Eggshell Membrane Proteins provide Innate Immune Protection

Cristianne Martins Monteiro Cordeiro

This Thesis is submitted to the
Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements
for the Doctorate in Philosophy degree in
Cellular and Molecular Medicine

Department of Cellular and Molecular Medicine
Faculty of Medicine
University of Ottawa

© Cristianne Martins Monteiro Cordeiro, Ottawa, Canada, 2015

Abstract

The microbiological safety of avian eggs is a major concern for the poultry industry and for consumers due to the potential for severe impacts on public health. Innate immune defense is formed by proteins with antimicrobial and immune-modulatory activities and ensures the protection of the chick embryo against pathogens. The objective of this project was to identify the chicken eggshell membrane (ESM) proteins that play a role in these innate immune defense mechanisms.

We hypothesized that ESM Ovocalyxin-36 (OCX-36) is a pattern recognition protein, and characterized purified ESM OCX-36. OCX-36 has antimicrobial activity against *S. aureus* and binds *E. coli* lipopolysaccharide (LPS) and *S. aureus* lipoteichoic acid (LTA). We additionally investigated the OCX-36 nonsynonymous single nucleotide polymorphisms (SNPs) at cDNA position 211. The corresponding isoforms (proline-71 or serine-71) were purified from eggs collected from genotyped homozygous hens. A significant difference between Pro-71 and Ser-71 OCX-36s for *S. aureus* LTA binding activity was observed. From these experiments, we confirmed the hypothesis that OCX-36 is a pattern recognition molecule. We also found that OCX-36 has anti-endotoxin properties and is a macrophage immunostimulator to produce NO and TNF-α. Digested OCX-36 down-regulated the expression of genes involved in LPS signaling and inflammatory responses. Moreover, OCX-36-derived peptides inhibited the production of LPS-induced pro-inflammatory mediators associated with endotoxemia *in vivo*.

Quantitative proteomics analysis of ESMs was performed to evaluate changes in ESM protein abundance during chick embryonic development. Bioinformatics analysis revealed enrichment of proteins associated with antimicrobial and immune protection, vascularization, calcium mobilization and lipid transport, which are vital for chick embryonic development. In unfertilized eggs, protease inhibitors and antimicrobial proteins were enriched.

In summary, the ESMs are enriched in proteins with antimicrobial, antioxidant and immune-modulatory properties, which aid in the development of the chick embryo and protect the embryo and unfertilized egg against pathogen invasion.

Table of Contents

Abstract………………………………………………………………….... ii
Table of contents……………………………………………………….... iv
List of tables……………………………………………………………… vii
List of figures.……………………………………………………….…..... ix
List of abbreviations.…………………………………………………….. xii
Acknowledgments.……………………………………………………….. xv

I. Chapter 1. Introduction………………………………………………… 1
11. Avian Egg…………………………………………………………….... 2
1.1. Formation and Structure of Avian Egg……………………………… 4
1.1.1. Egg yolk: Formation, Structure and Function…………………….. 6
1.1.2. Egg white: Formation, Structure and Function…………………..... 9
1.1.3. Eggshell Membranes: Formation, Structure and Function……..... 11
1.1.4. Eggshell: Formation, Structure and Function…………………….... 11
1.1.5. Embryo: Formation, Structure and Function……………………… 14
2. Defenses of the Avian Egg……………………………………………. 18
2.1. Physical Innate Immune Defenses…………………………………. 19
2.1.1. Eggshell……………………………………………........................ 19
2.1.2. Eggshell Membranes (ESMs)……………………………………… 20
2.2. Chemical Innate Immune Defenses……………………………….. 24
2.2.1. Lysozyme…………………………………………………………….. 25
2.2.2.Ovotransferrin………………………………………………………… 25
2.2.3. Avian Beta-Defensins (AvBDs)…………………………………….. 26
2.2.4.Bactericidal permeability-increasing protein (BPI)/ lipopolysaccharide-binding protein (LBP)/ palate, lung and nasal epithelial clone (PLUNCs) protein family……………………………………………………………………. 27

Hypotheses…………………………………………………………………….. 32
Thesis objectives…………………………………………………………..... 32
Thesis outline………………………………………………………………... 33

V. Chapter 2. Ovocalyxin-36 is a pattern recognition protein in chicken eggshell membranes…………………………………………………………….. 35
1. Abstract………………………………………………………………… 37
2. Introduction……………………………………………………………….. 38
3. Materials and Methods…………………………………………………... 41
4. Results……………………………………………………………………….. 51
5. Discussion………………………………………………………………… 77
6. References……………………………………………………………….. 85

VI. Chapter 3. Ovocalyxin-36 is an effector protein modulating the production of proinflammatory mediators membranes…………………. 94
1. Abstract………………………………………………………………… 98
2. Introduction……………………………………………………………….. 99
3. Materials and Methods…………………………………………………... 101
4. Results……………………………………………………………………….. 108
5. Discussion………………………………………………………………… 126
6. References……………………………………………………………….. 133

VII. Chapter 4. Quantitative proteomic analysis of eggshell membrane proteins in fertilized and unfertilized eggs during chick embryo development………………………………………………………………… 141
1. Abstract………………………………………………………………… 142
2. Introduction……………………………………………………………….. 144
3. Materials and Methods…………………………………………………... 146
4. Results……………………………………………………………………….. 153
5. Discussion………………………………………………………………… 172
6. References……………………………………………………………….. 187

VIII. Chapter 5. Discussion………………………………………………. 221
1. Avian Innate host defense…………………………………………… 222
1.1. Proteins involved in innate immune protection of chicken eggs and developing embryo……………………………………………………….. 227
1.2. OCX-36 as a pattern recognition protein in innate immune protection of chicken eggs………………………………………………. 229
1.3. Enhancing innate immune defenses using proteins that modulate the production of proinflammatory mediators…………………………….. 232
IX. Conclusions…………………………………………………………… 234
X. References…………………………………………………………….. 236
XI. Authorizations……………………………………………………… 269

List of Tables

Chapter 2

Table 1. Primer pairs used for boost/nested PCR……………….... 48

Table 2. Purification methods investigated for OCX-36 purification……. 57

Table 3. Merged proteomic results for purified OCX-36 samples using two independent samples……………………………………………….. 59

Table 4. Densitometry analysis of OