*Lophodytes cucullatus*) eggs were collected from man-made nest boxes setup along the Ottawa River in Kanata (Ontario, Canada). Mute swan (*Cygnus olor*) and Canada goose

(*Branta canadensis*) eggs were collected from birds breeding on the islands surrounding the Toronto inner harbour (Ontario, Canada). All eggs were collected during the first week of laying from each of the respective species. Eggs from the cavity nesting wood duck and hooded merganser as well as eggs from the open-top nesting Canada goose and mute swan were selected for their contrasting nesting conditions. The proper collection permits were obtained from the Canadian Wildlife Service (Permit No. CA 0149 and CA 0150) and Animal Care Committee (Permit No. CMM-96). After cleaning of eggshell surface with running deionised water, eggs were cracked open and egg whites sampled. McCabe and Deutsch (1952) reported that there is virtually no variation in egg white protein among the eggs of a clutch or between clutches of different chickens. Since the main interest of this study resides in the interspecific variation of egg white, albumen samples (12 per species) were pooled as previously conducted by Feeney et al., (1960) and McCabe and Deutsch (1952). Egg whites were dialyzed using cellulose dialysis tubing (MWCO 3500 Da, Fisher Scientific, Ottawa, ON), 6 times against a 100 fold greater volume of deionised water at 4 °C for 24 hour periods. Samples were then lyophilized and stored at -20 °C.

Analysis of Egg White Protein Extracts: SDS-PAGE and Western blot analysis of egg white extracts was conducted as described by Wellman-Labadie et al., (2008a). Lysozyme concentration of egg white extracts was evaluated by measurement of enzymatic activity against *Micrococcus* cell wall (suspended in 10 mM sodium phosphate buffer, pH 5.3 and 7.3) according to the method described by Wellman-Labadie et al., (2008a). Both bacteriostatic and bactericidal activity of egg white extracts was evaluated against two Gram-positive (*B. subtilis* ATCC 19659 and *S. aureus* ATCC 6538) and two Gram-negative (*P. aeruginosa* ATCC 15442 and *E. coli* D31) bacteria according to the method of Wellman-Labadie et al., (2008a). Data of antimicrobial assays was also evaluated for statistical significance according to the method described by Wellman-Labadie et al., (2008a).

Results:

Egg White Composition: SDS-PAGE analysis (Figure 1) revealed two major bands, at 45 kDa and 78 kDa, that appear to be shared across species and were identified as ovotransferrin and ovalbumin, respectively, by western blotting (Figure 2). Another

unidentified high molecular weight band of approximately 150 kDa was visible and shared across all species. Desert et al., (2001) also identified a high molecular weight band (~150 kDa) in albumen of the domestic chicken during SDS-PAGE and proposed that it corresponded to ovomucin or ovostatin. A 14 kDa band was present in wood duck and hooded merganser egg white and was identified as c-type lysozyme by western blotting (Figure 2). The immuno-positive bands were of similar intensity (Figure 2) and correlated well with the similar band intensity detected in Coomassie Blue stained SDS-PAGE gels (Figure 1). Canada goose and mute swan egg white samples did not possess a 14 kDa Coomassie Blue stained band; moreover, western blotting for c-type lysozyme was negative in these samples (Figure 2). The ovotransferrin band detected at 78 kDa showed almost equivalent band intensity across species by Coomassie Blue staining (Figure 1) and by western blotting (Figure 2). Similar levels of ovalbumin across species were revealed by SDS-PAGE (Figure 1) and by western blotting (Figure 2).

Enzymatic detection of lysozyme activity in egg white samples at pH 7.3 revealed that wood duck egg white had the highest activity (Table 1). Hooded merganser and mute swan egg white possessed similar and intermediate levels of lysozyme activity while Canada goose egg white had the lowest activity (Table 1). At pH 5.3, the estimated lysozyme content was again highest for wood duck followed by mute swan, hooded merganser and Canada goose, although activity was significantly greater than at pH 7.3 (Table 1). In contrast, *Gallus gallus* egg white activity at pH 5.3 did not vary from that of pH 7.3 (Table 1). This is expected since chicken c-type lysozyme possesses a broad pH range of 5.5 to 7.5 (Thammasirirak et al., 2001), while the enzymatic activity of goose egg white lysozyme is three times greater than an equimolar amount of hen egg white lysozyme at pH 6.2 (Canfield and McMurry, 1967). The pH optimum of g-type lysozyme of goose and ostrich was reported to be at pH 5.5-6.0 (Pooart et al., 2005). Increased lysozyme activity at pH 5.3 vis-à-vis pH 7.3 can therefore be used as an indication of the presence of g-type lysozyme since c-type lysozyme has a broad pH optimum while g-type lysozyme possesses an acidic and narrow pH optimum (Wellman-Labadie et al., 2008a).

Antimicrobial Activity of Egg White Samples: Antimicrobial activity is due to either a bacteriostatic effect, whereby bacterial growth is inhibited, or as a bactericidal effect,

whereby bacterial populations are reduced. For this reason, two different assays were utilized to distinguish between each type of antimicrobial activity.

In the presence of salts which enhance the binding of ovotransferrin and iron, the egg white samples demonstrated potent activity during the bacteriostatic assay. In the presence of 10 mM sodium citrate and 50 mM sodium bicarbonate, egg white samples (10 mg/ml) completely inhibited *B. subtilis* growth [ANOVA ($F = 30958.256$, $p < 0.001$), Pair-wise analysis (MSE < 0.001, $p < 0.001$)] but had no effect on that of *S. aureus* ($F = 3.844$, $p = 0.086$) (Figure 3a). In the presence of 50 mM sodium bicarbonate, all avian egg white samples inhibited the growth of *P. aeruginosa* [ANOVA ($F = 19.415$, $p = 0.001$), Pair-wise analysis (MSE = 0.001, $p = 0.003$ Canada goose, $p = 0.026$ mute swan, $p = 0.003$ wood duck, $p = 0.005$ hooded merganser) while all samples except wood duck egg white inhibited the growth of *E. coli* D31 [ANOVA ($F = 6.316$, $p = 0.022$), Pair-wise analysis (MSE = 0.002, $p = 0.019$ Canada goose, $p = 0.047$ mute swan, $p = 0.567$ wood duck, $p = 0.037$ hooded merganser) (Figure 3a). No Anseriform egg white inhibited the growth of *S. aureus* ($F = 1.104$, $p = 0.446$), *P. aeruginosa* ($F = 0.642$, $p = 0.656$) or E. coliD31 ($F = 1.777$, $p = 0.270$) in the absence of salts (Figure 3b). An ANOVA revealed that the growth of *B. subtilis* in the presence and absence of Anseriform egg white samples were significantly different ($F = 281.885$, $p < 0.001$). Pair-wise analysis demonstrated that mute swan (MSE = 0.003, $p < 0.001$), wood duck (MSE = 0.003, $p < 0.001$) and hooded merganser (MSE = 0.003, $p < 0.001$) egg white inhibited *B. subtilis* growth while Canada goose (MSE = 0.003, $p = 1.000$) had no significant effect. The complete inhibition of *B. subtilis* growth by these egg white samples, regardless of salt presence (Figure 3), suggests the action of a protein independent of the salt-dependent iron-binding mechanism of ovotransferrin. Contrarily to these egg white samples, Canada goose egg white completely inhibited the growth of *B. subtilis* only in the presence of salt (Figure 3) suggesting lower levels of the alternative antimicrobial agent than present in the other species.

We also evaluated the bactericidal activity of egg white samples at a lower concentration in order to distinguish species differences. At a concentration of 300 µg/ml, the Anseriform egg white samples did not exhibit significant toxicity to *S. aureus* ($F = 0.449$, $p = 0.801$), *P. aeruginosa* ($F = 2.688$, $p = 0.130$) and *E. coli* D31 ($F = 2.795$, $p = 0.122$) (Figure 4). ANOVA revealed that *B. subtilis* showed statistically significant differences

between bacterial survival in the presence and absence of egg white samples ($F = 41.386$, $p < 0.001$) (Figure 4). Wood duck egg white was found to demonstrate the greatest bactericidal activity (Figure 4). This sample also possessed the highest lysozyme content as measured by enzymatic activity (Table 1). Pair-wise analysis revealed that *B. subtilis* survival was significantly lower in the presence of all Anseriform egg white samples, except for Canada goose egg white at the concentrations evaluated [MSE = 2.26E+10; Canada goose ($p = 0.054$), mute swan ($p = 0.003$), wood duck ($p < 0.001$), hooded merganser ($p = 0.001$)]. In both bacteriostatic and bactericidal assays *B. subtilis* was most sensitive to egg white samples (Figures 3 and 4).

Discussion:

Wild waterfowl species often nest in conditions where high humidity and microbial contamination may influence egg survival and quality. Albumen is traditionally regarded as the major impediment to microbial contamination of eggs and its composition and activity may be selected by environmental pressures. Egg white protein from the eggs of wood duck (*Aix sponsa*), hooded merganser (*Lophodytes cucullatus*), Canada goose (*Branta canadensis*) and mute swan (*Cygnus olor*) was evaluated in order to compare the antimicrobial defences of these species.

Composition of Egg White Samples: SDS-PAGE analysis of egg white samples showed similarity of protein migration patterns across all species examined (Figure 1). This is in accordance with phylogeny which groups the 4 species investigated in the order Anseriform. Wood duck and hooded merganser egg white protein migration patterns appeared identical while sharing some similarity to Canada goose and mute swan egg white which themselves also showed great similarity. McCabe and Deutch (1952) noted that electrophoresis using the moving boundary method is effective at demonstrating protein variation in the Aves at the level of the family but is less effective at demonstrating differences between closer related groups. Miguel et al., (2005) noted that SDS-PAGE profiles of avian egg white, mainly chicken (*Gallus gallus*), quail (*Coturnix coturnix*), pheasant (*Phasianus colchicus*), duck (*Anas platyrhynchos*) and ostrich (*Struthio camelus*) egg white, vary across species in accordance with phylogenetic proximity.

Avian egg white lysozyme has been identified as either g-type or c-type lysozyme. The different classes of lysozymes have overall similarities in tertiary structure although their amino acid sequences are entirely different and antibodies directed against c-type lysozyme do not cross-react with the g-type lysozyme and vice versa (Hemmen et al., 1992; Pooart et al., 2005). Goose lysozyme is most active in acidic conditions and has 3 times more intrinsic activity than c-type lysozyme, while chicken c-type lysozyme possesses similar activity across a broad pH range (Thammasirirak et al., 2001). We observed a 3 to 4-fold increase in lysozyme activity at pH 5.3 compared to that measured at pH 7.3 in Canada goose and mute swan egg white samples, suggesting the presence of g-type lysozyme. In addition, we did not detect c-type lysozyme in mute swan or Canada goose egg white during SDS-PAGE and western blotting.

In accordance with the 14 kDa band observed during SDS-PAGE and western blotting, c-type lysozyme was identified in wood duck albumen as reported by Araki and Torikata (1999). The amino acid sequence of wood duck lysozyme shows high similarity to duck III c-type lysozyme (Araki and Torikata 1999). We also observed c-type lysozyme by SDS-PAGE and western blotting in the albumen of the hooded merganser. Both of these Anseriform species demonstrated enhanced lysozyme activity at pH 5.3. This suggests that some g-type lysozyme is present in the egg whites of these species. These predictions could not be confirmed due to the unavailability of antibodies to g-type lysozyme. The presence of lysozyme activity in all four species under both acidic and near neutral conditions demonstrates the large pH range under which Anseriform albumen would possess microbial protection against lysozyme sensitive micro-organisms.

Bacteriostatic Activity of Egg White Samples: The enhanced inhibitory activity of egg white samples in the presence of the sodium citrate and sodium bicarbonate salts is consistent with ovotransferrin being the egg white protein responsible for the inhibition of bacterial growth observed. Valenti et al., (1981a) demonstrated that these salts promote the binding of iron by ovotransferrin, thereby inhibiting the growth of micro-organisms. In Gram-negative micro-organisms, bicarbonate enhanced the antimicrobial action of ovotransferrin while citrate had an antagonizing effect since such bacteria possess an iron transport system mediated by citrate (Valenti et al., 1981a; 1985). At approximately 1 mg/ml, purified *Gallus gallus* ovotransferrin (Sigma-Aldrich, Oakville,

ON) can significantly inhibit microbial growth (Figure 5). Considering that raw chicken egg white contains 10 mg/ml of ovotransferrin and 100 mM sodium bicarbonate (Burley and Vadehra 1989), under natural conditions ovotransferrin should be involved in the antimicrobial defence of the embryo.

We observed that egg white from all four species inhibited the growth of the Gram-negative bacteria tested (excluding wood duck egg white which was ineffective against *E. coli* D31) as well as the Gram-positive *B. subtilis* in the presence of salts. The bacteriostatic activity of egg white samples did not appear to vary across Anseriform species, in accordance with the similar band intensity of ovotransferrin observed by both Coomassie Blue staining of SDS-PAGE gels and western blotting. Lack of differences in ovotransferrin activity across species may be explained by reports (Graham and Williams 1975) of similar amino acid composition but variable carbohydrate across transferrins. Alignment of ovotransferrin amino acid sequences from *Gallus gallus* (NCBI Accession #: CAA26040) and the duck, *Anas platyrhynchos*, (NCBI Accession #: P56410) demonstrates an 80 % similarity using ClustalW (http://www.ebi.ac.uk/clustalw/). Feeney et al., (1960) investigated the albumen composition of 17 Galliformes and 2 Anseriformes as well as other species from 4 different avian orders and reported that similar ovotransferrin levels are found in closely related avian species. Miguel et al., (2005) also reported similar ovotransferrin levels in albumen of the closely related hen, quail and pheasant.

Seviour et al., (1972) reported that coliform organisms were the principal contaminants of rotten or incubated eggs of 45 species of waterfowl from commercial duck hatcheries. Board and Board (1968) proposed that the dominance of Gram-negative bacteria in rotten chicken eggs was a result of their simple nutritional requirements and ability to grow in the presence of the iron-chelating ovotransferrin. For this reason, *E. coli* showed mild inhibition in the presence of most egg white samples while *P. aeruginosa*, a non-coliform Gram-negative bacterium, showed greater inhibition with all egg white samples in conditions favouring ovotransferrin activity.

S. aureus showed insensitivity to all 4 egg white samples, which can be explained by the ability of this organism to survive and grow in a low iron environment in addition to its lysozyme insensitivity. Valenti et al., (1983) demonstrated that *S. aureus* showed lower

sensitivity to ovotransferrin which was also observed in this study. Bera et al., (2005) reported that *Staphylococcus* species are completely lysozyme resistant due to *O*-acetylation of the muramic acid of the peptidoglycan cell wall. *Staphylococcus* spp., are known to significantly reduce hatching success and represent the majority of Gram-positive contaminants in commercial duck hatcheries (Seviour and Board 1972a). Pathogenic micro-organisms capable of growth in iron poor environments as well as pathogens demonstrating lysozyme insensitivity represent a greater risk to Anseriform reproductive success.

Bactericidal Activity of Egg White: Only *B. subtilis* demonstrated significant population reductions when treated with egg white samples. Bactericidal activity was found to correlate with the estimated lysozyme content measured by enzymatic activity, with wood duck showing the highest bactericidal activity and highest lysozyme content while Canada goose egg white showed the least. This observation implicates lysozyme as a bactericidal protein of egg white. The complete inhibition of *B. subtilis* growth, regardless of salt presence during the bacteriostatic assay, is likely due to lysozyme. Purified chicken egg white lysozyme concentrations as low as 2.5 µg/ml can significantly reduce populations of *B. subtilis* while higher levels have no effect on *S. aureus*, *E. coli* D31 and *P. aeruginosa* (Wellman-Labadie et al., 2008a). At the egg white concentrations used during the bacteriostatic assay, lysozyme levels of approximately 350 µg/ml could be expected (assuming similar composition to that of *Gallus gallus* egg white) and would certainly annihilate populations of *B. subtilis* and prevent the growth of any vegetative cells. Low lysozyme activity was detected in Canada goose egg white regardless of pH conditions during the turbidity assay. This would indicate low lysozyme levels and corresponds well with our observation that Canada goose egg white did not exhibit significant bactericidal activity against *B. subtilis*.

It is also interesting to note that c-type lysozyme, ovotransferrin and ovalbumin have also been identified as components of the avian eggshell matrix by Hincke et al., (2000), Gautron et al., (1997a) and Hincke (1995) respectively. The association of albumen proteins with the avian eggshell matrix, in particular antimicrobial proteins such as lysozyme and ovotransferrin, would reinforce the eggshell's role in antimicrobial defence.

The wood duck and hooded merganser are both cavity nesting birds while Canada goose and mute swan have open-top nests. Kern and Cowie (1995) demonstrated that nests in cavities or burrows had a higher relative humidity than open-top nests. In a study by Cook et al., (2003), *Gallus gallus* eggs placed under humid conditions had a significantly greater bacterial load than eggs placed in drier conditions. Messens et al., (2005) noted that the level of bacterial penetration to chicken egg contents increased with increasing temperature and relative humidity. Relatively high lysozyme content and activity may have evolved in the cavity nesting wood duck in order to prevent microbial contamination of their thin shelled eggs. The hooded merganser, in contrast to the wood duck, possess a much thicker eggshell with potent eggshell antimicrobial proteins (data not shown) which may represent an alternative strategy evolved in this species for successful development of embryos under the humid conditions of the cavity nest. Therefore, the respective microbial load and composition of nesting habitats may act through natural selection to affect the distribution of egg proteins and especially lysozyme in some avian species.

In conclusion, our study reveals that the content of the major egg white proteins does not vary considerably across Anseriform species. All species investigated possessed ovotransferrin and ovalbumin. C-type lysozyme was identified in wood duck and hooded merganser albumen while the presence of g-type lysozyme in mute swan albumen and Canada goose albumen is suspected but could not be confirmed due to the unavailability of antibodies against g-type lysozyme. Ovotransferrin, a bacteriostatic metal-binding protein, and lysozyme, a muridase, provide egg white with properties that are likely to significantly increase the potential survival of the developing embryo. Ovotransferrin content, as well as antimicrobial activity, was not found to vary across species. This investigation has revealed that Anseriform eggs are generally well protected by the antimicrobial components of egg white. While species nesting under humid conditions may be at greater risk from bacterial attack it also appears that such species have potentially evolved greater defences to compensate for the increased threat.

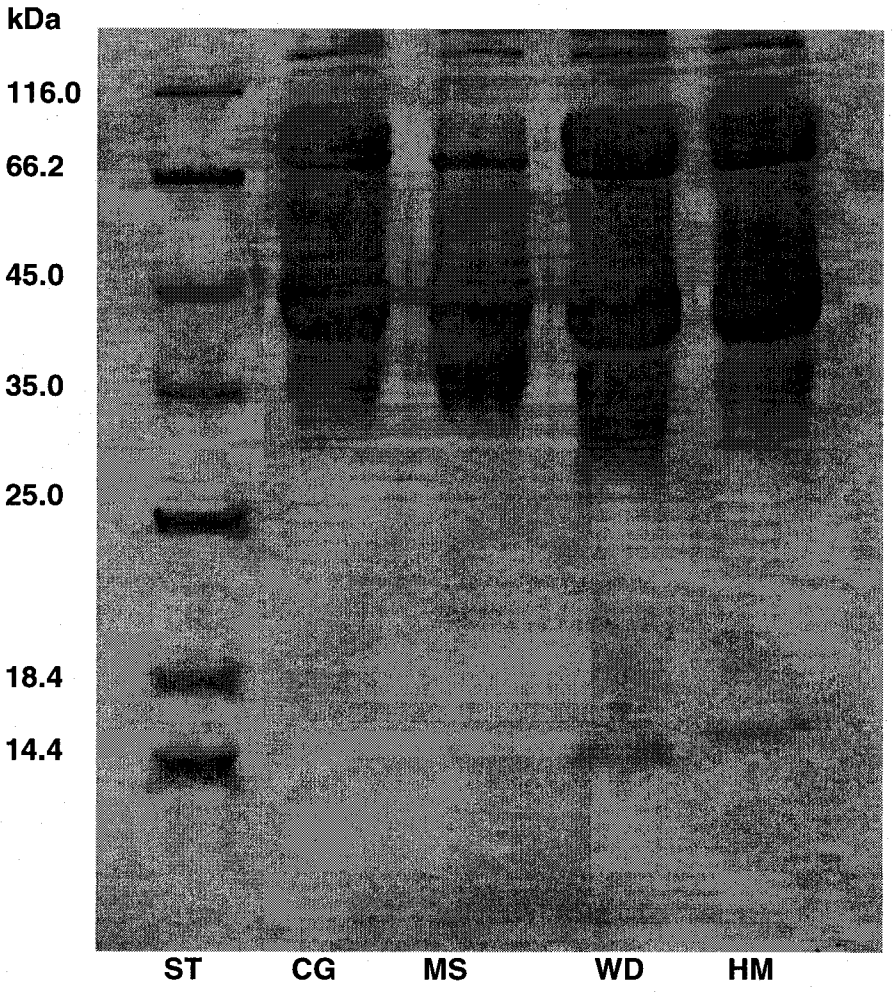

Figure 1: SDS-PAGE of avian egg white samples. Protein samples (20 μg) were loaded into each well of a 12 % polyacrylamide gel visualized by Coomassie Blue staining. Molecular weight of protein standards (ST) is indicated on the left. Egg white samples are labeled at the bottom (CG-Canada goose, MS-mute swan, WD-wood duck, HM-hooded merganser).

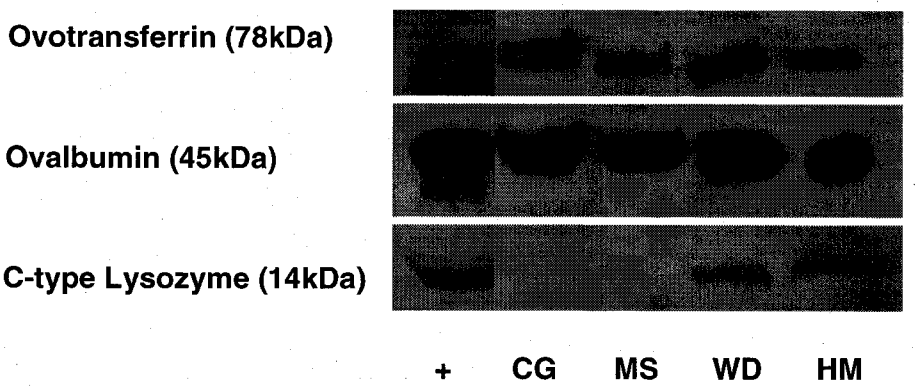

Figure 2: Western blot of avian egg white samples against selected chicken egg white proteins. Sample (1 μg) was loaded in each well. Purified chicken egg white lysozyme, ovotransferrin or ovalbumin (0.1 μg) was used as a positive control (+). Egg white samples are labeled at the bottom (CG-Canada goose, MS-mute swan, WD-wood duck, HM-hooded merganser).

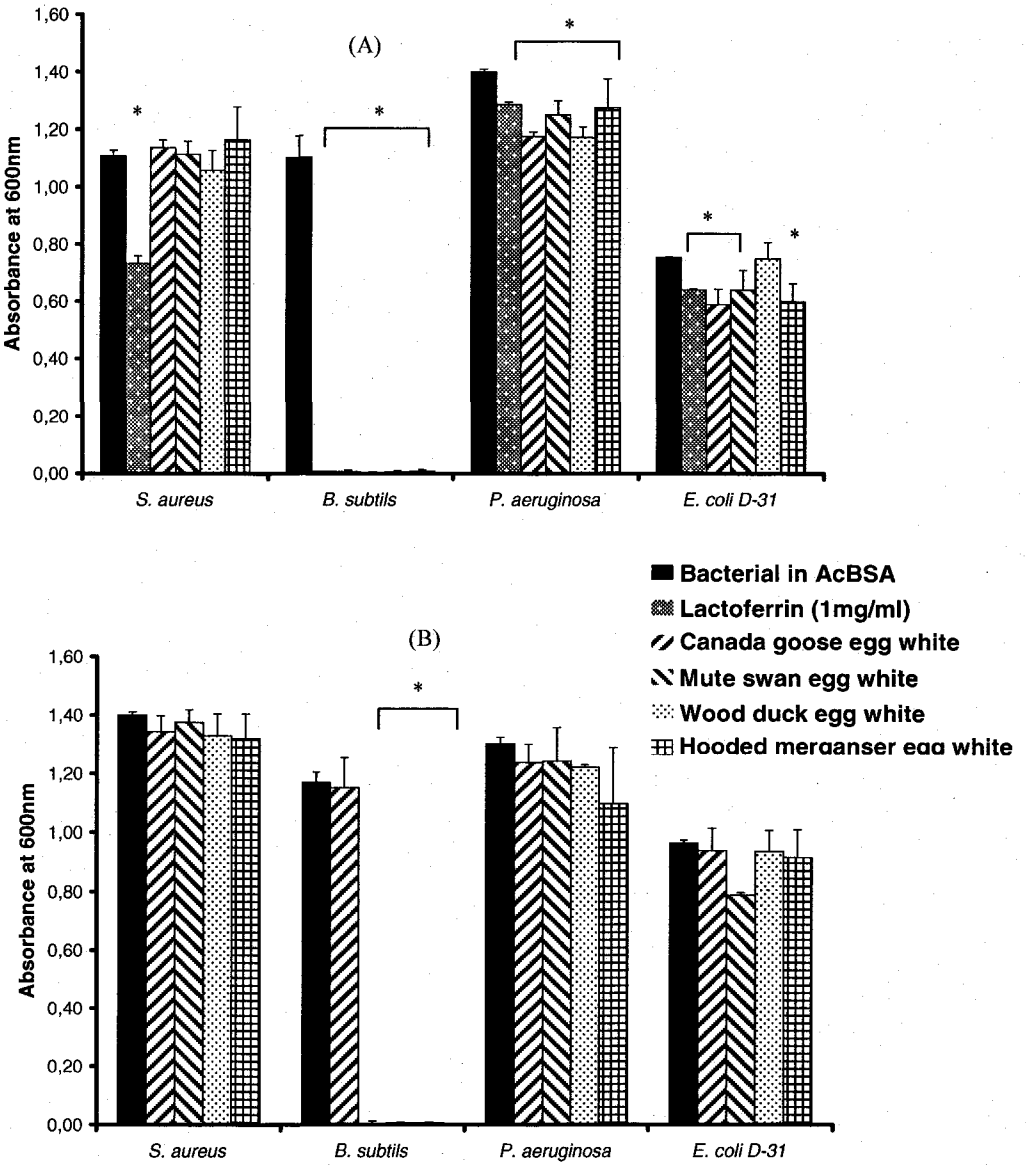

Figure 3: Bacteriostatic activity of 10 mg/ml egg white protein. Bacteria were incubated for 24 hours in LB broth with protein samples in the presence (A) and absence (B) of salt. When salt was used, 10 mM sodium citrate 50 mM sodium bicarbonate was used for Gram-positive bacteria and 50 mM sodium bicarbonate was used for Gram-negative bacteria. Bovine lactoferrin (1 mg/ml) was used as a positive control and 0.01 % acetic acid was used as a negative control. Experiment was performed on two separate occasions with triplicate readings. Average absorbance at 600 nm ± standard deviation is indicated. An ANOVA was performed to compare protein samples to control and statistically significant differences ($p < 0.05$) are indicated by an asterix (*).

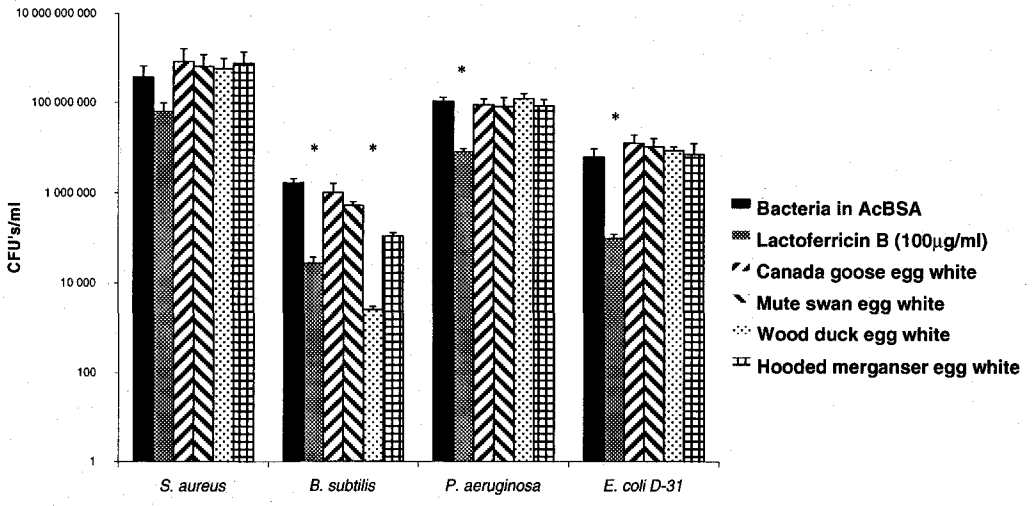

Figure 4: Bactericidal activity of purified chicken egg white c-type lysozyme against selected Gram-positive and Gram-negative bacteria. Bacteria in 10 mM sodium phosphate buffer (pH 7.3) were incubated in the presence of protein samples. Samples were plated on LB agar and CFU's/ml determined after over night incubation at 37 °C. Experiment was performed on two separate occasions with triplicate readings. Average CFU's/ml ± standard deviation is indicated.

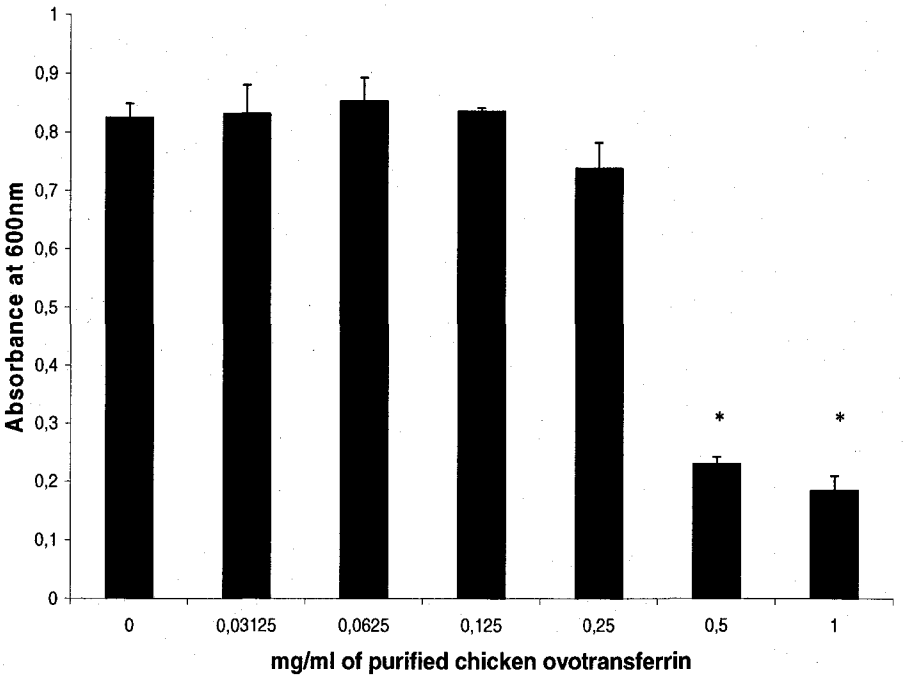

Figure 5: Inhibition of *E. coli* D31 growth in the presence of purified chicken egg white ovotransferrin after 24 hours incubation in LB broth supplemented with 50 mM sodium bicarbonate. Bacterial growth is indicated as the optical density at 600 nm ± standard deviation in triplicate for two independent trials. Statistically significant inhibition ($p < 0.05$) is indicated by an asterix (*).

Table 1: Determination of lysozyme content of pooled avian egg white samples by measurement of enzymatic activity against *M. lysodeikticus* cell walls. Lysozyme content is indicated as a percentage (± standard deviation) of total protein for each species at pH 5.3 and 7.3. Measurements were performed in triplicate during two independent trials.

Sample	% Lysozyme ± Standard Deviation		Ratio
	pH 7.3*	pH 5.3‡	pH 5.3 / pH 7.3
Chicken	2.44 ± 0.22	2.43 ± 0.21	1.0
Canada Goose	0.07 ± 0.01	0.23 ± 0.02	3.2
Mute Swan	0.10 ± 0.00	0.36 ± 0.02	3.6
Wood Duck	0.39 ± 0.09	0.73 ± 0.01	1.9
Hooded Merganser	0.10 ± 0.00	0.31 ± 0.01	3.1

* pH optimal of c-type lysozyme (*Gallus gallus*)
‡ pH optimal of g-type lysozyme (*Anser anser*)

Section III: Chapter 4

In the previous section of this thesis (Section 2), albumen from domestic and wild avian species was investigated for antimicrobial activity. This comparative approach permits an investigation of the effects of phylogeny and environmental pressures on the antimicrobial defences of the avian egg. While albumen may play an important role in the antimicrobial defences of the avian egg, antimicrobial components present in egg compartments other than albumen may also contribute significantly as suggested in section one of this thesis.

In section three of the thesis, the role of the eggshell in the antimicrobial defence of the avian egg is investigated. The avian eggshell, a structure directly in contact with the environment, appears to be a likely candidate for the presence of antimicrobial components as occurs in many of the physical barriers of animals. We therefore begin with chapter 4, an investigation of the antimicrobial activity of outer eggshell and cuticle protein extracts from domestic avian species. This experimental work was performed entirely by myself and is accepted for publication in the British Poultry Science Journal.

Chapter 4: Antimicrobial Activity of Cuticle and Outer Eggshell Protein Extracts from Three Species of Domestic Birds

Olivier Wellman-Labadie, Jaroslav Picman and Maxwell T. Hincke

Abstract:

The eggshell cuticle is the proteinaceous outermost layer of the eggshell which regulates water exchange and protects against entry of micro-organisms. In this study, we investigate the hypothesis that the cuticle may also reduce microbial contamination by providing a chemical defence. Outer eggshell and cuticle protein was extracted from domestic chicken (*Gallus gallus*), duck (*Anas platyrhynchos*) and goose (*Anser anser*) eggs by HCl and urea treatment, respectively. Antimicrobial activity of the extracts against Gram-positive and Gram-negative bacteria was evaluated. C-type lysozyme, ovotransferrin and ovocalysin-32 were identified in all extracts by Western blotting. All extracts from all species demonstrated lysozyme enzymatic activity. Immobilized c-type lysozyme retained some enzymatic activity. Protein extracts demonstrated activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis* suggesting the action of antimicrobial proteins in addition to lysozyme. The results suggest that the antimicrobial outer eggshell and cuticle proteins present in a number of avian species may be a mechanism which enhances avian reproductive success.

Introduction:

The avian egg is a reproductive structure that has been shaped through evolution to resist physical, microbial and thermal attack from an external and possibly aggressive environment, while satisfying the needs of the developing embryo (Hincke et al., 2008). The egg's natural defences have two components: one is the shell that acts as a physical barrier, and the other is a chemical system composed of endogenous antibacterial proteins that have been mainly identified in egg white and eggshell membranes (Hincke et al., 2008).

The egg is composed of a central yolk surrounded by the albumen, eggshell membranes, calcified eggshell and cuticle; it is assembled as it passes through specialized regions of the oviduct. During the 2-3 hour period where the yolk/ovum complex travels down the

largest portion of the oviduct, the magnum, it progressively acquires the albumen (Nys et al., 2004). Subsequently, the forming egg enters the proximal (white) isthmus, where the inner and outer shell membranes assemble during 1 to 2 hours. The inner membranes remain uncalcified, while the fibres of the outer shell membrane penetrate the mammillary cones of the calcified shell (Hincke et al., 2000; Nys et al., 2004). The membranes envelop the albumen, but are semi-permeable and allow the exchange of gases and water while retaining the proteins of the albumen. In addition, the eggshell membranes act as a physical and chemical barrier to bacteria (Gautron et al., 2007; Ahlborn et al., 2006; Ahlborn and Sheldon, 2006). Mineralization of the shell is initiated within the distal (red) isthmus, and is completed in the shell gland (uterus) during the subsequent 16-17 hours (Nys et al., 2004).

The calcitic eggshell is progressively composed of the inner mammillary cone layer, central palisades and the outer vertical crystal layers. The eggshell mineral is associated with an occluded organic matrix composed of proteins, glycoproteins and proteoglycans, which influence the fabric of this biomaterial during its deposition (Nys et al., 2004). The outermost layer is the eggshell cuticle, a noncalcified organic layer consisting mainly of protein, which is deposited on the mineral surface during the last 1.5 hours prior to oviposition (Baker and Balch, 1962). Eggs of some bird species, such as pigeons, lack a cuticle while in other species, such as chickens, the cuticle may be absent in a small proportion of eggs (Board, 1974; Board and Hall, 1973). The cuticle plays a role in controlling water exchange by repelling water or preventing its loss, and may function in limiting microbial colonization of the eggshell surface (Board and Tranter, 1986). Sparks and Board (1984) noted that chicken eggs lacking a cuticle readily absorbed water, and that newly laid fowl eggs with an incomplete cuticle, are less able to resist bacterial penetration of the shell.

Little is known about the protein constituents of the cuticle, or their possible contribution to the chemical aspect of antimicrobial defence. In this study, we extracted cuticle and outer eggshell surface proteins from three species of domesticated birds, chicken (*Gallus gallus*), duck (*Anas platyrhynchos*) and goose (*Anser anser*), in order to compare their protein constituents and to assess possible antimicrobial activity. The three species were selected since they are members of two differing avian orders which inhabit the same controlled environment as a result of domestication.

Materials and Methods:

Source of Eggs: Unfertilized, unwashed eggs of the domestic chicken (*Gallus gallus*), domestic duck (*Anas platyrhynchos*) and domestic goose (*Anser anser*) were obtained from a local farm in Perth, Ontario, Canada. Eggs were rinsed with running deionised water and left to dry for 1-2 hours at room temperature.

Measurement of Eggshell Thickness: After washing out the contents, 10 eggshells were processed for each species. The shells were dried at room temperature (22-25°C) for 48 hours, and shell fragments (about 1 cm^2) were selected from 4 random positions per shell. Eggshell thickness of all fragments was measured using a micrometer (Sino Science and Technology Company, Xiangcheng, China).

Liberation of Protein from the Eggshell Cuticle: Intact eggs were treated with 8 M urea 50 mM Tris-HCl pH 7.5 or 6 M Guanidine-HCl 50 mM Tris-HCl pH 7.5 to extract proteins from the outer eggshell surface (i.e. the cuticle) without decalcification of the eggshell. Within the exposure periods of 15, 30 and 60 minutes tested for Guanidine-HCl and urea, a treatment time of 60 min was found most suitable since this time gave the highest protein yield. The SDS-PAGE profile of the 6 M Guanidine-HCl extract was identical to that obtained with 8 M urea treatment (data not shown), and the latter was therefore adopted for reasons of economy and convenience.

Eggs (25 chicken and duck eggs, 16 goose eggs) were individually immersed in 8 M Urea 50 mM Tris-HCl (pH 7.5) for 60 minutes at room temperature (22-24 °C). Treated eggshells were rinsed with distilled water and dried at room temperature. The percentage of shell material removed was calculated from the difference in mass of dry eggshell before and after treatment divided by initial shell weight. Eggshell extracts were pooled and dialyzed 6 times, using cellulose dialysis membranes (MWCO 3500 Da; Fisher Scientific, Ottawa, ON), against a 100 fold greater volume of deionised water at 4 °C for 24 hour periods. Samples were lyophilized and stored at -20 °C until further analysis.

Liberation of Protein from Outer Eggshell Matrix and Cuticle: In preliminary trials, intact eggs were individually treated with solutions of ethylenediaminetetraacetic acid

(EDTA) (0.25, 0.5 and 1.0 M) in 50 mM Tris-HCl pH 7.5, using treatment times of 15, 30 and 60 min to partially decalcify eggshell and release proteins trapped within the outer eggshell matrix. Analysis of these materials by SDS-PAGE indicated that a treatment time of 60 min using 0.5 M EDTA was suitable (data not shown) since this extract had the highest protein yield and did not show any albumen contamination. Following dialysis and lyophilization, it was observed that EDTA was not completely removed. Previous reports indicated that EDTA interacts with proteins and is difficult to remove by conventional methods even after extensive dialysis (Wheeler et al., 1987; 1988). Contaminating EDTA interfered with the BCA protein assay as well as the antimicrobial assay. Therefore, treatment with 1 N HCl, as previously used to dissolve eggshell by Hincke et al., (2000; 2003), was tested and found to be suitable for liberation of proteins trapped within the outer eggshell matrix.

A treatment time of 5 min with 1 N HCl gave high protein yield and selective dissolution of the outer eggshell. In 15 % of *Gallus gallus* eggs, this treatment resulted in very rapid dissolution from small zones that completely penetrated the shell, which could lead to contamination of extracts with proteins from egg white (Hincke et al., 2003). For this reason, eggs were individually treated and extracts with potential albumen contamination were discarded. Treated eggshells were rinsed with distilled water, dried at room temperature and the percentage of shell dissolved was calculated. Eggshell extracts were pooled, dialyzed and lyophilized for storage at -20 °C until further analysis. Twenty five chicken and duck eggs as well as 16 goose eggs were used for this extraction.

Analysis of Proteins: Eggshell cuticle and outer eggshell protein extracts were analysed by SDS-PAGE and Western blot according to the method described by Wellman-Labadie et al., (2008a). Lysozyme activity of samples was evaluated by measurement of enzymatic activity against a *Micrococcus* cell wall suspension (10mM sodium phosphate buffer, pH 7.3) according to the method described by Wellman-Labadie et al., (2008a)

Enzymatic Activity of Immobilized Chicken Egg White Lysozyme: Serially diluted (0-1 mg/ml by 2 fold dilution) chicken egg white lysozyme (Sigma-Aldrich, Oakville, ON) in 0.01 % acetic acid (50 µl) was coated onto a medium binding 96 well EIA/RIA plate (Fisher Scientific, Ottawa, ON) in the presence of an equal volume of carbonate binding buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.5). Negative control

wells contained vehicle and bovine serum albumin (Bioshop, Burlington, ON) (1 mg/ml in vehicle). Following overnight incubation at 37 °C, the plate was washed (3 times, 5 minutes each at room temperature) with 10 mM sodium phosphate buffer (pH 7.3) to remove unbound lysozyme. A suspension of *Micrococcus lysodeikticus* ATCC 4698 cell walls (Sigma-Aldrich, Oakville, ON) (0.5 mg/ml in 10 mM sodium phosphate buffer, pH 7.3, 200 µl) was added to each well). The absorbance at 600 nm (turbidity) was measured at various times (0, 5, 10 and 22 hours) to monitor the hydrolysis of cell walls by lysozyme activity. The following control experiment was performed to assess the potential loss of bound lysozyme by solubilization from the plate. An identically coated plate was incubated overnight at 37 °C with 10 mM sodium phosphate buffer (pH 7.3, 200 µl/well). The solution was removed from each well for assessment of lysozyme activity (50 µl sample with 200µl *Micrococcus lysodeikticus* ATCC 4698 cell walls). No re-solubilized activity was detected, even with extended incubation (up to 24 hr). All conditions were tested in triplicate.

Antimicrobial Activity of Cuticle and Outer Eggshell Protein Extracts: A sample from an overnight bacterial culture was grown to log phase in Luria-Bertani (LB) broth (Bioshop, Burlington, ON). A bacterial inoculum was concentrated by centrifugation at 3000 g, 4 °C for 10 minutes and adjusted to ~1 x 10^5 CFU's/ml. Protein samples (200 µg) in 0.01 % acetic acid were added to bacterial inoculum and incubated at 37 °C, 250 rpm (Multitron HT Infors incubator, Rose Scientific, Mississauga, ON). Inhibition of growth was monitored by spectrophotometric readings at 600 nm (turbidity) after 24 hours incubation. Antimicrobial activity was evaluated in this manner for two Gram-positive (*Bacillus subtilis* ATCC 19659 and *S. aureus* ATCC 6538) and two Gram-negative (*Pseudomonas aeruginosa* ATCC 15442 and *E. coli* D31) bacteria. Concomitant negative controls (vehicle without protein sample) were performed in all experiments.

Statistical Analysis: Data was analysed using SYSTAT Version 8.0 (SPSS, Chicago, IL). Analysis of variance followed by pair-wise analysis (multiple comparisons test) was conducted to identify statistically significant differences. Bonferroni post hoc tests were employed. If the data could not meet the assumptions of the statistical model following transformation, a Kruskal-Wallis test followed by a Kolmogorov-Smirnov test was conducted. For analysis of eggshell mass loss across species and extracts, a two-way

model III (mixed model) ANOVA was employed. A one-way model II (random treatment) ANOVA was employed to analyse the variation of eggshell thickness across species. The same model was also employed for the analysis of the antimicrobial activity and lysozyme activity across extracts. For the analysis of the antimicrobial activity of extracts, multiple comparisons focused mainly on differences of extracts with the control.

Results:

Shell Thickness: The thickness of the domestic goose eggshell (0.63 ± 0.02 mm, N = 10 was greater ($p < 0.001$) than that of the domestic duck (0.41 ± 0.02 mm, N= 10). The domestic chicken had the thinnest shell (0.38 ± 0.01 mm, N = 10) of the three species ($p < 0.05$).

Extraction of Cuticle and Outer Eggshell Proteins: Various extraction methods and treatment times to solubilize proteins from the cuticle and from the outer eggshell matrix were evaluated during preliminary studies using *Gallus gallus* eggs (methods). Treatment of duck, goose and chicken eggs with 8 M urea 50 mM Tris-HCl pH 7.5 selectively extracted proteins from the cuticle without decalcification of the eggshell. Urea treatment removed less than 3.5 % of eggshell mass in all three species (Table 1). The percentage of eggshell mass removed by urea varied between species; pair-wise analysis demonstrated that this treatment removed a significantly larger percentage of eggshell mass from chicken than from duck and goose (Table 1).

Treatment of eggs with 1 N HCl, as previously used to dissolve chicken eggshell by Hincke et al., (2000; 2003), removed significantly more eggshell mass than urea treatment in all species (Table 1). The percentage of eggshell material removed by HCl treatment varied significantly across species. HCl treatment in goose removed less eggshell mass than in the chicken or duck.

SDS-PAGE of Cuticle and Outer Eggshell Protein Extracts: SDS-PAGE revealed multiple bands in each of the extracts which differed between the three species (Figure 1). However, bands of approximately 32-35 kDa appeared to be present in all extracts from all species (**). Additionally, urea extracts from chicken and goose appeared to

possess bands of approximately 20 and 25 kDa (*). Goose urea and goose HCl extracts apparently showed high similarity sharing most bands although some bands (14 and 35 kDa) were more intense in HCl extract.

Western Blotting of Cuticle and Outer Eggshell Protein Extracts: Western blotting with an antiserum raised against chicken ovocalyxin-32 revealed a 32 kDa protein (Figure 2) in extracts from all species. On the other hand, ovocalyxin-36 was detected solely in the chicken HCl extract (Figure 2). A number of egg white proteins were also detected. Ovalbumin (45 kDa) was detected in urea and HCl extracts of chicken and duck, but not in goose (Figure 2). A 14 kDa immunoreactive band was detected in extracts from all species (Figure 2) using antibodies to chicken egg white c-type lysozyme. This band was particularly intense in the duck HCl extract. We confirmed that goose albumen was negative for c-type lysozyme by Western blot (data not shown). Ovotransferrin was also detected in extracts from all species (Figure 2).

Antimicrobial Activity of Cuticle and Outer Eggshell Protein Extracts: All extracts possessed antimicrobial activity against one or more bacterial species. With respect to Gram-negative bacteria, growth of *E. coli* D31 was unaffected by any eggshell extract; however, all urea and HCl extracts significantly inhibited the growth of *P. aeruginosa* (Figure 3a, 3b). The observed decrease in optical density (0.4 units) of *P. aeruginosa* cultures, due to growth inhibition, would correspond to about 50 % reduction in CFU's/ml. Regarding Gram-positive bacteria, *B. subtilis* was inhibited by chicken urea and HCl extracts, and also duck and goose HCl extracts (Figure 3c). All extracts except for goose urea were found to inhibit the growth of *S. aureus* (Figure 3d).

Lysozyme Activity of Cuticle and Outer Eggshell Protein Extracts: Purified chicken egg white c-type lysozyme is inactive against *E. coli* D31, *P. aeruginosa* and *S. aureus* while demonstrating dose dependant activity against *B. subtilis* (Wellman-Labadie et al., 2008a). Lysozyme is a selective but effective antimicrobial and lysozyme immuno-reactivity was detected in all eggshell protein extracts (Figure 2). For this reason, lysozyme enzymatic activity of extracts was measured using the bioassay of Liao *et al.,* (2001). The minimal detection limit of this assay was approximately 11 μg/ml, whereas lysozyme concentrations as low as 2.5 μg/ml demonstrated potent antimicrobial activity against *B. subtilis* (Wellman-Labadie et al., 2008a). A standard curve, constructed using

purified chicken egg white lysozyme, was used to calculate equivalent lysozyme activity, which was expressed as a percentage of total protein for each sample. Analysis of the cuticle and outer eggshell protein extracts for lysozyme activity revealed that all extracts from all species possessed lysozyme activity (Table 2). Duck HCl extract showed the highest level of activity followed by goose HCl, duck urea, chicken urea, goose urea extract and chicken HCl extracts.

Enzymatic Activity of Immobilized Chicken Egg White Lysozyme: Outer eggshell and cuticle proteins are incorporated into the egg surface (Vadehra et al., 1972). An experiment was designed to simulate this condition and determine whether immobilized lysozyme demonstrated enzymatic activity, using purified chicken egg white lysozyme. Lysozyme was immobilized into the wells of an EIA/RIA polystyrene plate using a standard ELISA coating protocol. A suspension of *Micrococcus lysodeikticus* cell walls was added to serve as a substrate for lysozyme enzymatic activity. Immobilized lysozyme remained enzymatically active with *Micrococcus* cell walls; bound activity was effective in hydrolysing cell walls after an incubation time of 5 hours (Figure 4). In contrast to this assay, lysozyme in solution (5 μg; 5μl of 1 mg/ml solution), as used as a standard during the lysozyme activity assay, effects total hydrolysis within 1-2 minutes (data not shown) while 50 μg (50 μl of 1 mg/ml solution) of bound lysozyme (assuming all applied lysozyme is bound) totally hydrolyses the same cell wall suspension in 22 hours. In order to determine whether lysozyme was being solubilized during this prolonged incubation with bacterial cell wall substrate, we tested the effect of 24 hour pre-incubation of coated lysozyme with 10 mM sodium phosphate buffer (pH 7.3). Following this incubation period, contents of each well were removed to test for soluble lysozyme, the wells were washed and then cell wall suspension was added. No lysozyme activity was detected in the extraction buffer (6.5 ± 12.3 % decrease in optical density after 24 hr incubation for solution removed from wells containing 1 mg/ml immobilized lysozyme). This comparison demonstrated that bound lysozyme remained immobilized on the plastic during extended incubation, but still possessed lytic activity against bacterial cell walls.

Discussion:

The egg is essential for avian reproduction, and microbial contamination of its contents leads to reduced hatching success (Cook et al., 2003; 2005a). The antimicrobial defences of avian egg consist of two main components, a physical barrier provided by the intact shell, and a chemical defence arising from different components of the albumen, such as alkaline pH, ovotransferrin and lysozyme (Board and Tranter, 1986). The movement of water across the eggshell through the network of pores provides a pathway for bacterial contamination of the egg contents (Board and Tranter, 1986). The cuticle covering of the eggshell surface and the cuticular plugs that obstruct the pores are thought to exert a protective function by restricting water penetration (Baggott and Graeme-Cook, 2002). Indeed, the observation that bacterial contamination of egg contents is significantly higher in eggs with incomplete cuticle does suggest that the cuticle layer is an important element in antimicrobial protection of the egg (Sparks and Board, 1984). Recent studies have demonstrated that ovotransferrin and lysozyme, two components of the chemical defences of the albumen, are associated with the outermost eggshell layer in eggs of domestic chicken (Hincke et al., 2000; Gautron et al., 2001b). Such observations suggest that antimicrobial chemical defences may also be associated with the eggshell surface. The avian cuticle, and when the cuticle is incomplete, the outermost layer of mineralized shell, are directly in contact with the environment at a strategic location for antimicrobial defence. Therefore, in this study we tested the hypothesis that one or more of the cuticle and/or outer eggshell components possess antimicrobial activity, using eggs from 3 species of domesticated birds.

Protein was extracted from the cuticle alone using urea solutions, and from cuticle plus outer eggshell by selective acid dissolution of the outer mineralized eggshell. HCl treatment removed significantly more eggshell mass than urea treatment in all species due to decalcification of the outer eggshell, while urea treatment only solubilized proteins of the cuticle. Since HCl treatment times were the same for all species, less material relative to total shell weight was removed from species (duck and goose) with greater eggshell thickness. Analysis of the protein constituents of the extracts by SDS-PAGE revealed that goose urea and goose HCl extracts apparently showed high similarity sharing most bands although band intensity was greater in HCl extract. This suggests that the protein composition of goose outer eggshell is similar to that of its cuticle. However, this was not the case for chicken and duck since there were large differences between their respective urea and HCl extracts.

The antimicrobial activity of the extracts was evaluated against both Gram-positive and Gram-negative bacteria. The lysozyme-sensitive *Bacillus subtilis* and the lysozyme-insensitive *Staphylococcus aureus* were selected as Gram-positive bacteria. *Pseudomonas aeruginosa*, an opportunistic pathogen of plants and animals, and *Escherichia coli*, a fecal coliform, were selected as Gram-negative bacteria. Our results demonstrated that *P. aeruginosa* was inhibited by all eggshell extracts from all species. The growth of *B. subtilis* was inhibited by all outer eggshell protein extracts as well as the chicken eggshell cuticle extract, but not by duck or goose urea extract. In addition, all extracts from all species (excluding goose urea) inhibited the growth of *S. aureus.* On the other hand, none of these extracts were effective against *E. coli* D31. These results suggest that the cuticle and outer eggshell could play an active protective role against microbial contamination of the egg.

The cuticle of the chicken egg is a thin layer which is mainly composed of protein (>85%) (Baker and Balch, 1962). In the current study, a number of specific proteins that may be responsible for the demonstrated antimicrobial activity were identified in the cuticle / eggshell extracts by Western blotting. C-type lysozyme was detected in urea and HCl extracts from all species, indicating its general presence in the avian cuticle and outer eggshell as demonstrated previously for chicken (Hincke et al., 2000; 2003; Vadehra et al., 1972). Lysozyme enzymatic activity of extracts, excluding goose HCl extract, was found to roughly correlate with the intensity of the immunoreactive bands detected during Western blotting. Both duck and goose HCl extracts possessed greater lysozyme activity than their respective urea extracts. This likely indicates that lysozyme is distributed throughout the cuticle and outer eggshell matrix in the duck and goose but is predominately restricted to the cuticle in the chicken eggshell. Purified egg white lysozyme is effective against *B. subtilis* but inactive against the other bacteria tested. None the less, all extracts were active against the Gram-negative *P. aeruginosa*, and (except for goose urea extract), against Gram-positive lysozyme-insensitive *S. aureus*, suggesting that other antimicrobial proteins, shared between species, are present in these extracts.

C-type lysozyme was detected within the cuticle and outer eggshell of all species investigated, including goose. This result is surprising since goose albumen is known to

exclusively contain g-type lysozyme (Canfield and McMurray, 1967). The antibody which we used was specific for c-type lysozyme, since goose albumen from the same eggs was negative by Western blotting (Wellman-Labadie *et al.*, 2008a). This observation suggests that regionally selective expression of g-type / c-type lysozyme occurs in goose magnum and shell gland, respectively (Hindenburg *et al.*, 1974). In contrast to g-type lysozyme, c-type lysozyme demonstrates potent chitinase activity and is active across a wide pH range (Hindenburg et al., 1974; Thammasirirak et al., 2001). Chicken egg white lysozyme causes a rapid loss of viability among multiple *Candida albicans* isolates (Samaranayake et al., 2001). The protease and glycolytic activity of *Bacillus* spp., as well as that of fungi, can break down the eggshell cuticle and facilitate general microbial invasion by radically increasing the number of open pores (Cook et al., 2003; Baggott and Graeme-Cook, 2002). Mechanisms that reduce the loads of pioneer micro-organisms possessing the enzymes to destroy the cuticle would maintain the structural integrity of the egg surface and prevent entry of other, lysozyme-insensitive micro-organisms which could proliferate after reaching the albumen. Newly laid fowl eggs with an incomplete cuticle are less able to resist bacterial penetration of the shell (Sparks and Board, 1984). C-type lysozyme within the eggshell cuticle and outer eggshell may therefore reduce contamination from fungi and bacteria, if active in these locations, In order to assess this issue, we tested and found that lysozyme immobilized on a plastic surface still possessed lytic activity versus bacterial cell walls. Other workers have also demonstrated enzymatic activity, albeit reduced, of lysozyme immobilized on a variety of solid supports (Crapasi et al., 1993; Wu and Daeschel, 2007). The reduction in enzymatic activity is due, in large measure, to the diffusional limitations imposed by solid phase lysozyme and its substrate. Therefore, it is possible that eggshell lysozyme could provide antimicrobial protection *in situ*.

Low levels of ovotransferrin were detected in all extracts. Increasing HCl treatment time was found to result in greater shell decalcification and enhanced ovotransferrin extraction as detected by Western blotting (data not shown) indicating that the majority of ovotransferrin is found deeper within the eggshell matrix as described for chicken (Gautron et al., 2001a). Inhibition of bacteria by transferrins, such as ovotransferrin, at concentrations of 1 mg/ml and higher through sequestration of iron has been described (von Hunolstein et al., 1992). Ovotransferrin detected within the egg surface extracts could contribute to antimicrobial defence if concentrated in a thin layer or in specific

zones such as the vicinity of eggshell pores. Ovotransferrin immobilized on Sepharose 4B maintains its iron-binding capacity and its bacteriostatic activity (Valenti et al., 1982) indicating that eggshell-bound ovotransferrin could provide antimicrobial defence.

Ovocalyxin-32 (OCX-32) was detected in cuticle and outer eggshell extracts from all species and is a possible candidate for antimicrobial activity in avian eggshell extracts. Recombinant *Gallus gallus* ovocalyxin-32 inhibits the growth of *B. subtilis* and possesses carboxypeptidase inhibitory activity (Xing et al., 2007). OCX-32 shares homology with TIG1, a skin protein and latexin, a carboxypeptidase inhibitor of the rat (Hincke et al., 2003; Gautron et al., 2001a). Physiological barriers, such as skin and epithelial mucosa, are often associated with antimicrobial proteins; the outer eggshell and cuticle represent an equivalent barrier in the avian egg. A number of protease inhibitors, including the egg white protein ovomacroglobulin, are known to inhibit microbial proteases and demonstrate antimicrobial activity (Molla et al., 1987).

The presence of ovocalyxin-36 (OCX-36) solely in chicken HCl but not urea extracts indicates its presence within the eggshell matrix rather than in the cuticle. The protein shows homology to lipopolysaccharide-binding proteins, bactericidal permeability-increasing proteins as well as Plunc family proteins, leading to the proposal that it plays a role in antimicrobial defence within the chicken eggshell (Gautron et al., 2007). While equivalent versions of OCX-36 may be present in other avian species, the narrow epitope specificity (15 amino acid synthetic peptide) of the anti-OCX-36 antibody used in this study is a likely explanation for the lack of immunoreactivity in the other species.

A recent proteomic study of acid-soluble eggshell matrix proteins (after removing and discarding the cuticle and membranes) identified a number of potential antimicrobial proteins such as histones and avian beta-defensins (Mann et al., 2006). These may contribute to the antimicrobial activity that acetic acid extracts of eggshell matrix display against *P. aeruginosa, Bacillus cereus* and *S. aureus* (Mine et al., 2003). Histones with antimicrobial activity have been purified from chicken oviduct (Silphaduang et al., 2006). It is not known whether these proteins are also present in the outer eggshell or cuticle extracts of our study. In the future, proteomic analysis of cuticle may identify such potential antimicrobial constituents in addition to the ones that we have detected.

We have demonstrated that eggshell surface constituents possess antimicrobial activity when extracted from the cuticle and outer eggshell. Such immobilized antimicrobial proteins could also be released and solubilized from the cuticle during natural microbial contamination. Fungi as well as some bacteria produce enzymes capable of degrading the eggshell cuticle (Cook et al., 2003; Baggott and Graeme-Cook, 2002). Moreover, Gram-positive and Gram-negative bacteria produce acid metabolic wastes as a result of carbohydrate fermentation and bacterial oxidation (Murray et al., 2003). These metabolites may partially dissolve eggshell calcium carbonate and solubilize outer eggshell proteins. Moreover, proteolytic digestion of egg white lysozyme, ovotransferrin and ovalbumin generates peptides with bactericidal activity (Pellegrini et al., 2004). Thus, microbial attack, through limited physical damage of the outer eggshell and cuticle, could activate antimicrobial defences.

Eggshell proteins within the cuticle and outer eggshell are in contact with the external environment and represent the first line of defence for the avian embryo. Available studies have demonstrated that Gram-positive bacteria dominate the flora on the outside of eggs, possibly because of their tolerance of dry conditions, while Gram-negative bacteria dominate the interior of rotten eggs (Board and Tranter, 1986). It is likely that the antimicrobial activities present in the cuticle influence the selection of Gram-negative bacteria that occurs during the process of infection of the egg contents (Seviour and Board, 1972b). Our results indicate that eggshell cuticle proteins with similar antimicrobial activity are present in the eggs of species of differing phylogeny that are exposed to similar environmental pressures.

The identification of proteins within the eggshell cuticle with antimicrobial activity may have important implications for food handling and safety since egg washing chemicals damage the cuticle or modify the eggshell surface (Kim and Slavik, 1996). Processes which remove or damage the eggshell cuticle may therefore weaken intrinsic egg defences and potentially expose the consumer to increased health risks.

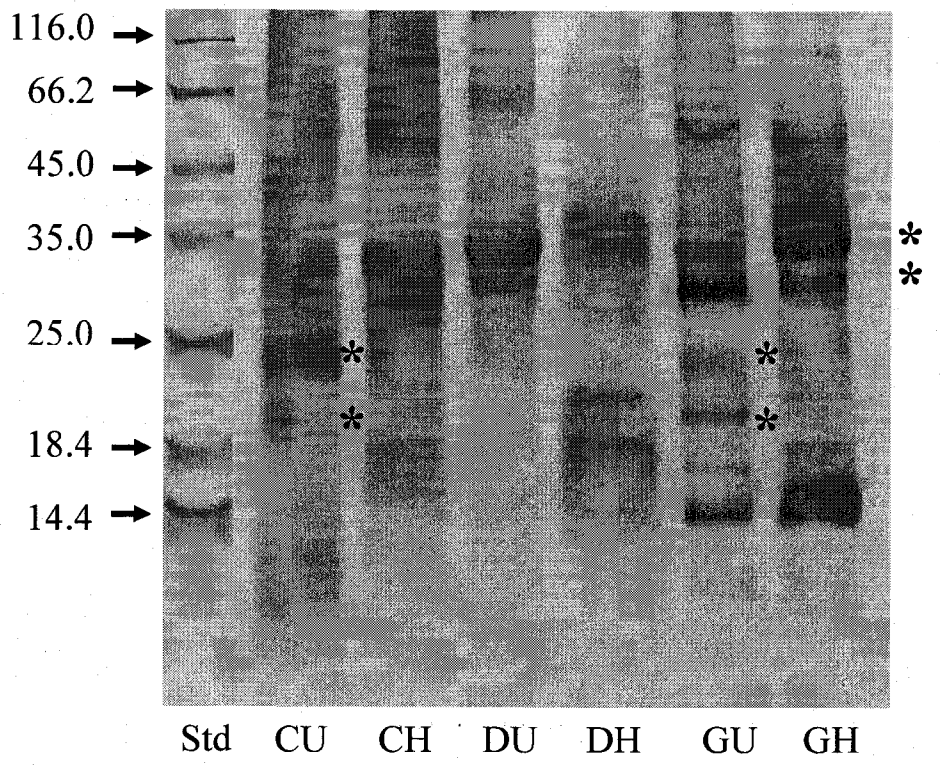

Figure 1: SDS-PAGE of avian eggshell extracts. Protein samples (20 μg) were resolved on 12 % polyacrylamide gel and visualized by Coomassie blue staining. Molecular weight standard (Std) is labelled on the left. The 20, 25 kDa bands in CU and GU samples are indicated (*), as are the 32, 35 kDa bands in goose extracts (**). Samples are labelled on the bottom (chicken extracts: CU - urea, CH - HCl; duck extracts: DU - urea, DH - HCl; goose extracts: GU - urea, GH - HCl).

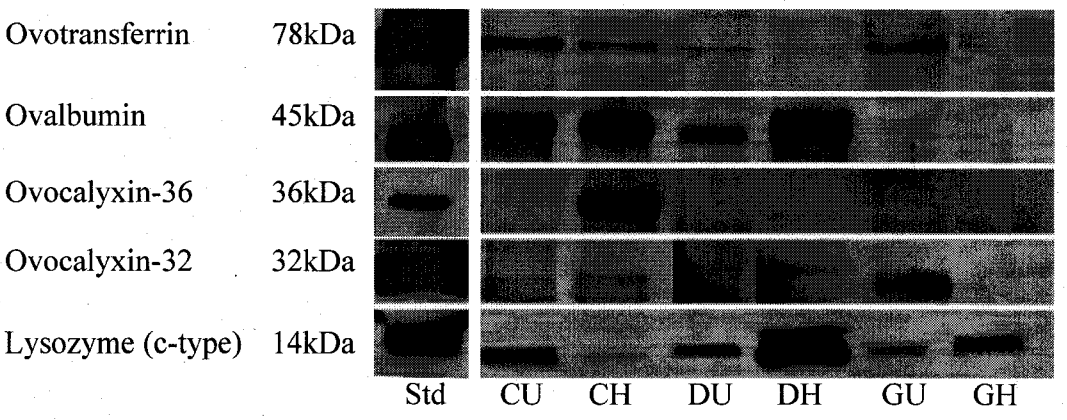

Figure 2: Western blot of avian eggshell protein extracted by Urea or HCl treatment. A 10 μg sample of protein was loaded in each well. Purified chicken egg white lysozyme, ovotransferrin or ovalbumin, and partially purified chicken eggshell OCX-32 and OCX-36, were used as positive controls (+). For clarity, the image of the dominant band obtained at the specified protein molecular weight is presented. Samples are labelled along the bottom as in Figure 1.

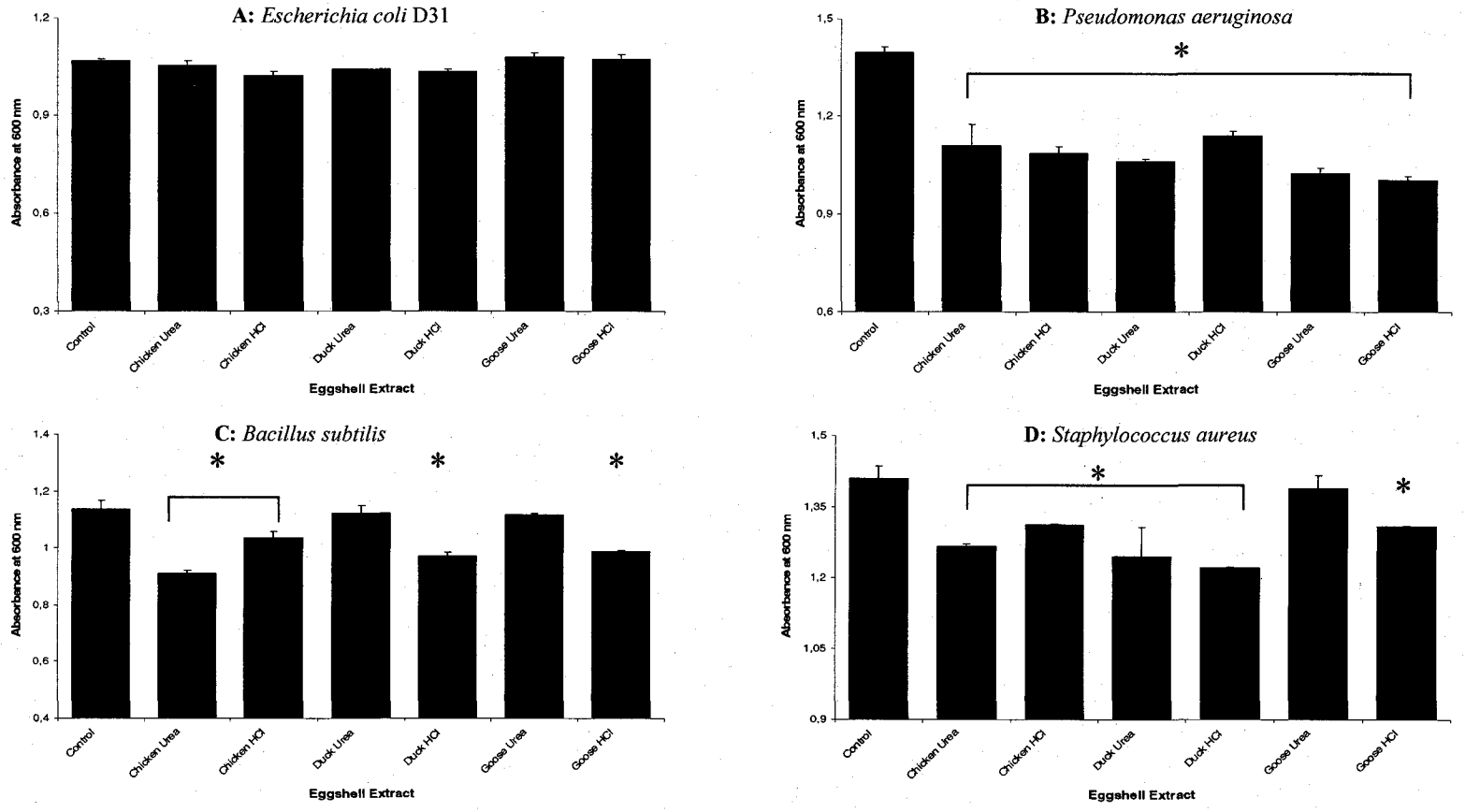

Figure 3: Inhibition of bacterial growth by eggshell extracts. Growth of *Escherichia coli* D31 (**A**), *Pseudomonas aeruginosa* (**B**), *Bacillus subtilis* (**C**) and *Staphylococcus aureus* (**D**) after 20 hrs incubation in the presence of chicken, duck or goose cuticle and outer eggshell protein extracts (200 µg/ml). Protein was extracted by immersion of eggs in 8 M urea 50 mM Tris-HCl pH 7.5 (60 min) or 1 N HCl (5 min) (methods). Bacterial growth is indicated as optical density at 600 nm ± standard error for two independent trials. Significant ($p < 0.05$) inhibition of growth relative to control is indicated (*)

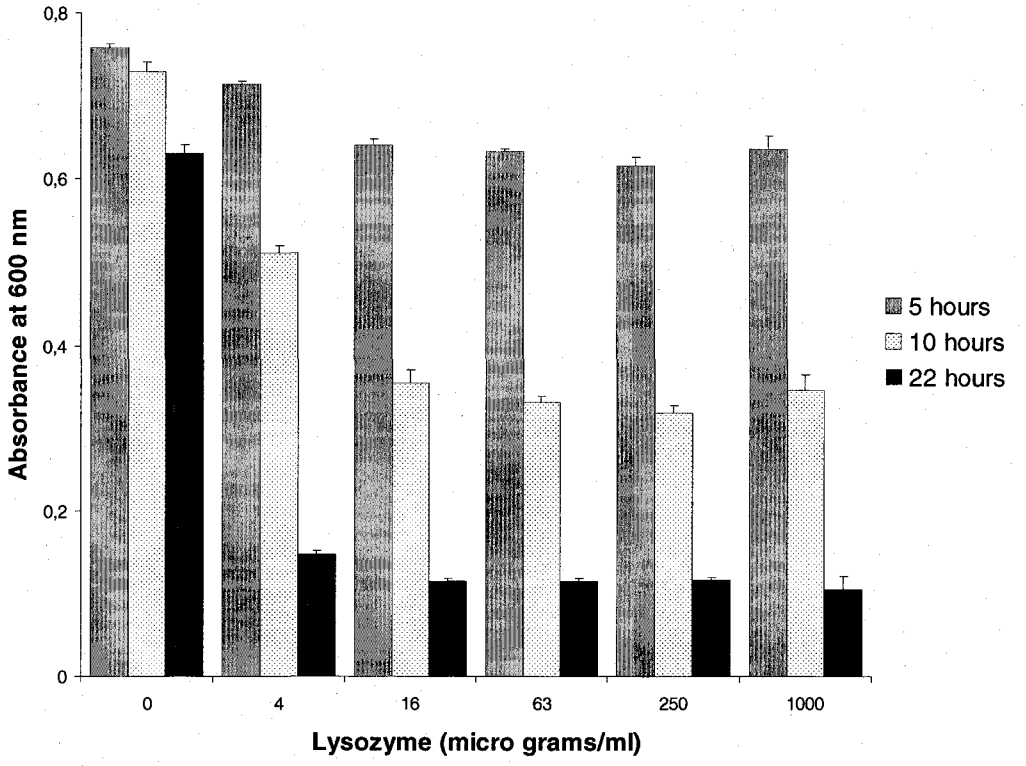

Figure 4: Enzymatic activity of immobilized chicken egg white lysozyme. Serially diluted lysozyme (0 -1 mg/ml in triplicate, 2 fold dilutions) was immobilized by overnight incubation in wells of an EIA/RIA plate. Following washing to remove unbound lysozyme, *Micrococcus* cell wall suspension in 10 mM sodium phosphate buffer (pH 7.3) was added to each well. Control wells had vehicle or BSA (1mg/ml) instead of lysozyme. Residual lysozyme activity due to immobilized enzyme was detected by measuring optical density at 600 nm after 5, 10 and 22 hr. In a parallel experiment, identical wells containing immobilized lysozyme were extracted overnight with 10 mM sodium phosphate buffer (pH 7.3). No lysozyme activity was detected in the resulting liquid phase (data not shown).

Table 1: Decrease in eggshell mass after immersion of individual, whole eggs in solutions of 8 M Urea 50 mM Tris-HCl pH 7.5 (60 min) and 1 N HCl (5 min).

Species	Extraction Method	Percent Eggshell Mass Loss ± SE	N
Chicken	8 M Urea/ 50 mM Tris-HCl pH 7.5	3.13 ± 0.206	25
Chicken	1 N HCl	7.99 ± 0.82	25
Duck	8 M Urea/ 50 mM Tris-HCl pH 7.5	1.81 ± 0.17	25
Duck	1 N HCl	7.18 ± 0.23	25
Goose	8 M Urea/ 50 mM Tris-HCl pH 7.5	2.38 ± 0.25	16
Goose	1 N HCl	4.81 ± 0.23	16

The percentage of eggshell mass removed by either urea or HCl varied between species ($p < 0.001$). Pair-wise analysis demonstrated that in the chicken, urea treatment removed a significantly larger percentage of eggshell mass than in duck ($p < 0.001$) and goose ($p < 0.05$), which did not differ from each other. Pair-wise analysis revealed that HCl treatment in goose removed a significantly lower percentage of eggshell mass than in the chicken ($p < 0.001$) and duck ($p < 0.01$), which did not differ from each other.

Table 2: Equivalent lysozyme activity of avian eggshell extracts against *M. lysodeikticus* cell walls. Experiment was conducted on two separate occasions. Lysozyme equivalency is expressed as a percentage of total protein extract for each species.

Species	Extraction Method	Lysozyme Activity ± Standard Error
Chicken	8 M Urea 50 mM Tris-HCl pH 7.5	0.33 ± 0.00
	1 N HCl	0.16 ± 0.00
Duck	8 M Urea 50 mM Tris-HCl pH 7.5	0.44 ± 0.04
	1 N HCl	1.33 ± 0.13
Goose	8 M Urea 50 mM Tris-HCl pH 7.5	0.20 ± 0.02
	1 N HCl	1.25 ± 0.01

Lysozyme activity varied between extracts ($p < 0.001$). Pair-wise analysis demonstrated that duck and goose HCl extracts demonstrated significant ($p < 0.001$) lysozyme activity when compared with the other extracts. Lysozyme activity of duck and goose HCl extracts did not differ significantly ($p > 0.05$) from each other.

Section III: Chapter 5

The previous chapter (Chapter 4) of section three demonstrated that antimicrobial proteins are present within the outer eggshell and cuticle of domestic avian species and enhance the antimicrobial defences of the avian egg. This investigation also demonstrated that proteins associated with the eggshell can retain their activity. In addition, this study permits an investigation of the effects of phylogeny on the antimicrobial defences of the avian egg.

In addition to phylogeny, environmental pressures may also influence the antimicrobial defences of the avian egg. Avian species that incubate eggs under humid conditions may lay eggs which contain enhanced antimicrobial defences. In the following chapter (Chapter 5), the outer eggshell and cuticle protein extracts of wild cavity and open-top nesting Anseriformes will be investigated for antimicrobial activity. This experimental work was performed entirely by myself and is published in Comparative Biochemistry and Physiology B (2008) 149(4): 640-649.

Chapter 5: Antimicrobial Activity of the Anseriform Outer Eggshell and Cuticle

Olivier Wellman-Labadie, Jaroslav Picman and Maxwell T. Hincke

Abstract:

The avian eggshell is a complex, multifunctional biomineral composed of a calcium carbonate mineral phase and an organic phase of lipids and proteins. The outermost layer of the eggshell, the eggshell cuticle, is an organic layer of variable thickness composed of polysaccharides, hydroxyapatite crystals, lipids and glycoprotein. In addition to regulating gas exchanges, the eggshell cuticle may contain antimicrobial elements. In this study, we investigated the antimicrobial activity of eggshell cuticle and outer eggshell protein extracts from four Anseriform species: wood duck (*Aix sponsa*), hooded merganser (*Lophodytes cucullatus*), Canada goose (*Branta canadensis*) and mute swan (*Cygnus olor*). Cuticle and outer eggshell protein was extracted by urea or HCl treatment of eggs. C-type lysozyme, ovotransferrin and an ovocalyxin-32-like protein were detected in all extracts. Cuticle and outer eggshell protein extracts inhibited the growth of *Staphylococcus aureus*, *Escherichia coli* D31, *Pseudomonas aeruginosa* and *Bacillus subtilis*. The presence of active antimicrobial proteins within the avian cuticle and outer eggshell suggests a role in antimicrobial defence. Protein extracts from the cavity nesting hooded merganser were especially potent. The unique environmental pressures exerted on cavity-nesting species may have led to the evolution of potent antimicrobial defences.

Introduction:

The avian eggshell is a complex multifunctional calcite biomineral, composed of highly structured calcium carbonate, containing a meshwork of proteins, glycoproteins and proteoglycans. The eggshell permits the exchange of gases and water while protecting the embryo against small predators and the crushing weight of the incubating hen. During the formation of the egg, within specialized regions of the oviduct, the eggshell is gradually acquired as uterine fluid components are deposited onto the eggshell membranes. Uterine fluid of the domestic hen changes in composition during the different stages of eggshell formation and influences calcite crystal growth in different zones of the calcified shell (Gautron et al., 1997b; Dominguez-Vera et al., 2000; Nys et al., 2004).

The eggshell cuticle is the outermost layer of the mineralized eggshell and is in direct contact with the environment. The cuticle is a thin layer of variable thickness (0.5 to 12.8 μm) composed of hydroxyapatite crystals, polysaccharides, lipids and glycoprotein (Whittow 2000; Fernandez et al., 2001). This organic layer is deposited on the eggshell surface and regulates water exchanges as well as the entry of micro-organisms through blocking of the eggshell pores (Chavez et al., 2002). The eggshell cuticle enhances the antimicrobial defences of the egg by physically preventing microbial contamination (De Reu et al., 2005; De Reu et al., 2006). Nonetheless, the chemical components of the cuticle, including egg white proteins, may also be involved in the antimicrobial defence of the egg.

Most studies of eggshell structure and function have been carried out using eggs of the domestic chicken, *Gallus gallus*. Mann et al., (2006) recently identified 520 proteins within the acid-soluble organic matrix of the chicken eggshell using MS-based technology. Multiple eggshell matrix proteins, including ovocleidin-17 (Hincke et al., 1995), ovocleidin-116 (Hincke et al., 1999; Panheleux et al., 1999), ovocalyxin-32 (Gautron et al., 2001a; Hincke et al., 2003) and ovocalyxin-36 (Gautron et al., 2007) have been identified with the *Gallus gallus* eggshell matrix. Ansocalcin, a homolog of ovocleidin-17, was isolated from the eggshell matrix of the domestic goose, *Anser anser*, and has been reported to influence calcium carbonate crystallization (Lakshminarayanan et al., 2002).

In addition to their involvement in eggshell formation, avian eggshell proteins may play additional roles. Lysozyme and ovotransferrin, two egg white proteins known for their antimicrobial properties, have been identified within the eggshell matrix of the chicken (Hincke et al., 2000; Panheleux et al., 1999; Gautron et al., 1997a). Mann et al., (2006) identified low levels of gallinacin-8 and β-defensin 11, as well as histones 2 and 4, as components of the chicken eggshell matrix using MS-based technology. Matrix proteins extracted from demineralised eggshell demonstrate antimicrobial activity against *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella enteritidis* (Mine et al., 2003). Histones H1 and H2B, two antimicrobial proteins demonstrating activity against Gram-positive as well as Gram-negative bacteria, were identified within ovary and oviduct tissue extracts of the domestic hen by

Silphaduang et al., (2006) suggesting that these proteins are incorporated during formation of the egg.

Eggshell matrix proteins and eggshell cuticle proteins, in particular, are strategically located directly in contact with the environment, leading to our hypothesis that they play a role in antimicrobial defence. In this study, the antimicrobial role of cuticle and outer eggshell matrix proteins from four wild avian species with alternative nesting strategies was investigated. The results suggest that proteins of the eggshell surface possess antimicrobial properties and provide defence against microbial attack.

Materials and Methods:

Sampling: Eggs from the cavity-nesting wood duck (*Aix sponsa*) and hooded merganser (*Lophodytes cucullatus*) were collected in Kanata (Ontario, Canada) from man-made nest boxes during the first week of laying. Eggs from the open-top nesting mute swan (*Cygnus olor*) and Canada goose (*Branta canadensis*) were collected during the first week of laying from pairs breeding on the islands of the Toronto inner harbour (Ontario, Canada). The proper collection permits were obtained from the Canadian Wildlife Service (Permit Number CA 0149 and CA 0150) and Animal Care Committee (Permit Number CMM-96). Species were selected for their alternative nesting strategies. Eggs were washed with deionised water and left to dry for 1-2 hours at room temperature.

Eggshell Thickness Measurements: The eggshell thickness of a sample of eggs from each species was measured according to the method described by Wellman-Labadie et al., (2008c). Data were analysed using SYSTAT Version 8.0 (SPSS, Chicago, IL). One-way analysis of variance (ANOVA) followed by pair-wise analysis was conducted to identify significant differences between species.

Liberation of Proteins from the Eggshell Cuticle and Outer Eggshell: Protein from the eggshell cuticle was extracted by immersion of whole eggs (7-30 eggs/species) in a urea solution as is described by Wellman-Labadie et al., (2008c). Outer eggshell protein was also extracted by immersion of whole eggs (7-30 eggs/species) in an HCl solution according to the method described by Wellman-Labadie et al., (2008c). Two-way mixed

model ANOVA, followed by pair-wise analysis, was employed for the analysis of eggshell mass loss across species and extracts.

Analysis of Proteins: SDS-PAGE and Western blot analysis of eggshell cuticle and outer eggshell extracts was conducted according to the method described by Wellman-Labadie et al., (2008c). Antimicrobial activity was evaluated against two Gram-positive (*Bacillus subtilis* ATCC 19659 and *Staphylococcus aureus* ATCC 6538) and two Gram-negative (*Pseudomonas. aeruginosa* ATCC 15442 and *Escherichia coli* D31) bacteria according to the method described by Wellman-Labadie et al., (2008c). Data were analysed using SYSTAT Version 8.0 (SPSS, Chicago, IL). One-way analysis of variance (ANOVA) followed by pair-wise analysis was conducted to identify any significant differences between the growth of bacteria in the presence and absence of protein samples.

Preparation of Eggshell Sections and Immunofluorescence: Samples of dried eggshell were immersed in a solution of 0.5 M ethylenediaminetetraacetic acid (EDTA), 4% paraformaldehyde with continuous shaking. Solutions were changed daily until samples were sufficiently decalcified. After washing of samples with phosphate buffered saline, the shells were embedded in 2% agar at 45°C. Agar blocks were left to solidify at room temperature and processed using an automatic tissue processor (Triangular Biomedical Systems, Durham, USA). During processing, samples were completely dehydrated with ethanol (three washes), cleared with toluene (two washes) and impregnated with molten paraffin (four washes). Paraffin blocks were prepared with a tissue embedding centre (Leica Microsystems, Richmond Hill, Ontario) and oriented to show a cross section of the eggshell. Four micron thick sections were cut with a manual microtome and mounted onto glass slides. Slides used for histological examination were stained with hematoxylin and eosin. Immunofluorescence was preformed as described by Hincke et al., (2000). Sections were dehydrated for 24 hours at 37°C. After de-waxing three times with xylene, sections were rehydrated through an ethanol series, immersed in distilled water and incubated in TBS (100 mM Tris/HCl pH 7.7, 0.15 M NaCl) with anti-serum (1: 100) raised against *Gallus gallus* egg white c-type lysozyme, ovalbumin or ovotransferrin. Primary anti-serum was omitted for control sections. After washing with TBS, sections were incubated 2 hrs at room temperature with the secondary antibody (anti-rabbit IgG–CY3, 1: 400, Sigma-Aldrich, Oakville, ON) and immunoreactivity was visualized by fluorescence microscopy.

Determination of Lysozyme Activity of Eggshell Cuticle and Outer Eggshell Extracts: Lysozyme activity of cuticle and outer eggshell extracts was evaluated by measurement of enzymatic activity against a *Micrococcus* cell wall suspension (10 mM sodium phosphate buffer, pH 7.3) according to the method described by Wellman-Labadie et al., (2008c).

Lysozyme Activity of Total Eggshell Protein Extracts: Eggs were rinsed with deionised water and dried at room temperature. The yolks and albumen were discarded after cracking eggs open. Membranes were removed and eggshells were washed thoroughly with deionised water before drying 48 hours at room temperature. Dried eggshell (50 grams) was pulverised into a fine powder and totally decalcified by immersion in 20% acetic acid (10 ml acid/gram eggshell) at 4°C with gentle stirring for 24 hours according to the method of Reyes-Grajeda et al., (2002). The resulting solution was centrifuged (22 000 g, 1 hour) and the supernatant dialyzed six times against a 10-fold volume of deionised water at 4°C. Resulting samples were lyophilized and analysed for c-type lysozyme by western blotting as described in the previous section. Lysozyme enzymatic activity was evaluated at pH conditions corresponding to the pH optimums of g-type (pH 5.3) and c-type (pH 7.3) lysozyme.

Results:

Extraction of Cuticle and Outer Eggshell Proteins: Various extraction methods and treatment times to solubilise proteins from the surface of the eggshell, as well as to liberate proteins incorporated into the outer eggshell matrix, were evaluated during preliminary studies using whole intact *Gallus gallus* eggs. Treatment with 8 M urea or 6 M Guanidine-HCl was found to extract proteins from the outer eggshell surface (i.e. the cuticle) without decalcification of the eggshell. Within the exposure periods of 15, 30 and 60 minutes tested, a treatment time of 60 min was found most suitable and provided a high protein yield. The SDS-PAGE profile of 6 M Guanidine-HCl extracts was identical to that obtained with 8 M urea treatment (data not shown), and the latter was adopted for economical reasons. Urea treatment removed less than 2.5% of eggshell mass in all four species (Table 1). The percentage of eggshell mass removed by urea varied across species ($p < 0.001$) Canada goose showed the greatest removal of eggshell mass

by urea treatment followed by mute swan, wood duck and hooded merganser (Table 1) with all species being significantly different ($p < 0.001$) from the others as confirmed by pair-wise analysis.

Solutions of 0.25, 0.5 and 1.0 M EDTA, 50 mM Tris/HCl pH 7.5, using treatment times of 15, 30 and 60 minutes, were used to partially decalcify outer eggshell and release proteins trapped within the outer eggshell matrix. Analysis of this material by SDS-PAGE indicated that a treatment time of 60 min using 0.5 M EDTA was suitable (data not shown). However, following lyophilization, it was observed that EDTA had not been completely removed by dialysis. Wheeler et al., (1987; 1988) also observed that EDTA is difficult to remove by conventional methods since it interacts with proteins and remains associated even after relatively extensive dialysis. This contaminant interfered with our BCA protein assay as well as the antimicrobial assay. Alternatively, treatment with 1 N HCl, as used by Hincke et al., (2000; 2003) to selectively dissolve eggshell, was suitable for liberation of proteins trapped within the outer eggshell matrix. A treatment time of 5 min with 1 N HCl gave high protein yield and selective dissolution of the outer eggshell. Hincke et al., (2003) reported that in 15% of *Gallus gallus* eggs, 1 N HCl treatment resulted in very rapid dissolution from small zones which completely penetrated the shell and contaminated the extracts with egg white proteins. For this reason, eggs were individually treated and egg white contaminated extracts were discarded.

During this study, 1 N HCl treatment was found to remove a significantly greater percentage of eggshell mass than 8 M urea, since urea treatment only solubilised proteins of the cuticle. For this reason, SDS-PAGE profiles of HCl extracts containing both outer eggshell and cuticle proteins differed from urea extracts (Figure 1). The percentage of eggshell removed by both HCl and urea treatment was found to vary across the species. Pair-wise analysis revealed that HCl treatment removed a similar percentage of eggshell mass ($p > 0.05$) from Canada goose and hooded merganser eggs (Table 1), but this was a significantly lower amount ($p < 0.001$) than in the other species (Table 1). Analysis of wood duck eggs demonstrated the highest percentage of eggshell mass decrease which differed significantly ($p < 0.001$) from that of mute swan eggs (Table 1). The eggshell thickness of eggs was measured using a micrometer and revealed that mute swan possessed the greatest eggshell thickness (0.759 ± 0.03 mm, $N = 11$), which differed significantly ($p < 0.001$) from the other species tested. Canada goose (0.542 ± 0.04 mm,

N = 20) and hooded merganser (0.534 ± 0.03 mm, N = 6) showed an intermediate eggshell thicknesses which did not differ significantly ($p > 0.05$) between these species. The wood duck (0.306 ± 0.02 mm, N =7) demonstrated the thinnest shells of the four species tested ($p < 0.001$). Since HCl exposure periods were the same for all species, less material relative to total shell weight was removed from species with greater eggshell thickness than that of chicken which was used as a model to develop the extraction protocols. This appears to be supported by the roughly negative relationship observed between the percentage of eggshell mass removed during HCl treatment and eggshell thickness.

Antimicrobial Activity of Eggshell Extracts: Cuticle and outer eggshell protein extracts were evaluated for their ability to inhibit the growth of Gram-positive and Gram-negative bacteria. The growth of both the Gram-positive *B. subtilis* and *S. aureus* was inhibited in the presence of wood duck HCl extract. *B. subtilis* and *S. aureus* growth was also completely inhibited in the presence of hooded merganser urea extract (Figure 2). In addition, *S. aureus* growth was inhibited in the presence of mute swan urea and hooded merganser HCl extracts while *B. subtilis* growth was inhibited in the presence of Canada goose HCl extract (Figure 2). Growth of both the Gram-negative *P. aeruginosa* and *E. coli* D31 was inhibited in the presence of hooded merganser urea extract (Figure 2). *P. aeruginosa* growth was also inhibited in the presence of Canada goose urea extract while *E. coli* D31 growth was inhibited in the presence of mute swan urea extract (Figure 2).

SDS-PAGE Analysis of Eggshell Protein Extracts: Protein samples were separated on a 12% polyacrylamide gel and visualized by Coomassie blue staining. Protein bands of approximately 14, 18 and 32 kDa were shared across all extracts and species (Figure 1). Proteins present within the urea extracts did not appear to vary significantly across species although the band intensities varied considerably. HCl extracts also demonstrated a similar pattern across the selected species although the protein migration profiles of urea extracts varied considerably from those of the HCl extracts (Figure 1).

Western Blot Analysis of Eggshell Protein Extracts: The presence of eggshell matrix-specific proteins as well as egg white proteins within the extracts was evaluated by western blotting. The 32 kDa band visualized during SDS-PAGE in all extracts was found to react with antibodies raised against chicken ovocalyxin-32 (Figure 3). Western

blotting revealed ovotransferrin immunoreactivity in all extracts; wood duck HCl extract demonstrated the most intense reaction (Figure 3). Ovalbumin was only detected in wood duck urea and HCl extracts while ovocalyxin-36 was absent from all the extracts (Figure 3). The 14 kDa band visible in all extracts during SDS-PAGE was immunoreactive with antibody for c-type lysozyme (Figure 3).

Eggshell Immunofluorescence: Within the eggshell sections, the mammillary layer and eggshell membrane of all species were immunoreactive for ovalbumin and ovotransferrin as well as c-type lysozyme (Figure 4). Ovalbumin immunoreactivity was detected within the outer eggshell matrix of Canada goose, mute swan, wood duck and hooded merganser eggs, while this was not observed in chicken eggs (Figure 4). In the chicken outer eggshell matrix, c-type lysozyme immunoreactivity appeared to be concentrated to the cuticle while in Canada goose, mute swan and hooded merganser, c-type lysozyme immunoreactivity was homogenously distributed throughout the outer eggshell matrix. In Canada goose and mute swan eggshells, ovotransferrin immunoreactivity was detected within the outer eggshell matrix while this was not detected in the other species (Figure 4).

Lysozyme Activity of Cuticle, Outer Eggshell and Total Eggshell Protein Extracts: Lysozyme is a potent antimicrobial and lysozyme immunoreactivity was detected in all extracts and eggshell sections. Therefore, lysozyme enzymatic activity was measured using a bioassay developed by Liao et al., (2001). A standard curve, constructed using purified chicken egg white lysozyme, was used to convert lysozyme activity into a percentage of total protein for each sample (Table 2). The minimum detection limit of lysozyme activity was approximately 11 μg/ml for this assay, and lysozyme concentrations as low as 1-2 μg/ml demonstrate potent antimicrobial activity against *B. subtilis* (Wellman-Labadie et al., 2008a). Due to the limited amounts of sample material available we were unable to assay the lysozyme activity of the hooded merganser urea extract as well as the mute swan and wood duck HCl extracts; all other urea and HCl extracts demonstrated lysozyme activity, with hooded merganser urea extract containing the highest lysozyme activity and mute swan urea the least (Table 2). C-type lysozyme was also detected in all total eggshell extracts during western blotting (Figure 5). Evaluation of the lysozyme activity of the total eggshell extracts at different pH's revealed that lysozyme activity at pH 7.3 did not differ from that measured at pH 5.3

(Table 2). Under both conditions, hooded merganser total eggshell extract demonstrated the highest lysozyme content followed by wood duck, Canada goose and mute swan eggshell extracts (Table 2).

Discussion:

The avian eggshell cuticle represents an accessible protective compartment of the eggshell, composed of glycoproteins, polysaccharides, hydroxyapatite and lipids (Whittow 2000, Fernandez et al., 2001), residing directly in contact with the environment at a strategic location for antimicrobial defence. Therefore, we have hypothesized that one or more of the cuticle and outer eggshell components would possess antimicrobial activity. During this study, outer eggshell protein as well as eggshell cuticle protein from four wild avian species was investigated. Hincke et al., (2000) previously used brief 1 N HCl treatment of eggs, as conducted in this experiment, to demonstrate the presence of c-type lysozyme within the cuticle and outer eggshell of the chicken, *Gallus gallus*. A similar approach was used to demonstrate that ovocalyxin-32, a chicken eggshell matrix protein, is associated with the cuticle and outer eggshell (Hincke et al., 2003).

At 50 μg/ml, extracts obtained by urea and HCl treatments affected both Gram-positive and Gram-negative bacteria. Urea extract from hooded merganser inhibited all bacteria tested, mute swan urea extract inhibited both *S. aureus* and *E. coli* D31 while Canada goose urea extract inhibited *P. aeruginosa*. HCl extract of wood duck inhibited both *S. aureus* and *B. subtilis*, hooded merganser HCl extract inhibited *S. aureus* while Canada goose HCl extract inhibited *B. subtilis*. The detection of antimicrobial activity with protein samples extracted from both the cuticle and outer eggshell suggests that the outer region of the eggshell plays a role in antimicrobial defence. Most protein extracts were active against Gram-positive bacteria indicating that avian eggshell defences may have evolved to effectively defend against this spectrum of bacteria. Seviour and Board (1972a) demonstrated that 92% of eggshell surface contaminants in duck hatcheries were Gram-positive micro-organisms. Environmental pressures may have therefore selected for cuticle and outer eggshell proteins possessing antimicrobial activity against a wide spectrum of Gram-positive bacteria.

Kern and Cowie (1995) demonstrated that nests in cavities or burrows have a higher relative humidity than open-top nests. The eggs of the cavity-nesting wood duck and hooded merganser may therefore be exposed to higher levels of humidity compared to the eggs of the open-top nesting Canada goose and mute swan. Messens et al., (2005) noted that the level of bacterial penetration into egg contents increased with increasing temperature and relative humidity. The most active protein extract was obtained by urea treatment of hooded merganser eggs. The presence of highly potent eggshell cuticle antimicrobial proteins may be a species-specific strategy of the hooded merganser since comparable activity was not observed in the cavity-nesting wood duck. On the other hand, the presence of high lysozyme levels and high enzymatic activity in albumen of the wood duck (Wellman-Labadie et al., 2008b) could be an alternative strategy limiting microbial contamination of eggs in this cavity-nesting species.

Ovocalyxin-32 (OCX-32) was detected in all extracts from all species. This eggshell matrix protein has been shown to share properties with latexin, a carboxypeptidase A inhibitor of the rat and TIG1, a skin protein (Hincke et al., 2003, Gautron et al., 2001a). Protease inhibitors, such as the egg white protein ovomacroglobulin, inhibit microbial proteases and demonstrate antimicrobial activity (Molla et al., 1987). Recombinant *Gallus gallus* ovocalyxin-32 demonstrated protease inhibition, as well as antimicrobial activity against *B. subtilis* (Xing et al., 2007). Although native OCX-32 may differ from recombinant OCX-32, the evidence available suggests that this protein is involved in the antimicrobial activity of the avian eggshell.

Ovotransferrin was detected in low levels in all eggshell extracts except for wood duck HCl which showed higher levels. The wood duck extracts showed the highest percentage of dissolved eggshell (Table 1) since the urea and HCl treatment periods were probably excessive relative to the thin wood duck eggshell thickness. This is supported by the detection of ovalbumin, only in the wood duck urea and HCl extracts. Ovalbumin is mainly localized to the mammillary bodies of the chicken eggshell matrix as indicated in Figure 4 and previously reported by Hincke (1995). The low ovotransferrin levels detected in the other extracts indicate that ovotransferrin is not a major component of the eggshell cuticle and outer eggshell, but is rather found deeper within the eggshell matrix as described for the *Gallus gallus* eggshell (Gautron et al., 2001b; Nys et al., 2001). This is also supported by ovotransferrin immunoreactivity which was predominately detected

within the mammillary layer of the avian eggshell sections. Von Hunolstein et al., (1992) demonstrated inhibition of bacteria by transferrins, such as ovotransferrin, at concentrations of 1 mg/ml and higher. The low levels detected in the extracts may nonetheless contribute to antimicrobial defence since contaminating microbial loads are usually low, and proteins may be concentrated in specific locations. Valenti et al., (1982) reported that ovotransferrin immobilized on Sepharose 4B maintains its iron-binding capacity and its bacteriostatic activity. Therefore, eggshell bound ovotransferrin may retain its bacteriostatic iron-binding properties and provide some antimicrobial defence to the developing embryo.

C-type lysozyme was detected in all extracts by western blotting and is likely to play a role in the antimicrobial activity of the eggshell. Vadehra et al., (1972) demonstrated that lysozyme enzymatic activity could be extracted from the chicken eggshell matrix and suggested that this protein is involved in antimicrobial defence of the egg. During this study, we demonstrated the presence of c-type lysozyme in cuticle and outer eggshell protein extracts of all species investigated. In addition, lysozyme activity roughly correlating with c-type lysozyme immunoreactivity was detected in the samples that could be tested. Total decalcification of eggshell from the four species and analysis by western blotting also revealed the presence of c-type lysozyme within eggshell of all species tested which correlated with lysozyme activity.

Total eggshell extracts demonstrated lysozyme activity which did not differ significantly between pH 5.3 and pH 7.3. Using the same method, domestic goose albumen was previously demonstrated to lose approximately 90% of its activity at pH 7.3 compared to that observed at pH 5.3 while domestic chicken albumen maintained maximal lysozyme activity under both conditions (Wellman-Labadie et al., 2008b). Pooart et al., (2005) reported a pH optimum of 5.5-6.0 for goose and ostrich g-type lysozyme. Canfield and McMurry (1967) reported that the enzymatic activity of goose egg white lysozyme is three times greater than an equimolar amount of chicken egg white lysozyme at pH 6.2. In contrast, chicken c-type lysozyme possesses a broad pH range of 5.5 to 7.5 (Thammasirirak et al., 2001). Our results therefore suggest that g-type lysozyme is absent from the cuticle and outer eggshell extracts, although this could not be confirmed immunochemically due to the unavailability of g-type lysozyme antibodies. In contrast to g-type lysozyme, c-type lysozyme demonstrates potent chitinase activity (Hindenburg et

al., 1974). Chicken egg white lysozyme causes a rapid loss of viability among multiple *Candida albicans* isolates (Samaranayake et al., 2001). The protease and glycolytic activity of *Bacillus* spp., as well as that of fungi, can break down the eggshell cuticle and facilitate microbial invasion by radically increasing the number of open pores (Cook et al., 2003; Baggott and Graeme-Cook, 2002). A reduction in loads of pioneer micro-organisms, which possess the enzymes to destroy the cuticle, would maintain the structural integrity of the egg and prevent entry of micro-organisms. Sparks and Board (1984) noted that chicken eggs lacking a cuticle readily absorbed water and that newly laid fowl eggs, where the cuticle was incomplete, were less able to resist bacterial penetration of the shell. C-type lysozyme within the eggshell cuticle and outer eggshell may therefore reduce microbial contamination.

The importance of c-type lysozyme in antimicrobial defence is well represented by its distribution in the avian egg. In species such as the domestic chicken and the wood duck, high levels of c-type lysozyme are present in the albumen. This is visualized in the eggshell sections of these two species by the high c-type lysozyme immunoreactivity within the inner eggshell membrane; a structure directly in contact with the albumen. Alternatively, in Canada goose and mute swan; two species which contain little c-type lysozyme in their albumen, immunoreactivity to c-type lysozyme is homogeneously distributed throughout the outer eggshell matrix. High c-type lysozyme levels in albumen or in eggshell may therefore represent alternative species-specific antimicrobial defence strategies. In any event, the consistent presence of c-type lysozyme, in either eggshell or albumen, implies an essential role for this enzyme in the microbial defences of avian eggs.

C-type lysozyme immobilized on a variety of solid supports has been reported to retain its enzymatic, bacterial cell wall degrading properties (Crapasi et al., 1993; Wu and Daeschel, 2007; Wellman-Labadie et al., 2008c). Hence, lysozyme within the avian eggshell is likely to be enzymatically active as an *in situ* antimicrobial defence. Antimicrobial proteins bound within the cuticle and outer eggshell may also be solubilised and released during microbial contamination. Fungi, as well as some bacteria, produce enzymes capable of degrading the eggshell cuticle (Cook et al., 2003; Baggott and Graeme-Cook, 2002). As a result of carbohydrate fermentation and bacterial oxidation, many Gram-positive and Gram-negative bacteria produce acid metabolic

wastes (Murray et al., 2003). Such metabolites may partially dissolve eggshell calcium carbonate thereby solubilising cuticle and outer eggshell proteins. In addition, proteolytic digestion of egg white lysozyme, ovotransferrin and ovalbumin, which have been identified as components of the cuticle and outer eggshell, can generate peptides that possess novel bactericidal activity (Pellegrini et al., 2004; Ibrahim et al., 1998; Ibrahim et al., 2000; Ibrahim et al., 2001b).

In conclusion, we have demonstrated that the eggshell cuticle contains proteins including c-type lysozyme, ovotransferrin and an ovocalyxin-32-like protein which are present in a broad range of species and are likely to be involved in the antimicrobial defence of the egg. Although, antimicrobial proteins of the cuticle and outer eggshell are embedded within the eggshell matrix, bound proteins may retain their activity and multiple mechanisms may liberate and activate eggshell antimicrobials. In order to successfully reproduce within the humid environment of the cavity nest, species such as the hooded merganser may have evolved especially potent antimicrobial proteins present within the eggshell cuticle. In other species, such as the Canada goose and mute swan, outer eggshells with homogeneously distributed c-type lysozyme may reinforce the antimicrobial defensive capability of their albumen.

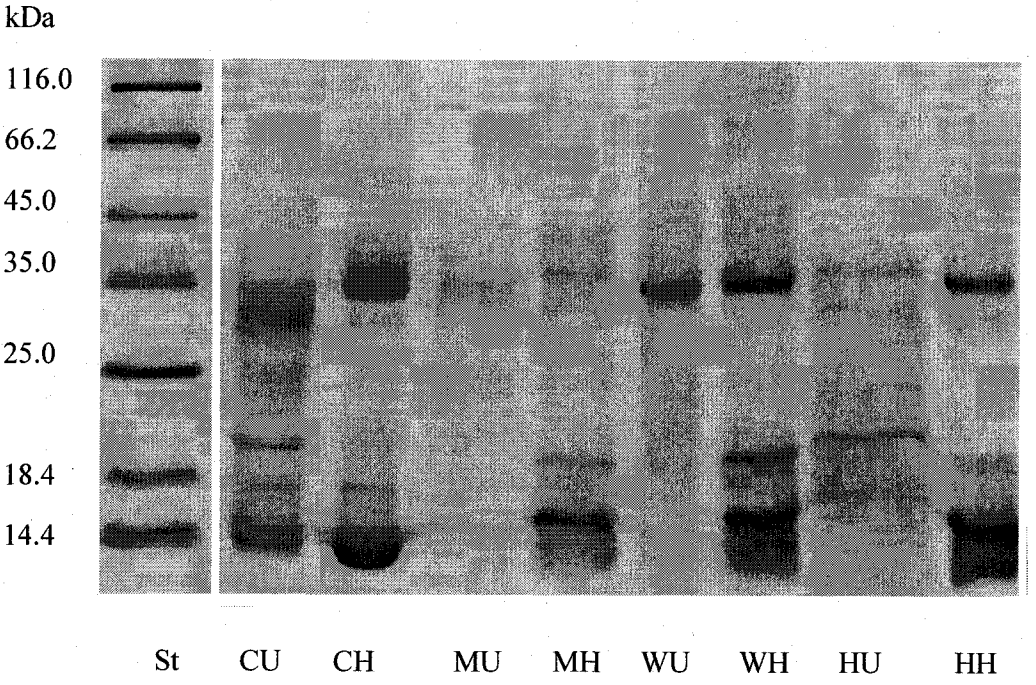

Figure 1: SDS-PAGE analysis of avian eggshell extracts. Protein samples (10 μg) were loaded into each well of a 12 % polyacrylamide gel and visualized by Coomassie blue staining. Molecular weight of standard (St) is indicated on the left. Samples are labelled on the bottom (CU-Canada goose urea extract, CH-Canada goose HCl extract, MU-mute swan urea extract, MH-mute swan HCl extract, WU-wood duck urea extract, WH-wood duck HCl extract, HU-hooded merganser urea extract, HH-hooded merganser HCl extract).

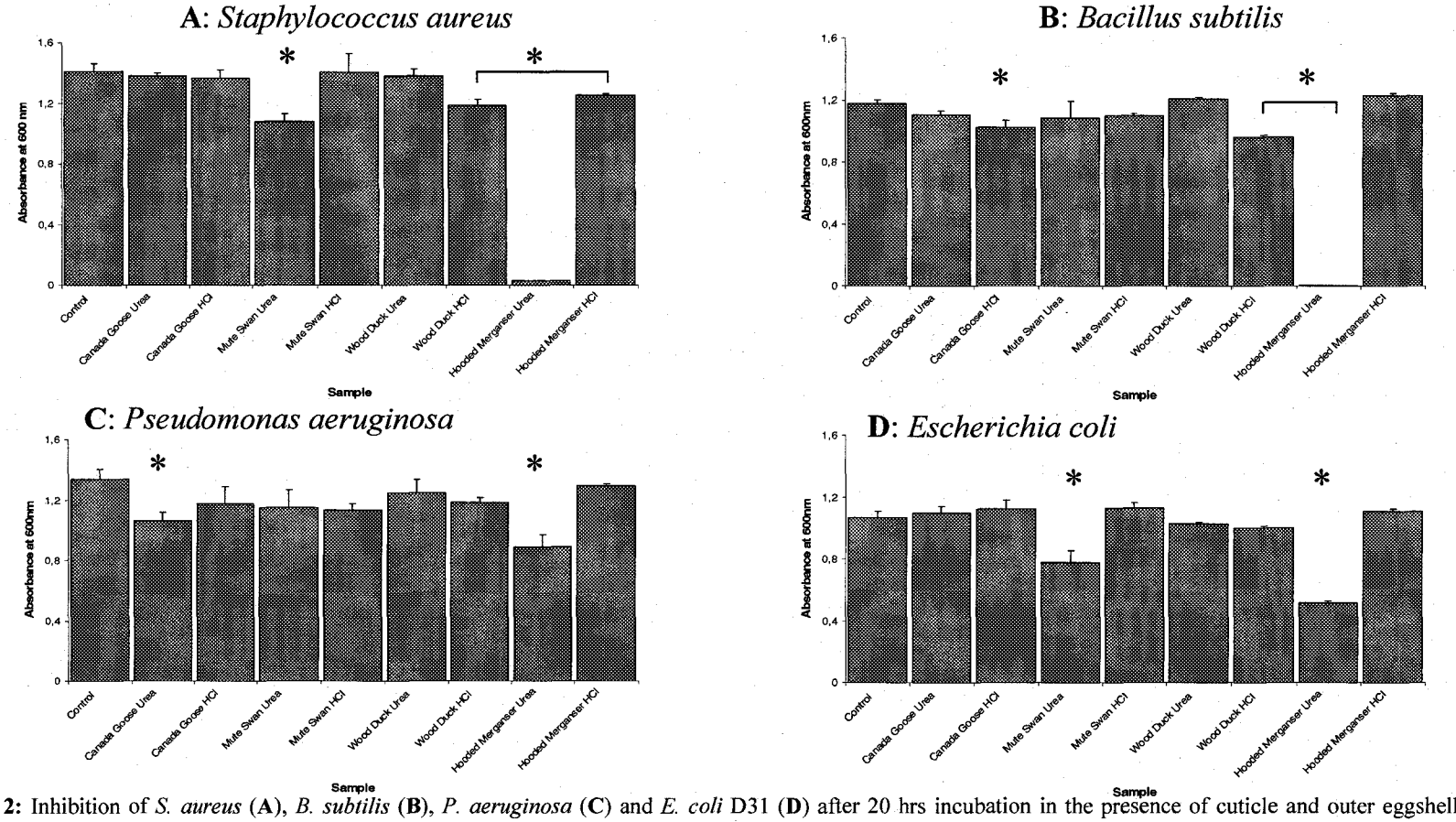

Figure 2: Inhibition of *S. aureus* (**A**), *B. subtilis* (**B**), *P. aeruginosa* (**C**) and *E. coli* D31 (**D**) after 20 hrs incubation in the presence of cuticle and outer eggshell protein extracts (50 µg/ml). Optical density at 600 nm ± standard error is indicated for two independent trials. Samples demonstrating significant inhibition of growth ($p < 0.05$) are indicated by an asterix (*).

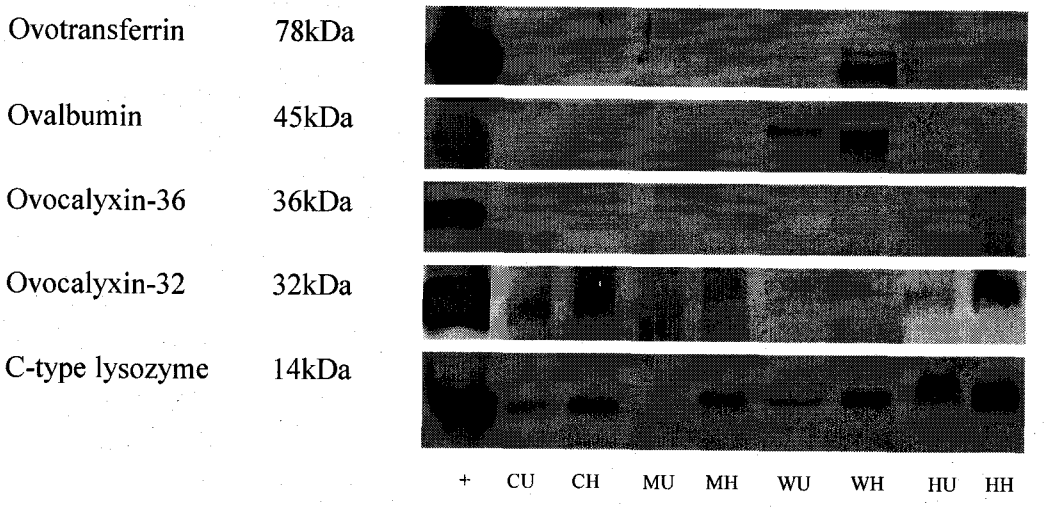

Figure 3: Western blot of avian eggshell protein extracted by 8 M urea 50 mM Tris-HCl pH 7.5 (60 min) or 1 N HCl (5 min) treatment. Purified chicken c-type lysozyme, ovotransferrin, ovalbumin and ovocalyxin-32 (0.1μg) was used as a positive control (+) and 1 μg of sample (10 μg for anti-ovocalyxin-32) was loaded in each well. Samples are labelled on the bottom (CU-Canada goose urea extract, CH-Canada goose HCl extract, MU-mute swan urea extract, MH-mute swan HCl extract WU-wood duck urea extract, WH-wood duck HCl extract, HU-hooded merganser urea extract, HH-hooded merganser HCl extract).

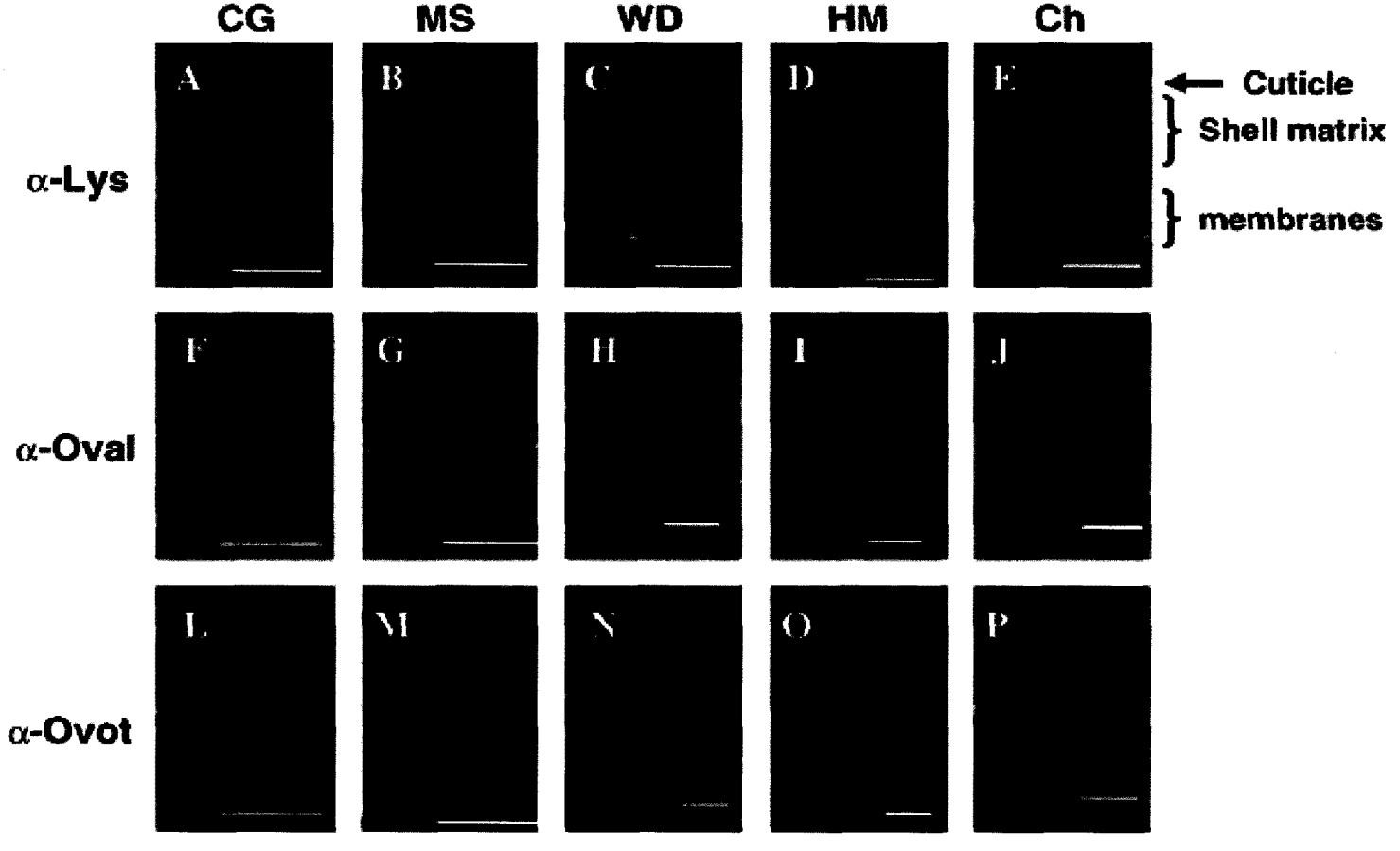

Figure 4: Immunofluorescence of decalcified avian eggshell matrix sections using anti-sera to purified chicken egg white c-type lysozyme (α-Lys), ovalbumin (α-Oval) and ovotransferrin (α-Ovot).

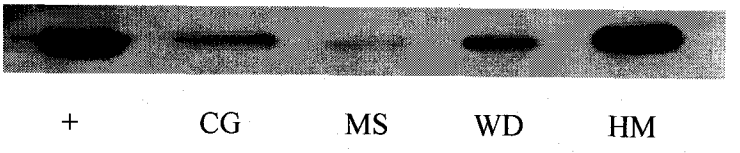

Figure 5: Western blotting of total eggshell protein extracts against c-type lysozyme. Chicken egg white c-type lysozyme (0.1μg) was used as a positive control (+) and 1 μg of sample was loaded into each well. Samples are labelled on the bottom (CG-Canada Goose, MS-Mute Swan, WD-Wood Duck, HM-Hooded Merganser).

Table 1: Eggshell mass loss during immersion of whole eggs in solutions of 8 M urea 50 mM Tris/HCl pH 7.5 and 1 N HCl. Loss of eggshell mass is expressed as percentage of total shell weight ± standard deviation. The number of nests sampled and the total number of eggs used for each treatment and species is indicated.

Species	Extraction Method	% Eggshell Dissolved ± Std. Dev.	No. Eggs	No. Nests Sampled
Canada Goose	8 M Urea 50 mM Tris-HCl pH 7.5	2.22 ± 0.17	7	7
Canada Goose	1 N HCl	3.73 ± 1.07	7	7
Mute Swan	8 M Urea 50 mM Tris-HCl pH 7.5	1.43 ± 0.16	7	7
Mute Swan	1 N HCl	10.54 ± 2.38	7	7
Wood Duck	8 M Urea 50 mM Tris-HCl pH 7.5	0.83 ± 0.42	30	11
Wood Duck	1 N HCl	15.27 ± 5.56	30	11
Hooded Merganser	8 M Urea 50 mM Tris-HCl pH 7.5	0.42 ± 0.09	12	5
Hooded Merganser	1 N HCl	2.56 ± 0.37	12	5

Table 2: Determination of lysozyme activity of avian cuticle (urea extract), outer eggshell (HCl extract) and total eggshell protein extracts by measurement of enzymatic activity against a *M. lysodeikticus* cell wall suspension in 10 mM sodium phosphate buffer (pH 5.3 or 7.3). Experiment was conducted on two separate occasions. Lysozyme content is expressed as a percentage (± standard deviation) of total protein and indicated for each species and extract.

Species	Extract	% Lysozyme ± Standard Deviation		Ratio
		pH 7.3	pH 5.3	pH 5.3 / 7.3
Canada Goose	Urea	0.109 ± 0.03		
	HCl	0.143 ± 0.05		
	Total Eggshell	0.334 ± 0.02	0.295 ± 0.05	0.9
Mute Swan	Urea	0.055 ± 0.02		
	HCl	---		
	Total Eggshell	0.095 ± 0.01	0.087 ± 0.00	0.9
Wood Duck	Urea	---		
	HCl	0.16 ± 0.03		
	Total Eggshell	0.690 ± 0.05	0.695 ± 0.02	1.0
Hooded Merganser	Urea	0.452 ± 0.06		
	HCl	---		
	Total Eggshell	1.021 ± 0.16	1.118 ± 0.03	1.1

Section III: Chapter 6

The previous two chapters of section three (Chapters 4 and 5), have investigated the antimicrobial activity of water-soluble outer eggshell and cuticle extracts from both domestic and wild avian species. These investigations have demonstrated that the protein content and antimicrobial activity of the avian eggshell may be influenced by phylogeny as well as environmental pressures.

In addition to water-soluble components, the avian eggshell also contains insoluble, lipophilic components. Lipophilicity is an important characteristic for antimicrobial activity, and lipophilic antimicrobial components have been identified in both plants and animals. In the following chapter (Chapter 6), the antimicrobial activity of lipophilic outer eggshell components from domestic avian species were investigated. This chapter is the result of entirely my experimental work.

Chapter 6: Chicken Outer Eggshell Surface Lipophilic Components Possess Antimicrobial Activity

Olivier Wellman-Labadie, Simon Lemaire, Jaroslav Picman and Maxwell T. Hincke

Abstract:

The eggshell cuticle is the outermost layer of the egg and is thought to play a role in the regulation of gas exchanges as well as in the antimicrobial defence of the avian egg. In this study, we report that ethyl acetate extracts from chicken (*Gallus gallus*) and goose (*Anser anser*) outer eggshell surface demonstrate antimicrobial activity against *Bacillus subtilis* and *Escherchia coli* D31. Using chicken eggs, an active fraction was purified by RP-HPLC. The crude and purified eggshell surface extracts were active against *B. subtilis*, *Staphylococcus aureus* and *E. coli* D31 while chicken ethyl acetate extracts from albumen as well as from fecal and uropygial gland material did not demonstrate this spectrum of activity. The results suggest that lipophilic eggshell surface components are involved in antimicrobial defence of the egg and are incorporated into the egg during eggshell formation. This represents the first study to demonstrate that lipophilic extracts from the outer eggshell of the domestic hen possess antimicrobial activity.

Introduction:

The eggshell cuticle is an organic layer deposited on the eggshell surface prior to ovoposition. This layer is also known as "bloom" since it gives the freshly laid egg a glossy appearance (Burley and Vadehra, 1989). At oviposition, the chicken eggshell cuticle has a moist lustre which disappears within 2-3 minutes and cannot be restored (Sparks, 1994). The cuticle, as well as the outer portion of the calcified shell, contains the eggshell pigments which serve as camouflage, temperature control and possibly in parental recognition (Sparks, 1994).

The function of the cuticle remains unclear. It is possible that the cuticle plays a role in controlling water exchanges by repelling water, preventing its loss, or may even function in preventing microbial attack (Whittow, 2000). It has been proposed that the cuticle limits water entering the pore canals and consequently reduces movement of bacteria into the egg (Sparks, 1994; Chavez et al., 2002; Baggott and Graeme-Cook, 2002). In mollusc

eggs, the cuticle has been reported to demonstrate antimicrobial activity (Benkendorff et al., 2004). The waxy tick cuticle has been reported to demonstrate antimicrobial activity and may also play a role in protecting the eggs from desiccation (Arrieta et al., 2006; Lees and Beament 1948, Schol et al., 2001).

The avian eggshell cuticle is a relatively thin layer of variable thickness (0.5 to 12.8 μm) composed of glycoprotein, polysaccharides, hydroxyapatite crystals and lipids (Whittow. 2000; Fernandez et al., 2001). Proteins known for their antimicrobial properties, such as lysozyme and ovotransferrin, have been identified within the chicken eggshell surface by Hincke et al., (2000) and Gautron et al., (1997a). Mine et al., (2003) demonstrated that chicken eggshell protein extracts possessed antimicrobial activity. Mann et al., (2006) identified low levels of gallinacin-8 and β-defensin 11 as components of the chicken eggshell matrix using MS-based technology.

Lipophilicity is closely associated with permeation of bacterial membranes and is an important parameter in the development of antibacterial agents (Tokuyana et al., 2001). However, no studies have focused on avian lipophilic eggshell components or on lipophilic eggshell cuticle components as potential sources of antimicrobial agents. In addition, the origin of the components present on the eggshell surface remains unclear. Cuticular components may be incorporated during eggshell formation but may also be subsequently applied to the egg after oviposition. Deeming (1987) reported that Muscovy ducks, as is the case with most ducks, often smear eggs with preening oil.

In this study, the lipophilic components of the eggshell cuticle from domestic chicken (*Gallus gallus*), duck (*Anas platyrhynchos*) and goose (*Anser anser*) were investigated for antimicrobial activity. This is the first study to successfully extract and demonstrate the presence of lipophilic antimicrobial agents on the outer surface of the avian egg.

Materials and Methods:

Ethyl Acetate Extraction: Eggs of the domestic chicken, *Gallus gallus*, the domestic duck, *Anas platyrhynchos*, and the domestic goose, *Anser anser*, were obtained from a local farm in Perth (Ontario, Canada). Unwashed chicken (25), duck (25) and goose (16) eggs were immersed in ethyl acetate for 1 hour. The extracts were filtered using

Whatman No. 3 filter paper and concentrated by rotary evaporation at 25 °C. Approximately 10 mg of a concentrated oily extract was obtained from each species.

Antimicrobial Activity of Ethyl Acetate Extracts: Antimicrobial activity of ethyl acetate extracts was evaluated using an assay adapted from the radial diffusion assay of Steinberg and Lehrer (1997). Bacteria were grown to log phase in Luria-Burtani broth (Bioshop, Burlington, ON) and concentrated by centrifugation. Pellet was washed and resuspended in 10 mM sodium phosphate buffer (pH 7.3) and further diluted. Molten culture medium (1.5 % low EEO agarose, 1 % biotryptone, 0.5 % yeast extract; Bioshop, Burlington, ON) was prepared in 10 mM sodium phosphate buffer. After cooling to 42 °C, culture medium was inoculated with bacteria to approximately 1 x 10^5 CFU's/ml. Plates were left to solidify at room temperature. A 10 mg sample of crude extract from each bird species was suspended in 1 ml of ethyl acetate. A sample (10 µl) of each extract was applied to Whatman No. 1 paper discs (6 mm diameter). After letting the solvent evaporate for ~1 hour at room temperature, discs were applied to bacterial plates. Discs treated with ethyl acetate or 10 mg/ml kanamycin in 70 % ethanol were used as negative and positive controls. Following the application of sample discs, plates were incubated at 37 °C for 6-18 hours. Bacterial plates were stained using 0.02 mg/ml Coomassie blue 27 % methanol 15 % formaldehyde according to the method of Silphaduang et al., (2006). Plates were photographed and diameter of clear zones measured. Antimicrobial activity was evaluated against *Bacillus subtilis* ATCC 19659 and *Escherichia coli* D31.

Identification of Active Fraction within the Chicken Ethyl Acetate Extract: A sample of 350 chicken eggs was treated with ethyl acetate, as described in the previous section, in order to obtain enough material for in-depth investigation. Approximately 230 mg of concentrated extract was obtained. Extract was resuspended in 1 ml of ethyl acetate and 5 µl aliquots were evaluated for antimicrobial activity against two Gram-positive (*Bacillus subtilis* ATCC 19659, *Staphylococcus aureus* ATCC 6538) and two Gram-negative (*Pseudomonas aeruginosa* ATCC 15442 or *Escherichia coli* D31) bacteria.

A sample of the crude extract was solubilized in methanol and further diluted with 15 % acetonitrile 0.1 % trifluoroacetic acid. Sample was fractionated on a C_{18} column (25 x

200 mm μ-Bondapak C18, Waters) by reverse phase high pressure liquid chromatography using an acetonitrile gradient. Elution of extract components at 4 ml/min was monitored spectrophotometrically at 240 nm and fractions individually collected every minute. Fractions were dried at 25 °C using an Eppendorf vacufuge concentrator (Fisher Scientific, Mississauga, Ontario) and evaluated for antimicrobial activity against *B. subtilis*.

Identification of Active Agent(s) from the Purified Chicken Ethyl Acetate Fraction: Fractions identified as possessing activity were pooled. A small sample of the purified fraction as well as the crude extract was individually suspended in 4 % sodium dodecyl sulphate 25 % glycerol 1.5 % Tris-HCl pH 6.8. Bromophenol blue (0.125 mg/ml) and 1, 4-dithiothreitol (7.7 mg/ml) was added to the samples before heating (5 min at 90 °C) and loading. A molecular weight marker (MBI Fermentas, Burlington, ON) was also loaded. SDS-PAGE was carried out on a 20 % polyacrylamide gel and visualized by Coomassie Blue staining. After photography of the gel, bands of interest within the purified fraction were excised and sent for peptide sequencing at the Centre de Séquençage de Peptides de l'Est du Québec (CRCHUL, Québec, QC). A sample of the purified fraction was also sent for ESI Mass spectroscopy analysis at the University of Ottawa without prior SDS-PAGE separation.

Investigation of Potential Sources for the Active Antimicrobial Agent: Antimicrobial agents present on the egg surface are likely to be incorporated during egg formation. Alternatively, the outer eggshell may come into contact with various surfaces and acquire material following ovoposition. For this reason, the antimicrobial activity of ethyl acetate extracts from chicken albumen, chicken uropygial gland material and chicken fecal material was investigated in order to determine if this activity corresponded to that of the eggshell surface ethyl acetate extracts.

Chicken Albumen:

A dozen chicken eggs were washed with running deionised water and cracked open. The egg whites were separated from the yolks and pooled. The pooled whites were homogenized by gentle shacking with a glass stirring bar. An equal volume of ethyl acetate was added to the egg whites and gently mixed by inversion at 4 °C. Resulting

solution was centrifuged (22 000 g, 4 °C, 30 min) and liquid phase was concentrated by evaporation. The extract was purified by RP-HPLC and analysed for antimicrobial activity as described above.

Uropygial Gland Material (Preen Oil):

Preen oil was collected from 12 laying hens at a commercial farm in Castleman (Ontario, Canada). Oil was extracted from hens by gentle massaging of the uropygial gland using a cotton swab as described by Reneerkens et al., (2005). Oil was extracted from swabs by immersion in ethyl acetate for 1 hour with gentle shacking. Sample was concentrated by evaporation, purified by RP-HPLC and analysed for antimicrobial activity as described in previous section.

Fecal Material:

Fecal material was obtained from a commercial laying hen flock in Castleman (Ontario, Canada). A 50 gram sample of fecal material was solubilized in 500 ml of ethyl acetate. Solution was filtered using Whatman # 3 filter paper and concentrated by evaporation at 25 °C. Fecal extract was purified by RP-HPLC and analysed for antimicrobial activity as described in previous section.

Results:

Antimicrobial Activity of Avian Egg Surface Ethyl Acetate Extracts: Through the immersion of whole eggs in ethyl acetate, lipophilic components were extracted from the surface of the eggs of domestic chicken, duck and goose. After concentrating the samples by evaporation at room temperature (22-25°C), a slightly yellow-greenish viscous material was obtained. In both chicken and goose, the crude extract was found to demonstrate antimicrobial activity against the Gram-positive *Bacillus subtilis* and the Gram-negative *Escherichia coli* D31 while the duck egg surface extract did not appear to possess this activity (Figure 1). The size of clear zones produced was similar in both the chicken and goose extracts with both species demonstrating greater antimicrobial activity against *B. subtilis* than against *E. coli* D31 (Figure 1, Table 1).

Purification of Active Fraction from Chicken Eggshell Surface Ethyl Acetate Extracts: In order to obtain sufficient material for subsequent experiments, an ethyl acetate extraction was conducted with a larger sample size. Extraction of lipophilic components from 350 chicken eggs resulted in approximately 230 mg of the viscous material. Analysis of the antimicrobial activity of a 2 fold serial dilution of the crude chicken eggshell surface ethyl acetate extract against *B. subtilis* revealed activity even after a 256 fold dilution (Figure 2).

The crude chicken extract was purified by RP-HPLC on a C_{18} column using an acetonitrile gradient. Spectrophotometric monitoring at 240 nm revealed over a dozen elution peaks during the period between 32 and 86 minutes corresponding to an acetonitrile concentration of 25-85 % (Figure 3). A major peak (at 66.953 minutes) was observed during the 20 minute period where the acetonitrile concentration was kept stable at 80 % (Figure 3).

During RP-HPLC purification, a fraction was collected every minute. Analysis of the antimicrobial activity of each individual fraction against *B. subtilis* revealed that fractions 66 and 67, corresponding to the major peak eluted at 66.953 minutes, were antimicrobially active (Figure 4a). SDS-PAGE analysis of this fraction revealed 4 bands after visualization by Coomassie blue staining (Figure 4b). Three low molecular weight bands (< 14 kDa) were visible as well as a band of ~17 kDa (Figure 4b). Coomassie blue stained bands of low molecular weight were visible in the SDS-PAGE profile of the crude extract but the ~17 kDa band was visible only in the purified extract (Figure 4b).

Identification of Potential Sources for the Chicken Eggshell Surface Antimicrobial Agent: In order to extract the antimicrobial agent from the eggshell surface, whole eggs were immersed in ethyl acetate. We were unable to determine the amount of material extracted from each egg since the eggs gained weight, rather than loosing mass, due to absorption of ethyl acetate by the albumen. In a few cases (N = 6), eggs absorbed enough ethyl acetate to cause the eggs to swell and the eggshell to crack. Such eggs as well as the resulting extracts were discarded.

Since ethyl acetate was able to penetrate the shell, albumen components may have inevitably been extracted during treatment. Therefore, albumen was treated with ethyl

acetate in order to extract potential antimicrobial agents. During the albumen extraction, ethyl acetate was found to react with the egg white and form a jelly-like material. A liquid phase was successfully extracted and was observed to be water and ethanol soluble but insoluble in ethyl acetate (data not shown). Antimicrobial analysis of this material failed to detect any antimicrobial activity (Figure 5) and the elution profile during RP-HPLC showed a marked contrast to that of the chicken eggshell surface extract (Figure 6). In agreement with the observed solubility of the crude albumen extract, the albumen extract was eluted at the beginning of the RP-HPLC purification (Figure 6) where the acetonitrile concentration was lowest (15 %).

The uropygial gland is a sebaceous gland which secretes a holocrine substance used to maintain feather condition that is spread over the plumage during preening. Preen oil, or uropygial gland oil, has been reported to demonstrate antimicrobial as well as antifungal activity (Bandyopadhyay and Bhattacharyya 1996; 1999; Shawkey et al., 2003). Deeming (1987) reported that Muscovy ducks often smear eggs with preening oil as is the case with most ducks. Since this substance may come into contact with the eggshell surface during incubation, *Gallus gallus* uropygial gland material was extracted and tested for antimicrobial activity.

During this study, analysis of the chicken uropygial gland material did not reveal any activity against *B. subtilis*, *S. aureus*, *E. coli* D31 or *P. aeruginosa* (Figure 5). The elution profile of the extract during RP-HPLC revealed over a dozen peaks although most of the extract was eluted at the beginning of the purification (Figure 6). A minor peak (at 61.219 minutes) was eluted within the 80 % acetonitrile zone (Figure 6) but did not demonstrate any antimicrobial activity (data not shown).

Jennes et al., (2000) reported that *Enterococcus gallinarum*, isolated from the intestinal tract of the ostrich, produced the bacteriocin enterocin 012. Since eggs may potentially come into contact with fecal material which may contain antimicrobial agents, ethyl acetate extracts of chicken fecal material were evaluated for antimicrobial activity. The chicken fecal ethyl acetate extract was found to demonstrate antimicrobial activity against *B. subtilis* and *S. aureus* while no activity was detected against the Gram-negative *E. coli* D31 or *P. aeruginosa* (Figure 5). In contrast to the fecal extract, the crude and purified chicken eggshell surface extracts were active against *E. coli* as well as

B. subtilis, and *S. aureus* (Figure 5). The crude and purified chicken eggshell surface extracts were equally active; demonstrating the most activity against *B. subtilis* while showing lower activity against *E. coli* followed by *S. aureus* (Figure 5, Table 2). In contrast, the fecal extract demonstrated similar activity against *B. subtilis* and *S. aureus* (Figure 5, Table 2). In addition, the majority of the fecal extract was eluted at the end of the RP-HPLC purification, near 90 % acetonitrile, and none of the fractions were antimicrobial.

Discussion:

In direct contact with the environment, the strategic location of the eggshell cuticle suggests a role in the antimicrobial defence of the avian egg. Hincke et al., (2000) as well as Gautron et al., (1997a) identified lysozyme and ovotransferrin, two egg white proteins known for antimicrobial activity, within the chicken eggshell matrix. Mann et al., (2006) identified low levels of gallinacin-8 and β-defensin 11 as components of the chicken eggshell matrix using MS-based technology. Mine et al., (2003) demonstrated that the acid-soluble protein fraction of demineralised chicken eggshell possessed antimicrobial activity. In this study, we demonstrate that the lipophilic components from the outer eggshell of the domestic chicken and goose possess antimicrobial activity.

In both the chicken and the goose, ethyl acetate outer eggshell extracts were antimicrobial against *B. subtilis*, *S. aureus* and *E. coli* D31. No such activity was detected within the duck extract. Fragmentation of the chicken, duck and goose extracts by thin layer chromatography using 20 % ethyl acetate and 80 % hexane followed by visualization with 2.5 % ammonium molybate 1 % cerium sulphate 10 % sulphuric acid revealed similar profiles for goose and chicken while the duck extract contained a significantly higher number of constituents (data not shown). The greater complexity of the duck extract may have in effect diluted the relative proportion of the active outer eggshell antimicrobial agent. The antimicrobial activity of the ethyl acetate extracts from the outer eggshell of the domestic chicken, goose and possibly duck may suggest that lipophilic outer eggshell components are involved in the antimicrobial defence of many and possibly all avian species. In the chicken, < 250 fold dilution of 5 µl of the crude extract obtained from ethyl acetate extraction of 350 eggs (~230 mg resuspended in 1 ml of ethyl acetate) revealed significant antimicrobial activity. The detection of activity,

even after such significant dilution, would suggest that the material present on a single egg could demonstrate activity and play a role in the antimicrobial defence of the egg.

Being present directly on the surface of the egg, the antimicrobial agent(s) may have been acquired from contact of eggs with other materials. During incubation, material may be transferred from the hen to the egg. The uropygial gland is located at the base of the tail and secrets a holocrine waxy substance, termed preen oil, which the bird spreads over its plumage during preening in order to maintain feather condition. Muscovy ducks, as is the case with most ducks, often smear eggs with preening oil (Deeming 1987). Bandyopadhyay and Bhattacharyya (1996; 1999) reported that secretions from the uropygial gland of the chicken inhibited the growth of bacterial and fungal species. Shawkey et al., (2003) reported that uropygial oil of the house finch, *Carpodacus mexicanus*, inhibited the growth of feather-degrading bacterial species. Martin-Platero et al., (2006) reported the characterisation of a bacteriocin demonstrating antimicrobial activity against both Gram-positive and Gram-negative bacteria from *Enterococcus faecalis* MRR 10-3 isolated from the uropygial gland of the hoopoe (*Upupa epops*). During this experiment, ethyl acetate extracts from uropygial gland material were not found to demonstrate antimicrobial activity. Never the less, the role of preen oil in antimicrobial defence remains unclear as reports have also indicated that this secretion is ineffective against some bacteria while stimulating the growth of others (Shawkey et al., 2003; Bandyopadhyay and Bhattacharyya 1996; 1999). Preen oil of most birds is composed mainly of monoesters while diesters were secreted by incubating hens (Reneerkens et al., 2005). Lipids, wax alcohols and acids as well as triglycerides and hydrocarbons have been attributed with the antimicrobial activity of chicken uropygial gland oil (Bandyopadhyay and Bhattacharyya 1996; 1999).

Fecal material of the hen is another material which may come into contact with the egg surface. Jennes et al., (2000) reported that *Enterococcus gallinarum* isolated from the ostrich intestinal tract secreted a bacteriocin. Gallinacin-3 expression has been reported in chicken large intestine by Zhao et al., (2001) as well as Townes et al., (2004). In this study, ethyl acetate extracts of chicken fecal material were found to be antimicrobial against *B. subtilis* and *S. aureus*. None the less, the antimicrobial spectrum of this extract did not correspond to that of the crude or purified chicken eggshell surface ethyl acetate extract. In addition, the elution profile of the fecal extract during RP-HPLC was

significantly different from that of the eggshell surface extract. The activity of the fecal extract was also lost during purification. As suggested by the elution of the majority of the fecal extract at high acetonitrile concentrations, the antimicrobial component of the fecal extract may have been retained by the column during purification. Therefore, it would appear that the chicken fecal material is not the source of the antimicrobial activity detected in the eggshell surface ethyl acetate extract.

During the chicken eggshell surface extractions, ethyl acetate was found to easily penetrate through the eggshell and may have therefore extracted components from the albumen. Avian egg white is well known for its ability to inhibit bacterial growth due to multiple antimicrobial proteins including lysozyme and ovotransferrin (Baggott and Graeme-Cook, 2002). In this study, ethyl acetate extracts of chicken albumen failed to demonstrate antimicrobial activity. In addition, the albumen extract, in contrast to the eggshell extract, was water-soluble and insoluble in ethyl acetate suggesting that albumen is not the source of the outer eggshell antimicrobial activity.

Following RP-HPLC purification, the active fraction from the chicken eggshell surface ethyl acetate extract was separated by SDS-PAGE. Coomassie blue staining revealed four bands with molecular weights below 20 kDa suggesting the presence of proteonaceous components. Silphaduang et al., (2006) recently reported that chicken oviduct and ovary extracts contained histone H1 as well as histone H2B. These two proteins were reported to demonstrate antimicrobial activity against *B. subtilis* and *E. coli* (Silphaduang et al., 2006). In similarity with the SDS-PAGE profile of the purified chicken eggshell surface ethyl acetate extract, histone H2B has a molecular weight of approximately 17 kDa. Histone H2B from the pacific white shrimp, *Litopenaeus vannamei*, is eluted at high acetonitrile concentrations during RP-HPLC purification (Patat et al., 2004). None the less, MALDI analysis of the four Coomassie blue stained bands excised from the SDS-PAGE gel of the purified chicken eggshell surface ethyl acetate fraction obtained during this study failed to demonstrate hits with any protein databases suggesting the presence of undetectable proteins or non-proteinaceous components. Coomassie blue staining has also been reported to stain lipids (Abe, 1998; Nakamura et al., 1996; Nakamura and Handa, 1984). LC trace ESI Mass spec analysis of the purified eggshell surface ethyl acetate extract revealed significant background as expected from samples obtained with a time-based RP-HPLC fractionation. None the

less, peaks of interest were found in the 600-1000 region. High m/z peaks were detected within this region from successive trials (m/z of 722.6 and m/z of 766.8). CID mass spectrum of these peaks revealed identical mass fragments below 340 suggesting that the compounds within the 600-1000 region share a common molecular skeleton with side chains of variable size. Under NH^{4+} ionization, a distribution of peaks separated by 14 amu was detected as would be expected with a fatty acid.

In conclusion, this is the first study to report that lipophilic components of the outer eggshell of domestic chicken, goose and possibly duck demonstrate antimicrobial activity and could contribute to antimicrobial defence of the avian embryo. A partially purified ethyl acetate extract was obtained from chicken outer eggshell surface and demonstrated a wide spectrum of antimicrobial activity affecting both Gram-positive and Gram-negative bacteria. Antimicrobial activity as well as the elution profile at 240 nm obtained during RP-HPLC purification of the chicken eggshell surface ethyl acetate extract differed significantly from that of the albumen, fecal material and uropygial gland material ethyl acetate extracts. The data presented suggests that lipophilic antimicrobial components are incorporated into the outer eggshell surface during egg synthesis and enhance the antimicrobial defences of the avian egg.

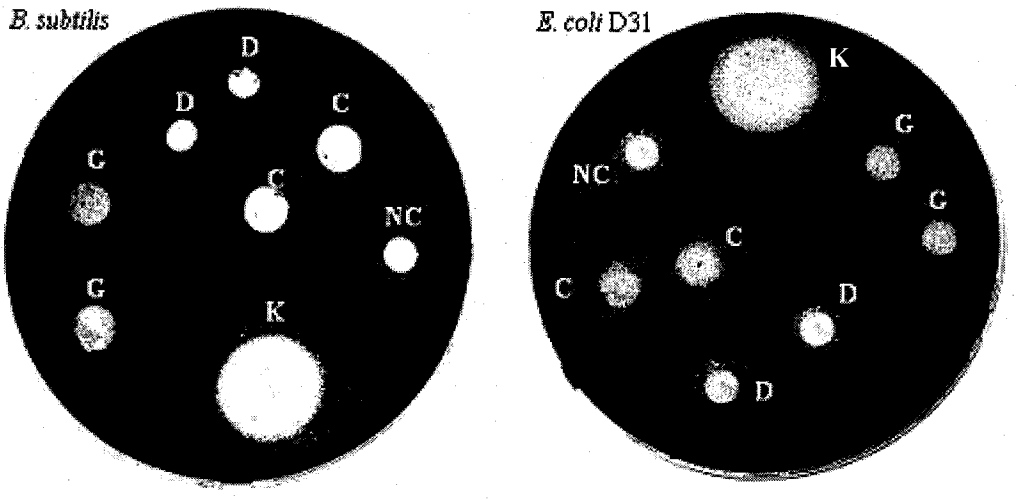

Figure 1: Antimicrobial activity of crude avian egg surface ethyl acetate extracts against *Bacillus subtilis* and *Escherichia coli* D31. Samples (K: kanamycin, C: chicken ethyl acetate extract, D: duck ethyl acetate extract, G: goose ethyl acetate extract, NC: ethyl acetate negative control) were applied to paper discs and placed onto an agarose plate inoculated with bacteria. After overnight incubation at 37 °C, plates were stained and discs removed. Plates were photographed and clear zone diameters measured.

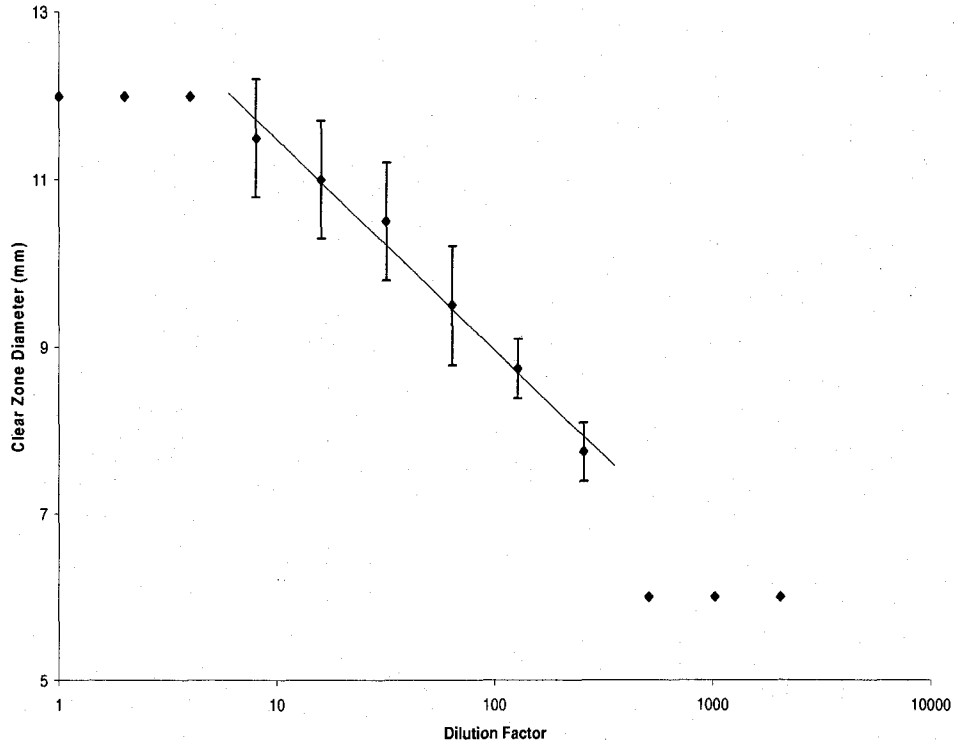

Figure 2: Dose response of crude chicken egg surface ethyl acetate extract against *B. subtilis*. A sample (5 µl) from a 2 fold serial dilution of crude chicken ethyl acetate extract was applied to Whatman # 1 paper disc (6 mm diameter) and placed onto a plate inoculated with *B. subtilis*. The diameter of the clear zones was measured after 24 hours incubation. Experiment was performed on two separate occasions.

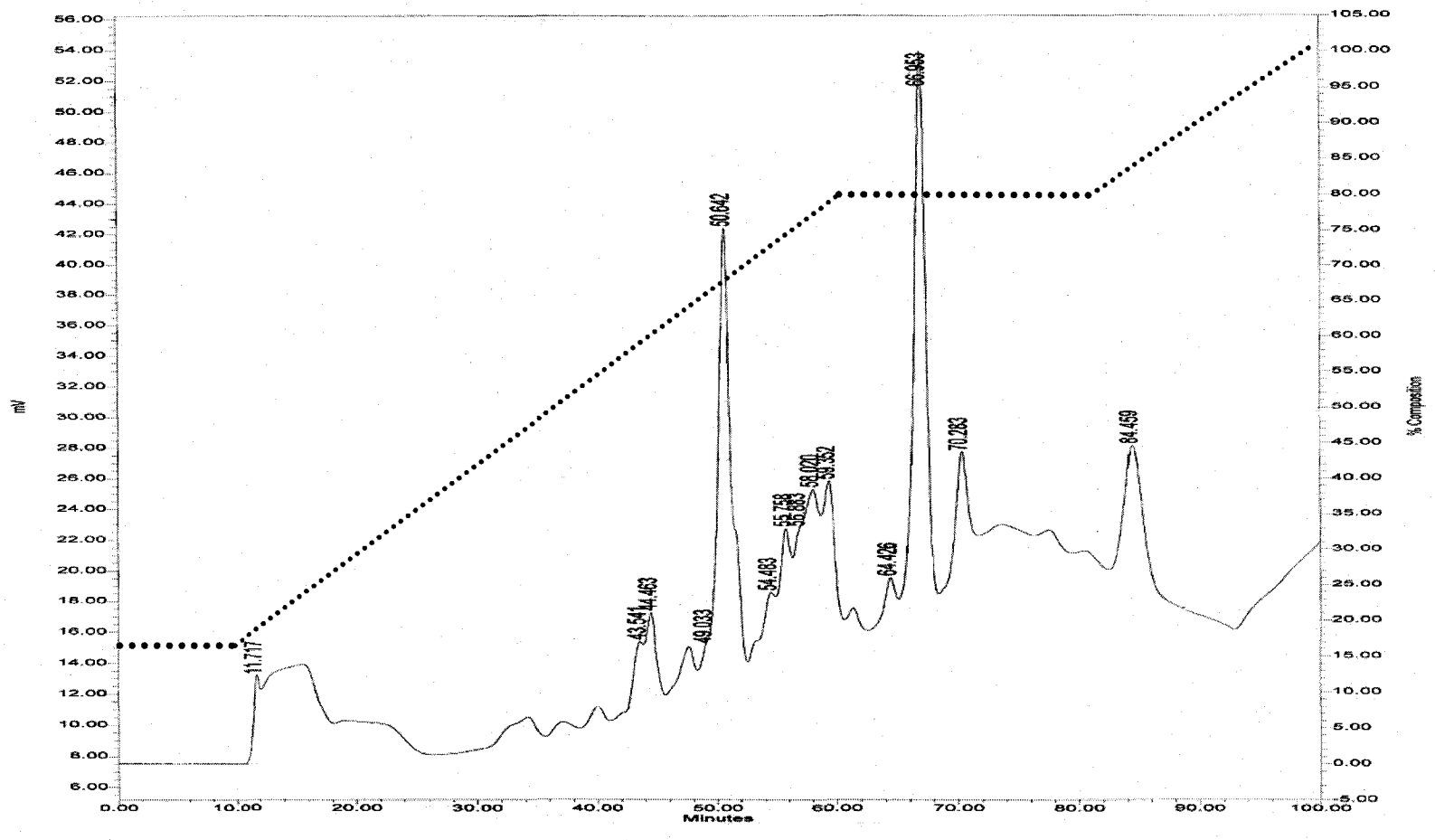

Figure 3: Analysis of crude chicken egg surface ethyl acetate extract by reverse phase high pressure liquid chromatography using an acetonitrile gradient on C_{18} column. Elution at 4 ml/min was monitored at 240 nm and fractions individually collected every minute.

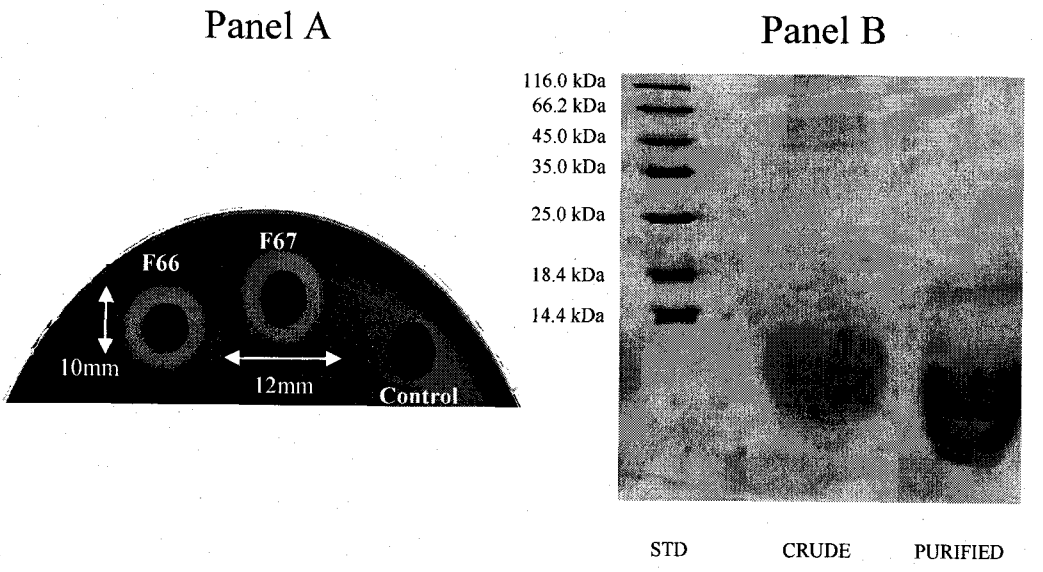

Figure 4: Antimicrobial activity (Panel A) and SDS-PAGE analysis (Panel B) of fractions corresponding to peak eluted at 66.953 minutes during reverse phase high pressure liquid chromatography of crude chicken egg surface ethyl acetate extract. Antimicrobial activity was evaluated against *Bacillus subtilis*. Crude extract as well as the purified active fraction was run on a 20 % SDS-PAGE gel and visualized by Coomassie blue staining.

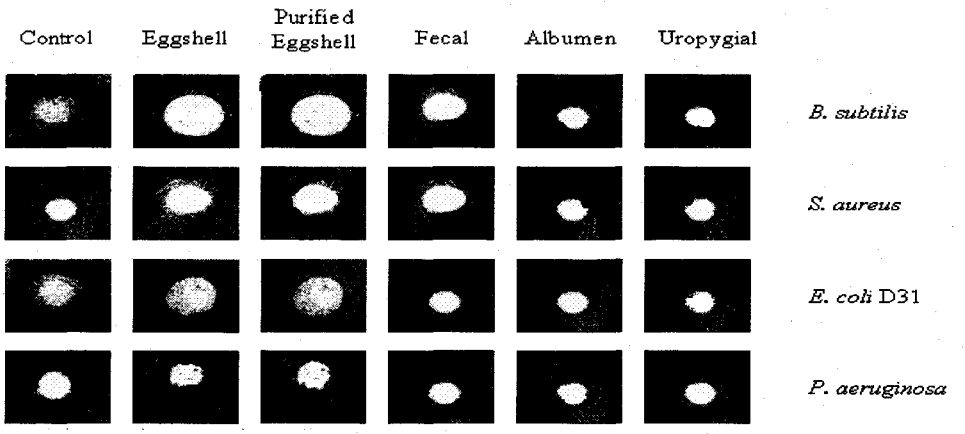

Figure 5: Comparative antimicrobial spectrum of crude and purified chicken egg surface ethyl acetate extracts with ethyl acetate extracts of chicken albumen, chicken fecal material and chicken uropygial gland material. Samples (N =2) of each extract were applied to paper discs and placed onto agarose plates inoculated with Gram-positive or Gram-negative bacteria.

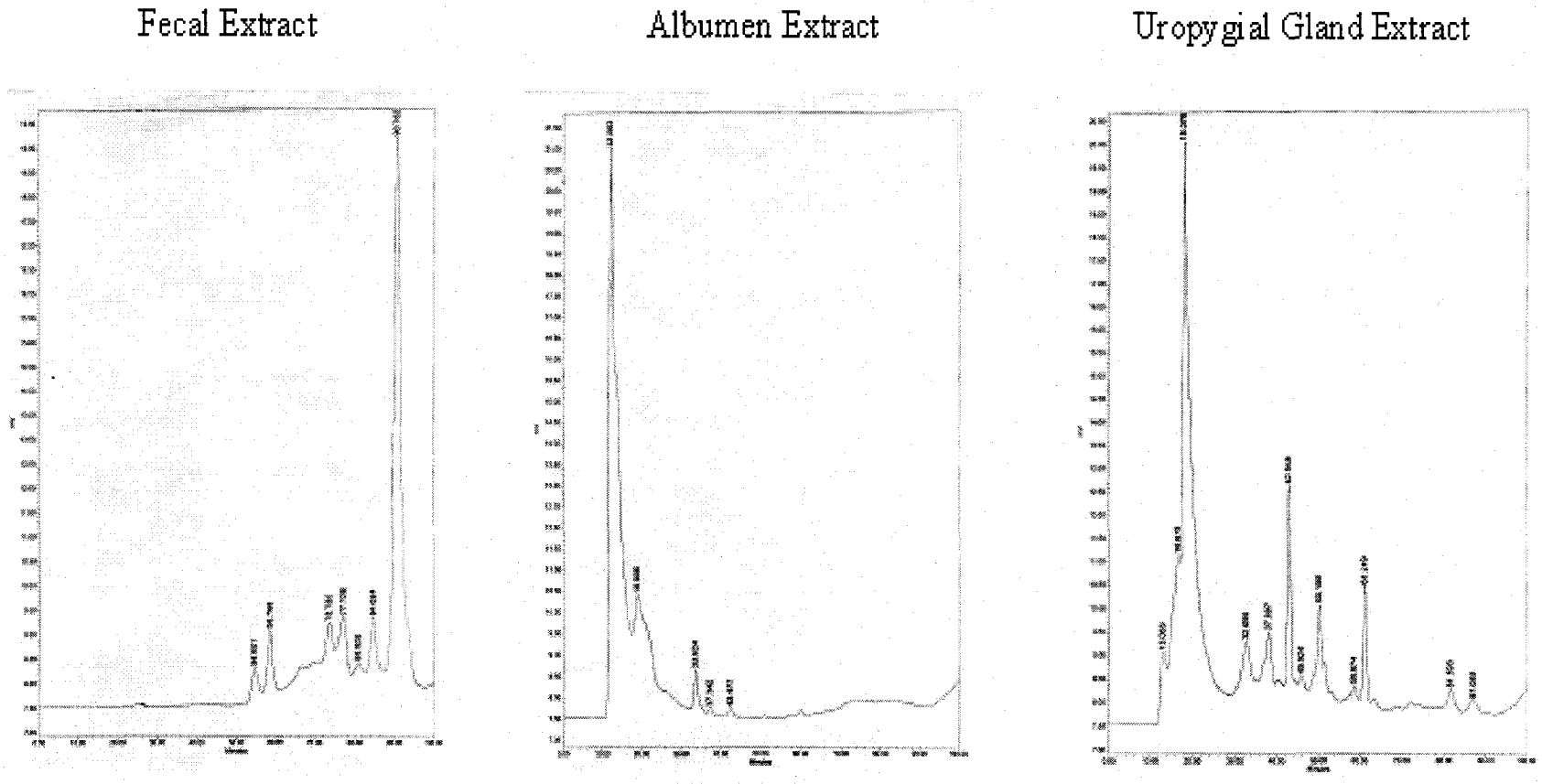

Figure 6: Elution profile at 240 nm of ethyl acetate extracts from chicken albumen, chicken fecal material and chicken uropygial gland material during reverse phase high pressure liquid chromatography using an acetonitrile gradient on C_{18} column.

Table 1: Clear zone diameter of crude avian eggshell surface ethyl acetate extracts and controls after application onto *B. subtilis* or *E. coli* D31 plates.

Sample	Sample Symbol	Clear Zone Diameter (mm) *Bacillus subtilis*	*Escherichia coli D31*
Kanamycin 10 mg/ml in 70 % Ethanol	K	19, 19, 19	21, 21, 21
Ethyl Acetate Negative Control	NC	6, 6, 6	6, 6, 6
Chicken Ethyl Acetate Extract	C	12, 12, 12	10, 10, 10
Duck Ethyl Acetate Extract	D	6, 6, 6	6, 6, 6
Goose Ethyl Acetate Extract	G	12, 12, 12	10, 10, 10

Table 2: Clear zone diameter of chicken ethyl acetate extracts applied on plates inoculated with Gram-positive or Gram-negative bacteria.

Bacteria	Sample	Clear Zone Diameter (mm)
Bacillus subtilis	Crude eggshell extract	12, 12
	Purified eggshell extract	12, 12
	Ethyl acetate negative control	6, 6
	Fecal extract	9, 9
	Egg white extract	6, 6
	Uropygial extract	6, 6
Staphylococcus aureus	Crude eggshell extract	8, 8
	Purified eggshell extract	8, 8
	Ethyl acetate negative control	6, 6
	Fecal extract	9, 9
	Egg white extract	6, 6
	Uropygial extract	6, 6
Escherichia coli D31	Crude eggshell extract	10, 10
	Purified eggshell extract	10, 10
	Ethyl acetate negative control	6, 6
	Fecal extract	6, 6
	Egg white extract	6, 6
	Uropygial extract	6, 6
Pseudomonas aeruginosa	Crude eggshell extract	6, 6
	Purified eggshell extract	6, 6
	Ethyl acetate negative control	6, 6
	Fecal extract	6, 6
	Uropygial extract	6, 6

Section IV: Chapter 7

Section three of this thesis demonstrated that components of the outer eggshell and cuticle of both domestic and wild avian species enhance the antimicrobial defences of the avian egg. The results of these investigations support the claims of the previous thesis sections indicating that egg components, other than albumen, are involved in antimicrobial defence of the avian egg. Section one of the thesis demonstrated the potential of the egg as a source of novel antimicrobial components. In the following section of this thesis (Section 4), selected avian eggshell proteins are investigated for their antimicrobial properties. Chapter seven describes an investigation of the activity of a family of major eggshell matrix-specific proteins present in multiple avian species. This experimental work was primarily conducted by myself. Figure 8 of this chapter was contributed by Dr. R. Lakshminarayanan (University of Singapore). Purification of ovocleidin-17 and ansocalcin was conducted by Dr. S. Valiyaveettil and Dr. R. Lakshminarayanan (University of Singapore). Its is published in FEBS Letters 2008 Mar 5;582(5):699-704.

Chapter 7: Antimicrobial Properties of Avian Eggshell-specific C-type Lectin-like Proteins

Olivier Wellman-Labadie, Rajamani Lakshminarayanan and Maxwell T. Hincke

Abstract:

C-type lectin-like proteins are major components of the calcified eggshell of multiple avian species. In this study, two representative avian C-type lectin-like proteins, ovocleidin-17 and ansocalcin, were purified from decalcified chicken and goose eggshell protein extracts and investigated for carbohydrate binding activity as well as antimicrobial activity. Purified ovocleidin-17 and ansocalcin were found to bind bacterial polysaccharides, and were bactericidal against Bacillus subtilis, Staphylococcus aureus and Pseudomona aeruginosa. Bactericidal activity was found to be enhanced in the presence of calcium but was not dependent on its presence. The results suggest that avian C-type lectin-like proteins may play an important antimicrobial role in defence of the avian embryo.

Introduction:

Ovocleidin-17 (OC-17) and ansocalcin are the major eggshell-specific matrix proteins from chicken and goose, respectively (Mann and Siedler, 1999; Hincke et al., 1995; Lakshminarayanan et al., 2003; Lakshminarayanan et al., 2005; Reyes-Grajeda et al., 2004). They share significant identity with C-type lectin-like (CTL) proteins, but lack the consensus QPD motif that is required for binding to simple sugars. Similar CTL proteins are present in eggshell of various domesticated birds (Panheleux et al., 1999). Interestingly, two families of CTL proteins have been identified within eggshells of ratite species; the amino acid sequence of one form aligns better with ansocalcin (group I) while the other is more similar to OC-17 (group II) (Mann and Siedler, 2004; Mann, 2004; Mann and Sieder, 2006). The presence of CTL proteins in the eggshells of multiple avian species suggests a common biologically important role.

The C-type lectin superfamily is a large group of extracellular proteins with diverse functions in multicellular organisms (Zelinsky and Gready, 2005). Recently, RegIIIγ, a mouse epithelial C-type lectin (Group VII CTL) and its human counterpart, HIP/PAP,

were shown to inhibit the growth of Gram-positive bacteria (Cash et al., 2006). Ansocalcin (33%) and OC-17 (28%) possess significant identity to the Reg family of C-type lectins. Given their widespread presence and abundance within the eggshell matrices of various species, we sought to investigate the antimicrobial properties of avian eggshell CTL proteins. Our strategy was to study the binding of ansocalcin and OC-17 to bacterial cell wall polysaccharides, and investigate their antimicrobial properties against Gram-positive and Gram-negative bacteria. The results show that avian eggshell CTL proteins have excellent antimicrobial as well as bacterial polysaccharide binding properties. This is the first report on the antimicrobial properties of eggshell-specific CTL proteins and highlights the multiple roles played by these proteins.

Materials and Methods:

Protein Extraction and Purification: Eggshells were ground into a fine powder and decalcified using 20 % acetic acid (10 ml of acetic acid/ gram eggshell) according to the method of Reyes-Grajeda et al., (2004). Purification of ansocalcin and OC-17 was performed as previously described by Lakshminarayanan et al., (2003; 2005). The concentration of proteins used for SDS-PAGE, carbohydrate binding and antimicrobial assays was determined by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) using BSA (Bioshop, Burlington, ON) as a standard.

Carbohydrate Binding Activity: The ability of ansocalcin and OC-17 to bind to various complex carbohydrates was analyzed by a pull-down assay adapted from Cash et al., (2006). Dry protein samples were suspended in 0.01 % acetic acid and further diluted with 0.01 % acetic acid and 0.1 % bovine serum albumin (BSA). Samples were incubated in the presence or absence *of Micrococcus lysodeikticus* ATCC 4698 cell walls (Sigma-Aldrich, Oakville, ON), *B. subtilis* peptidoglycan (Sigma-Aldrich, Oakville, ON), crab shell chitin (Sigma-Aldrich, Oakville, ON), corn starch (Best Foods Canada, Etobicoke, ON) or *E. coli* 0127:B8 lipopolysaccharide (Sigma-Aldrich, Oakville, ON) suspended in 0.01 % acetic acid at 4 °C with gentle shaking for 24 hours. After incubation, samples were centrifuged for 5 min (13 000 rpm) at 4 °C, and the supernatant carefully removed. Supernatant and pellet were analyzed by SDS-PAGE.

Antimicrobial Activity of Purified Ansocalcin and Ovocleidin-17: Antimicrobial activity was evaluated by the micro-broth dilution assay adapted from Steinberg and Lehrer (1997). Vehicle was BSA (0.1 %) in acetic acid (0.01 %), and positive control was 100 μg/ml bovine lactoferricin B (Sigma-Aldrich, Oakville, ON). *Micrococcus lysodeikticus* ATCC 4698 cell walls or *E. coli* 0127:B8 lipopolysaccharide (0.5-5 mg/ml) in the presence and absence of ansocalcin or OC-17 was also investigated for potential inhibition of antimicrobial activity. Antimicrobial activity was evaluated against two Gram-positive (*B. subtilis* ATCC 19659 and *S. aureus* ATCC 6538) and two Gram-negative (*P. aeruginosa* ATCC 15442 *and E. coli* D31) bacteria. Each antimicrobial assay was conducted in triplicate for two independent trials (N=2). Antimicrobial activity, in the presence and absence of 1 mM $CaCl_2$ (bacteria suspended in 25 mM HEPES buffer pH 7.3), was also evaluated in order to investigate the effect of calcium on the activity of the avian C-type lectin-like proteins. Data were analysed using SYSTAT Version 8.0 (SPSS, Chicago, IL). T-tests were conducted to identify any significant differences between bacterial populations in the presence and absence of protein samples. For morphological experiments, 200 μg/ml of ansocalcin or OC-17 was incubated in the presence of *B. subtilis* bacterial suspension for 1 hour as described for the antimicrobial assay. Following the incubation period, samples were individually centrifuged (3000 g, 4°C, 10 min), washed and resuspended in an equal volume of 10 mM sodium phosphate buffer (pH 7.3). Bacterial pellets (20 μl) were heat fixed to microscope slides and Gram-stained for examination under oil-immersion light microscopy.

Results and Discussion:

SDS-PAGE analysis of the crude domestic chicken and domestic goose eggshell protein extracts revealed that OC-17 (17 kDa) and ansocalcin (15 kDa), respectively, are major proteins of the shell matrix as we have previously reported and were greatly purified by RP-HPLC (Hincke et al., 1995; Lakshminarayanan et al., 2003) (Figure1).

We investigated the ability of highly purified ansocalcin and OC-17 to bind to bacterial polysaccharides. The binding of OC-17 and ansocalcin to peptidoglycan (PGN, a polymer that forms the thick layer of Gram-positive bacteria and a thin layer in Gram-negative bacteria), lipopolysaccharide (LPS, the outer cell membrane of Gram-negative bacteria), chitin, and *Micrococcus lysodeikticus* cell wall was examined. Since complex

polysaccharides are insoluble in aqueous solution, a pull-down assay was used to evaluate the polysaccharide binding ability of the avian C-type lectin-like proteins (Cash et al., 2006). Figure 2 shows the results obtained from the pull-down assay. OC-17 and ansocalcin remained in the supernatant solution of crab shell chitin. Similar results were obtained with corn starch (not shown) indicating lack of binding to these polysaccharides. However, both OC-17 and ansocalcin were greatly retained in the pellet when incubated in the presence of *Micrococcus cell wall*, lipopolysaccharide or peptidoglycan, indicating their strong binding to the bacterial polysaccharides. This effect was more pronounced for PGN pellet than for LPS. OC-17 is almost completely removed from the supernatant of PGN, indicating that it binds more strongly to this bacterial polysaccharide than ansocalcin.

We evaluated these C-type lectin-like proteins for their antimicrobial activity against various bacteria. When incubated with Gram-negative bacteria, both OC-17 and ansocalcin exhibited a weak antimicrobial activity against *P. aeruginosa* (Figure 3 and Table 1). OC-17 exhibits greater bactericidal activity than ansocalcin against *P. aeruginosa* (Figures 3a, 3b). The antibacterial activity of both CTL proteins was diminished in the presence of added lipopolysaccharide (Figures 3a, 3b, and Table 1). However, lipopolysaccharide alone did not demonstrate any antibacterial activity against either of the Gram-negative bacteria. No statistically significant reductions in *E. coli* bacterial populations were detected at a 200 μg/ml concentration of the avian C-type lectin-like proteins. On the other hand, in accordance with the pull-down assay, OC-17 and ansocalcin exhibited strong antimicrobial activity against the Gram-positive *S. aureus* and *B. subtilis* (Figures 3c, 3d and Table 1). For both avian CTL proteins, the antimicrobial activity was higher against *B. subtilis* than *S. aureus*, and the activity against both Gram-positive bacteria was reduced in the presence of *Micrococcus* cell walls (Figures 3c, 3d, 4; Table 1). *Micrococcus* cell wall alone had no antibacterial activity against either of the Gram-positive bacteria (Figures 3c, 3d, 4).

The concentration of CTL proteins was varied from 12 to 200 μg/ml to examine the dose dependency of the antimicrobial activity (Figure 5). Bacteria incubated with OC-17 exhibited a greater decrease in population compared to ansocalcin, further confirming the more potent antimicrobial action of OC-17. Morphological examination following incubation with avian C-type lectins revealed that few intact bacilli remain following

incubation with ansocalcin (200 μg/ml) (Figure 6). Moreover, in the presence of OC-17, the vast majority of *B. subtilis* cells were lysed as a result of cell wall damage and only unstained bacterial debris was detected reflecting loss of Gram-staining cellular contents (Figure 6). Since many CTL proteins bind polysaccharides in the presence of Ca^{2+}, the effect of calcium on the antimicrobial activity of ansocalcin and OC-17 was investigated. For both ansocalcin and OC-17, bactericidal activity against *B. subtilis* was enhanced in the presence of 1 mM $CaCl_2$ (Figure 7). The antimicrobial activity of ansocalcin showed ~3 fold increase in the presence of Ca^{2+} whereas OC-17 showed ~10 fold increase. In the absence of avian CTL's, $CaCl_2$ did not affect the viability of *B. subtilis* bacterial populations.

Previous studies have investigated the effect of pH and / Calcium ion upon the conformation and dimerization of OC-17 and ansocalcin. Circular dichroism and dynamic light scattering measurements reveal that OC-17 conformation is not affected by calcium ion, and that the protein would be essentially monomeric at the concentrations used in our antimicrobial assay (Lakshminarayanan et al., 2005). Ansocalcin, however, dimerizes above 100 μg/ml and this property is independent of the calcium and pH; below this concentration it is monomeric (Lakshminarayanan et al., 2003; 2005). It is possible that the enhanced antimicrobial activity of OC-17 is due to its monomeric state at the assay concentration, compared to dimeric ansocalcin (Figure 5).

Structure-function studies of broad-spectrum antimicrobial peptides reveal that key requirements are cationic charge and an ability to fold into amphiphilic or amphipathic conformations (Powers and Hancock, 2003). The crystal structure of OC-17 and the molecular model of ansocalcin (modeled from the crystal structure of OC-17) indicate that the former has widespread distribution of positive charges compared to the latter (Figure 6) (Reyes-Grajeda et al., 2004). On one side of the OC-17 molecule the surface of the protein displays an extended solvent exposed basic stretch consisting of 17 of the 21 arginine / lysine residues. This feature is reminiscent of the multiple arginine and lysine residues that cationic antimicrobial peptides exhibit (Powers and Hancock, 2003), and probably contributes to the increased activity of OC-17 compared to ansocalcin.

In addition to the mineral phase, which provides a primary physical barrier against microorganisms, the avian egg is equipped with a number of chemical defences, such as

lysozyme, avidin, ovotransferrin, ovomucoid, and β-N-acetylglucosaminidase that exhibit varying degree of antimicrobial activities (Wellman-Labadie et al., 2007). These components are abundant in the egg albumen and scarce in the eggshell. During embryonic development, selective decalcification of the inner eggshell occurs due to protons secreted by the cells of the chorioallantoic membrane. This process would concomitantly release occluded avian CTL proteins from the inner shell into a calcium-rich environment and thereby up-regulate antimicrobial defences surrounding the embryo as the eggshell becomes progressively weaker in preparation for hatching. We mimicked this situation by conducting the pull-down assay in an acidic milieu, where an interaction with bacterial cell wall polysaccharides was observed.

In conclusion, this study reports that OC-17 and ansocalcin demonstrate strong binding preference for bacterial polysaccharides and especially peptidoglycan. Both OC-17 and ansocalcin are potently bactericidal against Gram-positive bacteria such as *B. subtilis* and *S. aureus*, and exhibited enhanced activity in the presence of calcium. CTL proteins are major eggshell matrix components in multiple avian species and have been proposed to play a role in eggshell calcification (Mann and Siedler, 1999; Hincke et al., 1995; Lakshminarayanan et al., 2005; Reyes-Grajeda et al., 2004). Avian C-type lectin-like proteins may therefore be involved in both eggshell calcification and antimicrobial defence. It will be interesting to compare the antimicrobial properties of eggshell CTL proteins from diverse avian species that are subject to a variety of microbial environments. Our results further indicate that proteins of potential pharmaceutical interest can be obtained from the avian eggshell; an inexpensive and readily available source of bioactive molecules.

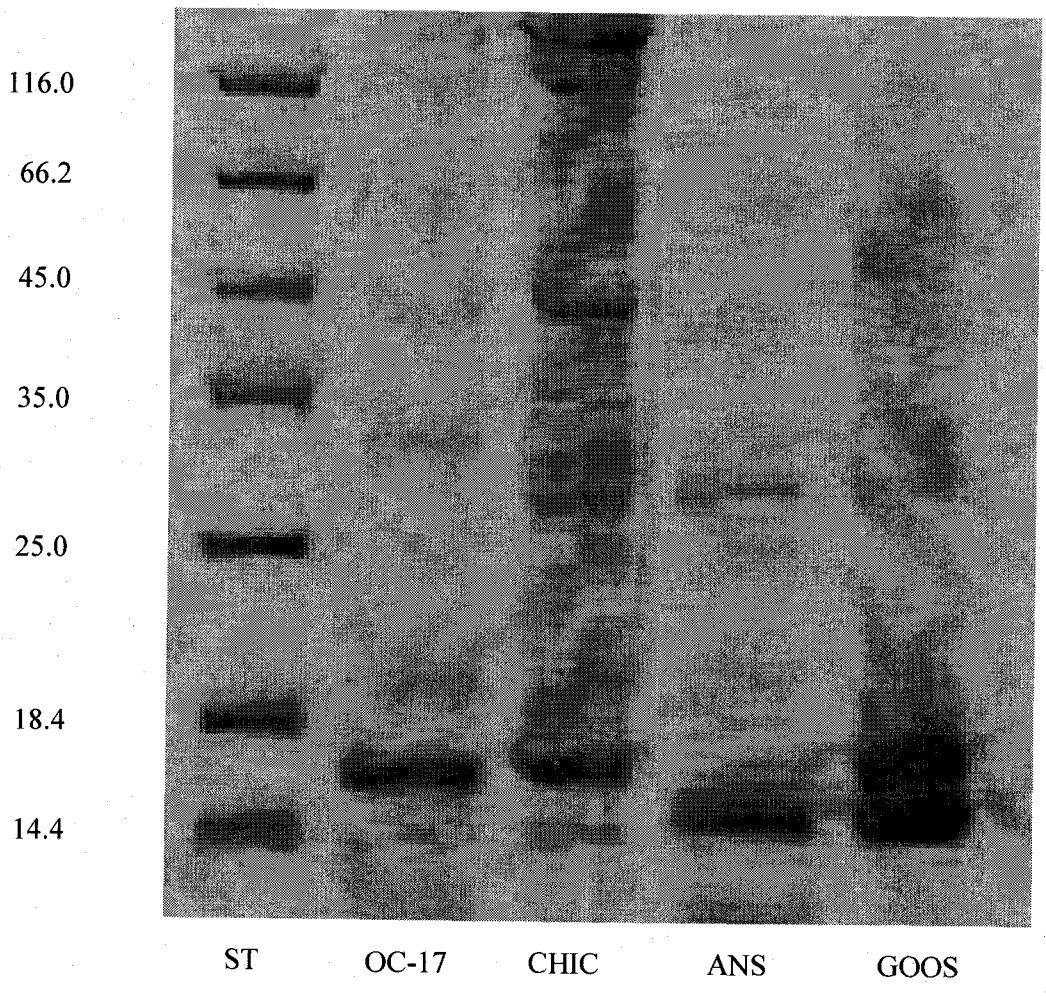

Figure 1: SDS-PAGE analysis of crude and RP-HPLC purified eggshell protein extracts. Samples (2.5μg of purified and 20μg of crude extract) were separated on a 12 % SDS polyacrylamide gel and visualized by Coomassie blue staining. Molecular weight of standards (STD) is indicated on the left. Samples are labelled at the bottom (OC-17: Purified ovocleidin-17; CHICK: crude chicken eggshell extract; ANSO: Purified ansocalcin; GOOSE: crude goose eggshell extract).

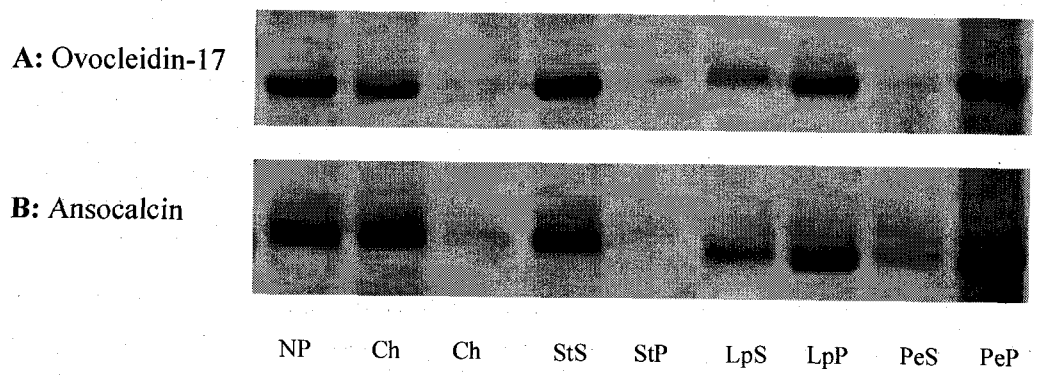

Figure 2: Eggshell CTL proteins pull-down assay. For clarity, SDS-PAGE of the ansocalcin and OC-17 bands alone are shown. Abbreviations: NPS - No polysaccharide supernatant; ChS - Crab chitin supernatant; ChP - Crab chitin pellet; CwS – *Micrococcus* cell wall supernatant; CwP – *Micrococcus* cell wall pellet; LpS - *E. coli* lipopolysaccharide supernatant; LpP - *E. coli* lipopolysaccharide pellet; PeS - *B. subtilis* peptidogylcan supernatant; PeP - *B. subtilis* peptidoglycan pellet.

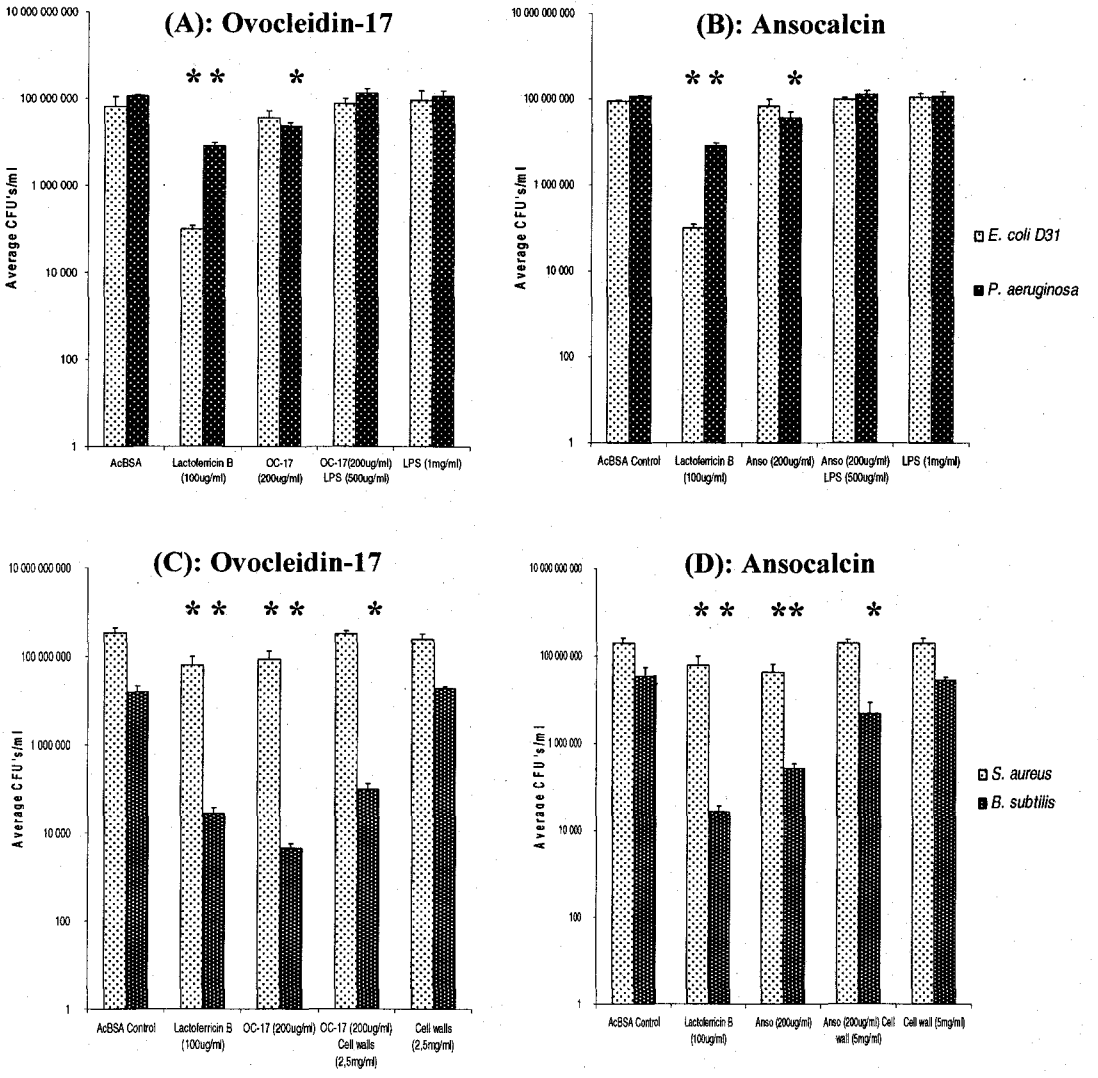

Figure 3: Antimicrobial properties of eggshell CTL proteins. OC-17 and ansocalcin were tested against selected Gram-negative (**A**, **B**) and Gram-positive (**C**, **D**) bacteria. The grey and black color in the panels **A** and **B** represent *E. coli* and *P. aeruginosa*, respectively, whereas the checked grey and black shading in the panels **C** and **D** represent *S. aureus* and *B. subtilis*, respectively. Bovine serum albumin (0.1 %) in 0.01 % acetic acid (AcBSA) was used as a negative control and bovine lactoferricin B (100 μg/ml) served as a positive control. The average colony counts were obtained after plating multiple dilutions on LB agar. Each experiment was conducted in duplicate. Significant reductions in bacterial populations ($p < 0.05$) are indicated by an asterix (*).

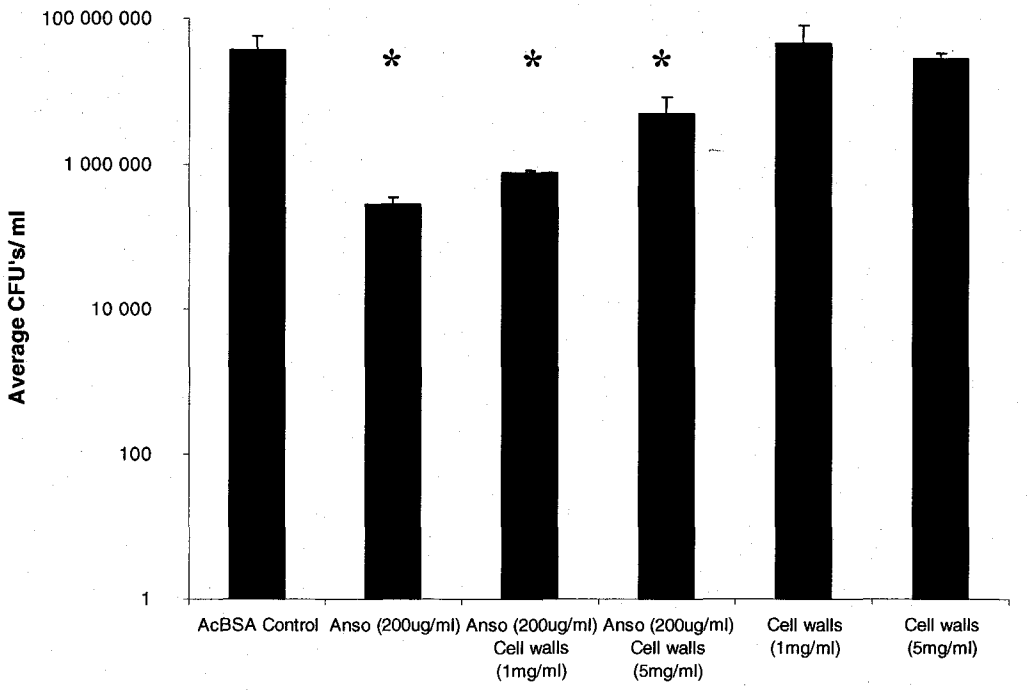

Figure 4: Inhibition of ansocalcin bactericidal activity in the presence of *Micrococcus* cell walls. *Bacillus subtilis* suspended in 10 mM sodium phosphate buffer (pH 7.3) was incubated 1 hr in the presence or absence of the avian C-type lectin-like protein and *Micrococcus* cell walls. Bovine serum albumin (0.1 %) in 0.01 % acetic acid (AcBSA) was used as a negative control. Bovine lactoferricin B (100 μg/ml) was used as a positive control. The average colony counts were obtained after plating on LB agar. Each experiment was conducted in duplicate. Significant reductions in bacterial populations ($p < 0.05$) are indicated by an asterix (*).

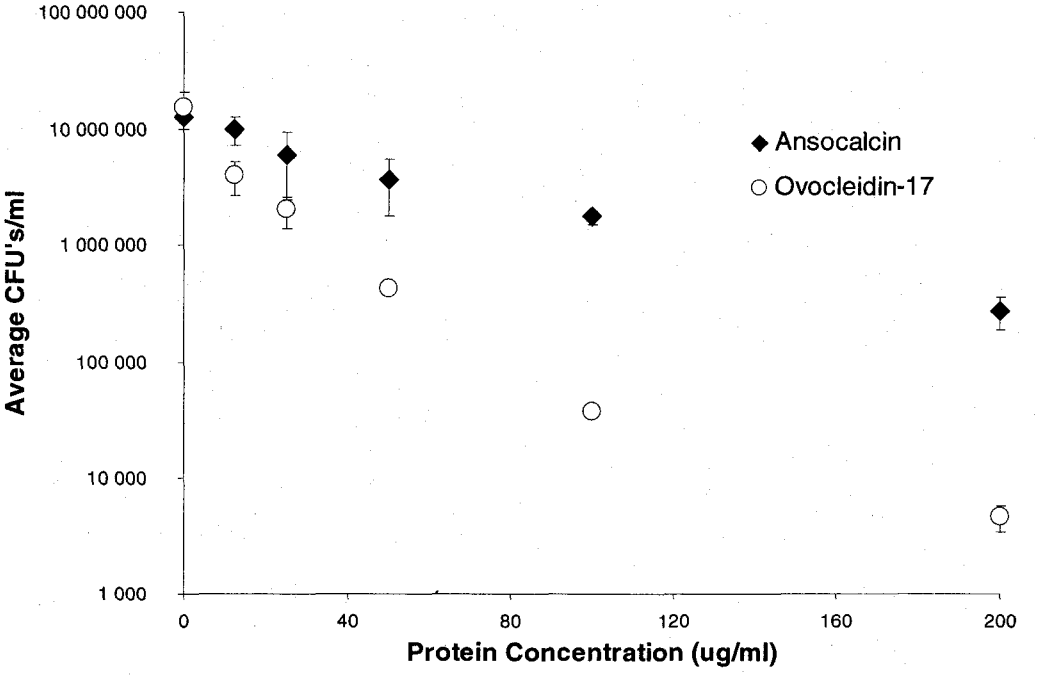

Figure 5: Dose – response of antimicrobial activity of eggshell CTL proteins. The ability of different concentrations of OC-17 and ansocalcin to reduce bacterial populations of *B. subtilis* was evaluated.

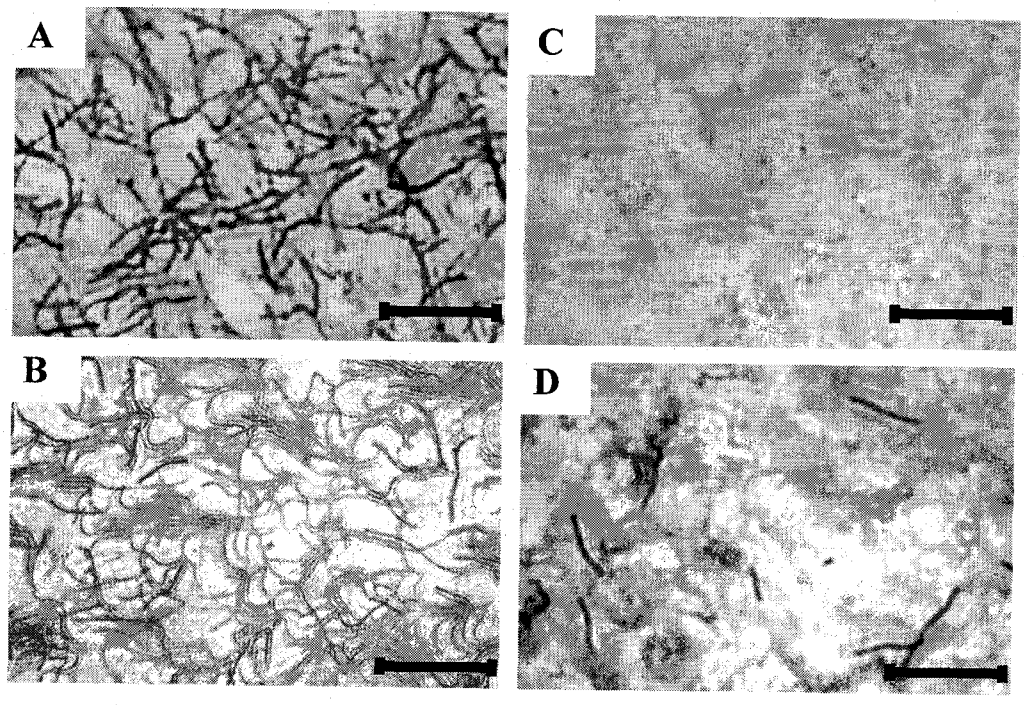

Figure 6: Bacterial morphology of *B. subtilis* in the presence of eggshell CTL proteins. Bacteria were incubated 1 hour in the presence of vehicle alone (a), 100 μg/ml lactoferricin B (b), 200 μg/ml OC-17 (c) or 200 μg/ml ansocalcin (d). Bacterial smears were Gram-stained and photographed under light microscopy. These images are representative of multiple fields of view for triplicate slides for each condition. Scale bar 12.5 μm.

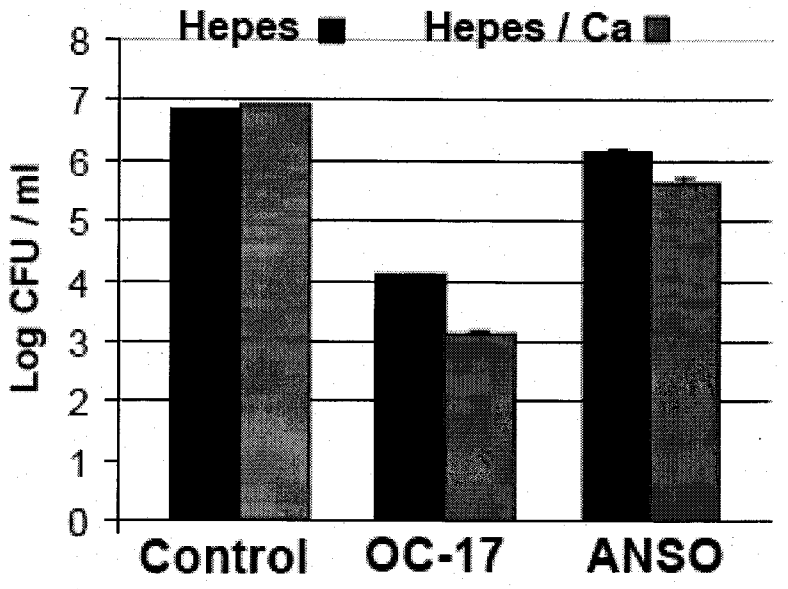

Figure 7: Effect of calcium on the antimicrobial properties of OC-17 and ansocalcin. The presence of 1 mM $CaCl_2$ did not affect the bacterial populations in the absence of avian CTL proteins. Average colony counts were obtained after plating on LB agar in triplicate. Each experiment was conducted in duplicate.

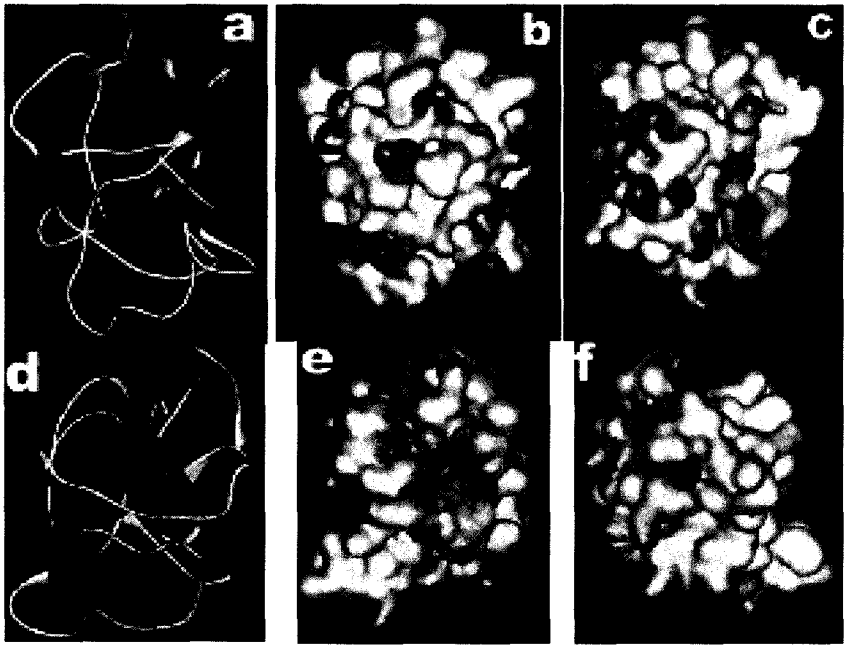

Figure 8: Three-dimensional structures of avian eggshell CTL proteins. (a) Crystal structure of OC-17. (b) Surface structure of OC-17 in the same orientation as in (a). (c) Surface structure of OC-17 that is rotated 180° clockwise around the Y-axis of (a). (d) Molecular model of ansocalcin. (e) Surface structure of ansocalcin model in the same orientation as in (d). (f) Surface structure of ansocalcin that is rotated 180° clockwise around the Y-axis of (d). In Figures a) and d) protein secondary structures are indicated in blue (β-strand) and red (α-helix). The blue and red colors in the surface plot indicate the distribution of positive and negative charges, respectively.

Table 1: Effect of lipopolysaccharide and *Micrococcus* cell walls on the bactericidal activity of ansocalcin and ovocleidin-17. Activity is expressed as the percent reduction of bacterial population (± standard deviation) for two independent trials. The absence of reductions in bacterial populations is indicated by a horizontal line (---).

Bacteria	Protein Sample	% Reduction ± Standard Deviation	P-value
E. coli D31	Lactoferricin B (100 µg/ml)	99.89 ± 0.03	0.035
	Ovocleidin 17 (200 µg/ml)	45.23 ± 1.42	0.295
	Ovocleidin (200 µg/ml) Lipopolysaccharide (500 µg/ml)	---	>0.05
	Ansocalcin (200 µg/ml)	25.27 ± 9.32	0.064
	Ansocalcin (200 µg/ml) Lipopolysaccharide (500 µg/ml)	---	>0.05
P. aeruginosa	Lactoferricin B (100 µg/ml)	92.93 ± 0.02	0.027
	Ovocleidin 17 (200 µg/ml)	75.60 ± 3.79	0.05
	Ovocleidin (200 µg/ml) Lipopolysaccharide (500 µg/ml)	---	>0.05
	Ansocalcin (200 µg/ml)	67.80 ± 5.85	0.05
	Ansocalcin (200 µg/ml) Lipopolysaccharide (500 µg/ml)	---	>0.05
S. aureus	Lactoferricin B (100 µg/ml)	73.50 ± 9.74	0.024
	Ovocleidin 17 (200 µg/ml)	79.78 ± 3.38	0.031
	Ovocleidin (200 µg/ml) cell walls (2.5 mg/ml)	7.18 ± 4.06	>0.05
	Ansocalcin (200 µg/ml)	78.00 ± 2.38	0.048
	Ansocalcin (200 µg/ml) cell walls (2.5 mg/ml)	2.00 ± 1.19	>0.05
B. subtilis	Lactoferricin B (100 µg/ml)	99.92 ± 0.03	0.004
	Ovocleidin 17 (200 µg/ml)	99.97 ± 0.01	0.007
	Ovocleidin (200 µg/ml) cell walls (2.5 mg/ml)	99.37 ± 0.05	0.007
	Ansocalcin (200 µg/ml)	99.25 ± 0.14	0.004
	Ansocalcin (200 µg/ml) cell walls (2.5 mg/ml)	86.44 ± 0.85	0.004

Section V: Conclusion

The previous sections of this thesis have described the potential of the egg as a source of antimicrobials, illustrated the protective nature of the albumen and eggshell, and described the effects of environmental pressures and phylogeny on egg defences as well as identifying proteins which contribute to the antimicrobial defences of the egg.

In this concluding section, the major findings and contributions of this thesis are reviewed. The data presented during this thesis is compared to the available literature allowing a new understanding of the role of albumen and the eggshell in the antimicrobial defences of the avian egg. In addition, the direction of future research into the antimicrobial defences of the avian egg and the antimicrobial components of the egg will de discussed.

The Integrated Antimicrobial Defences of the Avian Egg

Olivier Wellman-Labadie, Jaroslav Picman, and Maxwell T. Hincke

Avian Albumen and Antimicrobial Activity of the Egg:

Chicken albumen, while providing water and nutrients to the developing embryo, prevents the growth of microorganisms. Raw chicken egg white inhibits the growth of *Staphylococcus aureus*, *Shigella dysenteriae*, *Escherichia coli* and *Saccharomyces cerevisiae* (Schade and Caroline, 1944). Wang and Shelef (1991) reported that *Listeria monocytogenes* strain Scott A and Brie-1 are highly sensitive to raw chicken albumen. Sahin et al., (2003) reported that the viability of inoculated *Campylobacter jejuni* was dramatically reduced in albumen while bacteria were able to survive up to 14 days in chicken egg yolk. The alkaline pH of the albumen and the presence of antimicrobial proteins such as lysozyme and ovotransferrin significantly reduce the growth of microorganisms (Baggott and Graeme-Cook, 2002).

In support with these findings, Wellman-Labadie et al., (2008a) reported that the growth of *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* D31 was inhibited by domestic chicken egg white protein extracts. In addition, Wellman-Labadie et al., (2008a; b) investigated the activity of domestic turkey, duck and goose albumen as well as that of wild Canada goose, mute swan, wood duck and hooded merganser albumen. These are among the few comparative studies which investigate avian albumen and are the first to analyze the antimicrobial activity of egg white protein extracts from domestic and wild avian species. The activity of domestic turkey egg white protein extracts was similar to that of domestic chicken; inhibiting the growth of *P. aeruginosa*, *B. subtilis*, *S. aureus* and *E. coli* D31 (Wellman-Labadie et al., 2008a). The growth of *P. aeruginosa* and *B. subtilis* was inhibited by domestic duck and domestic goose egg white protein extracts as well as by Canada goose, mute swan, wood duck and hooded merganser egg white protein extracts (Wellman-Labadie et al., 2008a; b).

Composition of Avian Egg White:

The protein composition of avian albumen appears to be highly conserved across avian species. As proposed by Miguel et al., (2005), the comparative protein composition of

egg white is qualitatively and quantitatively similar in species of high phylogenetic proximity. Ovalbumin, ovotransferrin, lysozyme and other egg white proteins of the hen are present in many avian species. Simultaneous expression of both c-type and g-type lysozyme is observed in the albumens of some Anseriformes (Florkin and Scheer, 1978; Hemmen et al., 1992). Domestic chicken, turkey, duck and goose as well as wild Canada goose, mute swan, wood duck and hooded merganser all contain ovalbumin, ovotransferrin and either c-type or g-type lysozyme in their albumens (Wellman-Labadie et al., 2008a; b). C-type lysozyme was detected in domestic chicken, turkey and duck as well as in wood duck and hooded merganser (Wellman-Labadie et al., 2008a; b). Enhanced lysozyme activity under acidic conditions (a characteristic of g-type lysozyme) and the lack of c-type immunoreactivity in domestic goose, Canada goose and mute swan albumen suggest the presence of g-type lysozyme in the albumens of these species (Wellman-Labadie et al., 2008a; b).

C-type lysozyme has been demonstrated in the albumens of Galliformes and Anseriformes whereas g-type lysozyme was found in the albumen of species from nine avian orders including the Anseriformes (Florkin and Scheer, 1978; Hemmen et al., 1992). Canfield and McMurry (1967) reported that the enzymatic activity of goose egg white lysozyme is three times greater than an equimolar amount of hen egg white lysozyme at pH 6.2. Wellman-Labadie et al., (2008a) reported a 9 fold increase in the lysozyme enzymatic activity of goose albumen protein extracts at pH 5.3 when compared to that observed at pH 7.3. Goose, ostrich, rhea and cassowary g-type lysozyme have a narrow and acidic pH optimum (~5.0) which contrasts with the broad pH range (5.5 to 7.5) of hen egg white c-type lysozyme (Thammasirirak et al., 2001; Pooart et al., 2005). At its normal pH of 9.6, the buffering capacity of chicken albumen is limited and bacterial contamination has been shown to cause the pH of albumen to drop to near 6 (Cotterill et al., 1958; Seviour and Board, 1972a). This would in effect create ideal conditions for optimal g-type lysozyme activity. Therefore, microbial contamination of albumen may result in the activation of g-type lysozyme and prevent bacterial proliferation. This mechanism may explain the much wider distribution of g-type lysozyme in avian species.

The ovotransferrin content of domestic chicken albumen was found to be similar to that of domestic turkey (Wellman-Labadie et al., 2008a). Ovotransferrin levels also appear to

be similar among domesticated and wild Anseriformes but these where found to be lower than in the Galliformes (Wellman-Labadie et al., 2008a; b). In support with these findings, Feeney et al., (1960) reported little inter-specific variation in the ovotransferrin content of albumen among various species of Galliformes, Anseriformes, Casuariformes or Columbiformes. In addition, Miguel et al., (2005) reported similar ovotransferrin content for hen, pheasant and quail albumen.

None the less, some variation in the content of egg white protein is observed when comparing avian species and especially when these are from different orders. Feeney et al., (1960) reported variations as high as 30 fold in the c-type lysozyme content of albumen from different bird species. Prager and Wilson (1974) reported that the g-type lysozyme content of albumen from different bird species span more than a 200-fold range. Wellman-Labadie et al., (2008a) reported that albumen from domestic Galliformes (chicken and turkey) contained higher levels of c-type lysozyme than in the albumens of domestic Anseriformes (duck and goose).

Environmental pressures may also influence the protein content of avian albumen. Kern and Cowie (1995) reported that the humidity levels of open-bowl nests are much lower than those of cavity nests or underground burrow nests. The probability and magnitude of microbial contamination is positively related to exposure period and relative humidity (Cook et al., 2003). Board et al., (1979) noted that the shells of domestic hen egg are frequently colonized by bacteria when stored under humid conditions. Avian species which reuse nests for multiple breeding attempts or nest in enclosed sites or cavities are exposed to higher levels of micro-organisms (Cook et al., 2003). It can therefore be hypothesized that species exposed to abnormally humid conditions and the associated increased risk of microbial attack would evolve more potent antimicrobial defences. Wellman-Labadie et al., (2008b) reported that within four species of wild Anseriformes tested, albumen protein extracts from the cavity nesting wood duck demonstrated the greatest bactericidal activity, which correlated positively with egg white c-type lysozyme content. Enhanced c-type lysozyme content in wood duck albumen may therefore be a strategy employed by some cavity nesting species in order to protect the developing embryo from the harsh conditions associated with cavity nesting.

Parallels of the Avian Albumen and Vertebrate Innate Immune System:

Tranter and Board (1986) reported that hen egg albumen was more toxic for Gram-negative bacteria at 39.6°C than at 30°C. In addition, bacteria and fungi have been reported to grow rapidly on the shells of newly laid eggs exposed to ambient conditions but decline to low levels on the shells of incubated eggs (Cook et al., 2005b). Cook et al., (2005a) reported that eggs that were incubated did not become infected suggesting that among its many functions, the increase in egg temperature during incubation minimizes infection. Tranter and Board (1986) suggested that the antimicrobial action of hen egg albumen may be akin to mammalian serum in that a reduction in iron content (due to the presence of transferrins) coupled with an elevation of temperature (fever in mature vertebrates and incubation in the case of the egg) is a coordinated host-defence mechanism. In addition to transferrins, lysozyme is another antimicrobial protein present in avian egg white which is also protein present in multiple biological fluids of mature vertebrates.

Bacterial proteolytic enzymes can degrade egg white protein components during microbial infection. Pellegrini et al., (2004) noted that *in vitro* proteolytic digestion of ovalbumin by tryptic and chymotryptic digestion produced fragments with strong antimicrobial activity against *B. subtilis* and weak activity against *Candida albicans.* Ovomucin-derived peptides have been found to show activity against Newcastle disease virus, bovine rotavirus and human influenza virus *in vitro* (Tsuge et al., 1996a; 1996b; 1997a; 1997b; Watanabe et al., 1998). Clostripan digestion of lysozyme produced an antimicrobial peptide with potent activity against both Gram-positive and Gram-negative bacteria as well as the fungus *C. albicans* (Ibrahim et al., 2001b). Recently, an antimicrobial region, termed ovotransferrin antimicrobial peptide 92 (OTAP-92), was isolated by trypsin-nicking of ovotransferrin and demonstrated activity against *S. aureus* and *E. coli* K12 (Ibrahim et al., 1998; Ibrahim et al., 2000). Limited bacterial contamination resulting in proteolytic cleavage of avian albumen proteins may therefore liberate antimicrobial peptides and enhance the antimicrobial defences of the egg. This is reminiscent of the inflammatory response of the vertebrate immune system whereby damage stimulates antimicrobial protein secretion and activation.

The Avian Eggshell and Antimicrobial Activity of the Egg:

The avian eggshell is a complex, multifunctional biomineral composed of a mineral phase (95 %) predominantly containing calcium carbonate as well as an organic phase (3 %) of lipids and proteins (Parsons, 1982; Solomon, 1991; Nys et al., 1999). Multiple eggshell matrix proteins are suspected to play a role in eggshell formation but the role of individual proteins remains unclear. Mann et al., (2006) recently identified 520 proteins within the acid-soluble organic matrix of the chicken eggshell using MS-based technology suggesting that the avian eggshell is a relatively rich source of diverse proteins. Mann et al., (2006) identified low levels of gallinacin-8 and β-defensin 11 as components of the chicken eggshell matrix using MS-based technology. Histones were also detected during this investigation (personal communication).

Panheleux et al., (1999) reported that the general structural organisation of different avian eggshells is generally similar with some matrix components common to the eggshells of various species but with specific differences in the ultrastructure of the mammillary layer. It was proposed that these particularities may be responsible for some of the modifications in the structural organisation of the eggshell of the different species. Panheleux et al., (1999) also reported that species of the same taxonomic family could be grouped according to their eggshell structural analogies and that some of the organic matrix components appear universal as expected for components involved in conserved biologically important roles. Comparative investigations, using antibodies against hen eggshell and egg white proteins, revealed that ovotransferrin, ovalbumin, lysozyme, osteopontin and ovocleidin-17 are apparently widespread among the eggshell matrix of neognathe birds (Panheleux et al., 1999).

Mine et al., (2003) noted that acid-soluble protein extracts from pulverized eggshell of the domestic chicken demonstrated activity against *Pseudomonas aeruginosa, Bacillus cereus, Staphylococcus aureus, Escherichia coli* and *Salmonella enteritidis*. In support with these findings, Wellman-Labadie et al. (2008c) found that eggshell protein extracts of domestic chicken inhibited the growth of *P. aeruginosa*, *S. aureus* and *B. subtilis*. The novelty of this study resides in its investigation of the role of outer eggshell and cuticle in the antimicrobial defences of the avian egg. Outer eggshell and eggshell cuticle protein extracts from wild as well as domesticated Anseriformes were also found to inhibit bacterial growth (Wellman-Labadie et al., (2008c; d). These investigations were the first to demonstrate that the outer eggshell and cuticle enhance the antimicrobial defences of

the egg in domestic as well as in wild avian species. Remarkably, cuticle and outer eggshell extracts of domestic chicken, duck and goose demonstrated identical antimicrobial activity, regardless of their phylogenic differences (Wellman-Labadie et al., 2008c).

None the less, it appears that the outer eggshell and cuticle of some avian species demonstrate unusually high antimicrobial activity. Cuticle extracts of the cavity nesting hooded merganser demonstrated total inhibition of *B. subtilis* and *S. aureus* growth while demonstrating much milder inhibition of *P. aeruginosa* and *E. coli* D31 (Wellman-Labadie et al., (2008d). Enhanced antimicrobial activity of the hooded merganser outer eggshell and cuticle appears to be another species-specific antimicrobial defence strategy present in some cavity nesting species which represents an alternative to a high c-type lysozyme content as present in the cavity nesting wood duck albumen (Wellman-Labadie et al., 2008d). Therefore, it appears that antimicrobial defences of the egg, including eggshell and albumen, may be adapted to the environmental niche of the respective species.

Role of Eggshell Proteins in Antimicrobial Defence of the Egg:

Hincke et al., (2000) demonstrated that c-type lysozyme is a component of the eggshell matrix of the domestic chicken, *Gallus gallus*. In support with this finding, Wellman-Labadie et al., (2008c; d) reported the presence of c-type lysozyme in cuticle and outer eggshell protein extracts of domestic chicken, duck and goose as well as in cuticle and outer eggshell protein extracts of wild Anseriform species. It appears that c-type lysozyme is a widespread eggshell protein of the Galliformes and Anseriformes, and is likely to be involved in antimicrobial defence. This protein has also been detected in the eggshell cuticle of some Anseriform species which contain g-type rather than c-type lysozyme in their albumens (Wellman-Labadie et al., 2008c; d). Hincke et al., (2000) reported a specific eggshell matrix localization pattern for c-type lysozyme is that is not detected for other egg white proteins. Immunofluorescence of chicken eggshell matrix using antibodies against hen egg white lysozyme verified that c-type lysozyme is largely concentrated to the cuticle and shell membranes (Wellman-Labadie et al., 2008c). In contrast to chicken, immunofluorescence of Canada goose and mute swan eggshell matrix demonstrated homogeneous distribution of c-type lysozyme throughout the

thickness of the eggshell. This difference reflects alternative species-specific antimicrobial strategies. The chicken and wood duck, with their high c-type lysozyme albumen content, may require a lower eggshell matrix c-type lysozyme content concentrated to the cuticle; a structure directly in contact with the environment and microbial threats. Alternatively, species which contain low or absent levels of c-type lysozyme within their albumens, such as Canada goose and mute swan, rely on homogeneously distributed c-type lysozyme within their eggshell matrices that may eliminate bacteria before they reach the albumen.

Hincke et al., (2000) proposed that the resistance of the chicken eggshell cuticle, calcified shell and eggshell membranes to bacterial penetration may be due in part to the presence of c-type lysozyme and that this enzyme may play an important role in the protection of the embryo against microbial contamination. Lysozyme has been successfully extracted from the chicken eggshell surface and shell membranes (Vadehra et al., 1972; Hincke et al., 2000). Vadehra et al., (1972) demonstrated that lysozyme extracted from the chicken outer eggshell retained its enzymatic activity. Wellman-Labadie et al., (2008c; d) also reported lysozyme enzymatic activity in cuticle and outer eggshell protein extracts. In addition, Wellman-Labadie et al., (2008c; d) demonstrated that immobilized hen egg white lysozyme, as occurs naturally within the eggshell matrix of many avian species, retains its enzymatic activity. Ovotransferrin, another antimicrobial egg white protein identified within the avian eggshell matrix, has also been found to retain its activity when immobilized on Sepharose 4B (Valenti et al., 1982; Gautron et al., 2001b). It is therefore likely that avian eggshell matrix proteins, including ovotransferrin and lysozyme, retain their antimicrobial activity *in situ* and are involved in the antimicrobial defence of the avian egg.

Wellman-Labadie et al., (2008c; d) reported the presence of c-type lysozyme within the eggshell matrix of the Galliformes and Anseriformes investigated (seven species in total) including within the eggshells of those species previously demonstrated to contain g-type lysozyme in their albumens. C-type lysozyme may be present within the eggshell matrix of Galliformes and Anseriformes due to its potent chitinase activity. In contrast to g-type lysozyme, c-type lysozyme possesses potent chitinase activity (Hindenburg et al., 1974). Chicken egg white lysozyme causes a rapid loss of viability among multiple *Candida albicans* isolates (Samaranayake et al., 2001). Fungal growth on the eggshells can break

down the cuticle and facilitate microbial invasion by greatly increasing the number of open pores (Cook et al., 2003; Board et al., 1964; Board and Halls, 1973; Board et al., 1979). Therefore, eggshell matrix c-type lysozyme may be an especially important antifungal protein which limits further microbial egg contamination by preventing eggshell cuticle degradation.

Outer eggshell and cuticle protein extracts of domestic (chicken, duck and goose) as well as that of wild (Canada goose, mute swan, wood duck and hooded merganser) avian species are immunoreactive against antibodies for ovoclayxin-32 (Wellman-Labadie et al., (2008c; d). Ovocalyxin-32 is an abundant chicken eggshell matrix-specific protein, localized mainly in the outer eggshell, sharing identity with the mammalian carboxypeptidase inhibitor, latexin, and TIG1, a human skin protein (Gautron et al., 2001a; Hincke et al., 2003). Recombinant *Gallus gallus* ovocalyxin-32 has recently been found to demonstrate carboxypeptidase inhibitory activity as well as inhibiting the growth of *B. subtilis* (Xing et al., 2007). Xing et al., (2007) proposed that the role of carboxypeptidase inhibitors in innate immunity as inhibitors of bacterial proteases would provide an explanation for the wide distribution of these proteins. Ovocalyxin-32 may therefore be an eggshell matrix-specific protein which enhances the antimicrobial defences of the egg (Xing et al., 2007).

Ovocleidin-17, ansocalcin, struthiocalcin, dromaiocalcin, and rheacalcin are the predominant soluble proteins of the calcified layer of the chicken, goose, ostrich, emu and rhea, respectively (Hincke et al., 1995; Mann and Siedler, 1999; Lakshminarayanan et al., 2002; Mann and Siedler, 2004; Mann and Siedler, 2006; Mann, 2004). Panheleux et al., (1999) reported immunoreactivity within the eggshell matrix of laying hen, breeder hen, pheasant, turkey, guinea fowl, duck and goose using antibodies raised against chicken eggshell ovocleidin-17. These major avian eggshell-specific matrix proteins demonstrate significant homology to C-type lectin proteins. Cash et al., (2006) reported that epithelial C-type lectins from mice and human demonstrate antimicrobial activity through peptidoglycan binding and bacterial cell wall damage.

Wellman-Labadie et al., (2008e) reported that purified ovocleidin-17 and ansocalcin bind peptidoglycan and lipopolysaccharide. This investigation is the first to demonstrate the role of purified eggshell matrix proteins in antimicrobial defence of the egg. In addition,

the purified C-type lectin-like proteins demonstrated bactericidal activity against *B. subtilis*, *S. aureus* and *P. aeruginosa* (Wellman-Labadie et al., 2008e). Ovocleidin-17 demonstrated greater bactericidal activity than ansocalcin. The bactericidal activity of both avian C-type lectin-like proteins was inhibited in the presence of bacterial polysaccharides while demonstrating enhanced activity in the presence of Ca^{2+} (Wellman-Labadie et al., 2008e). Therefore, it appears that avian eggshell specific C-type lectin-like proteins, through their bacterial polysaccharide binding activity, are likely to be involved in antimicrobial defence of the egg.

In contrast to many other eggshell antimicrobial proteins which are present within the outer eggshell, ovocleidin-17 appears to be localized mainly to the inner regions of the chicken eggshell matrix (Hincke et al., 1995). During embryonic development, the bases of the mammillary cones (calcium reserve body) are partially decalcified in order to liberate calcium subsequently used in embryonic growth and development. Through decalcification of the inner eggshell, avian eggshell matrix-specific C-type lectin-like proteins would be liberated in a Ca^{2+}-rich environment optimal for the activation of these bactericidal proteins. Therefore, avian eggshell matrix-specific C-type lectin-like proteins may enhance antimicrobial defences of the egg at the specific period where embryonic development weakens the integrity of the eggshell and exposes the embryo to microbial contamination.

Wellman-Labadie et al., (2008f) reported that ethyl acetate outer eggshell surface extracts of domestic chicken, as well as those of domestic goose, inhibited the growth of *B. subtilis* and *E. coli* D31. This is the first study to investigate lipophilic outer eggshell surface components and their role the antimicrobial defence of the avian egg. Using chicken eggs, the lipophilic antimicrobial agent was partially purified by RP-HPLC and found to inhibit the growth of *B. subtilis*, *S. aureus* and *E. coli* D31 while lacking activity against *P. aeruginosa* (Wellman-Labadie et al., 2008f). Recent MS data suggests that an antimicrobial protein sharing sequence similarity with histones was present within the purified ethyl acetate extract (Dr. K. Mann, personal communication). Histones H1 and H2B have been previously identified in ovary and oviduct tissue extracts of the domestic hen and were reported to be antimicrobial against both Gram-positive and Gram-negative bacteria (Silphaduang et al., 2006). Therefore, histone or histone derived antimicrobial

proteins may be present within the outer eggshell and enhance the antimicrobial defences of the avian egg.

The Integrated Antimicrobial Defences of the Egg and Future of Avian Egg Research:

The avian egg possesses physical and chemical mechanisms to protect against microbial invasion (Board, 1966; Board and Fuller, 1974; Board and Tranter, 1986). The greatest protection is provided by high levels of antimicrobial egg white proteins, mainly ovotransferrin and lysozyme, which are present in the albumen of avian species and prevent the proliferation of foreign microorganisms. Gautron et al., (2007) proposed that the antimicrobial activity of the egg involves the synergistic effect of many antimicrobial proteins. Reports have indicated that hen egg white lysozyme can demonstrate synergistic antibacterial activity in the presence of other antimicrobials (Yan and Hancock, 2001; Nishiyama et al., 2001; Chun and Hancock, 2000; Leitch and Willcox, 1998).

The antimicrobial properties of avian albumen are reinforced by the avian eggshell and eggshell membranes which act as a mechanical barrier. The cuticle, in direct contact with the environment, is the final component of this system. It contains antimicrobial components which, through their strategic location, provide a first line of defence. This system may be enhanced in the eggs of some species which nest under harsh environmental conditions through an increased antimicrobial content. The effectiveness of the defences of the egg depends on the environmental conditions and attributes of invading organisms (Board, 1966; Board and Fuller, 1974; Board and Tranter, 1986). Under evolutionary selection from environmental pressures experienced by different species, female birds may contribute eggshell and egg white components that compensate for greater microbial threats present under certain conditions, such as high humidity environments, and/or develop more potent antimicrobials.

Contrary to common belief, the avian egg possesses a complex, multi-component, effective defence system showing great similarity to the innate immune system of vertebrates. The eggshell and eggshell membranes are reminiscent of vertebrate skin through their common roles as primary physical barriers to infection. The eggshell is in

direct contact with the environment and contains multiple antimicrobial proteins. The egg and vertebrate innate immune system share in common antimicrobial proteins such as lysozyme and transferrins. The egg white fluid, with its high levels of transferrins and lysozyme, is reminiscent of vertebrate serum and other biological fluids providing chemical defence.

In a process akin to the inflammatory response of mature vertebrates, microbial contamination can activate antimicrobial defences since proteolytic digestion and acidification of the albumen as well as physical damage of the eggshell results in the localised release and activation of antimicrobial proteins. In addition, the increased temperature during incubation of the egg results in enhanced antimicrobial activity and further limits infection as occurs during the vertebrate inflammatory response. As suggested by Ahlborn et al., (2006), the complex interactions among all of the physical and chemical components within the intact egg provide protection to the developing embryo.

A clearer insight into the antimicrobial defences of the avian egg would have important implications for the poultry industry. The demonstration of the role of the eggshell and cuticle in antimicrobial defence may lead to changes in the current processing and handling of eggs, as egg washing chemicals may damage the eggshell and weaken intrinsic egg defences (Kim and Slavik, 1996). In addition, antimicrobial proteins of the egg, such as lysozyme and ovotransferrin, represent ideal conservation agents which are already in use in the food industry. Similar antimicrobial egg components are likely to be identified in the near future and represent molecules of economic and medical importance.

Further studies investigating the biological activity of individual egg and eggshell components are needed. Research should also be conducted on the eggs of species other than chicken since, although a significant model organism, the chicken represents a miniscule fraction of the available species. Such studies would demonstrate the diversity of the antimicrobial components present in the egg and allow a better understanding of the effects of environmental pressures on the antimicrobial defences of the avian embryo. In addition, these studies would identify optimal sources for the most active therapeutical components present in eggs. Future developments in the field of proteomics,

transcriptomics and bioinformatics will facilitate the identification of these antimicrobial agents which represent an appealing alternative to conventional antibiotics.

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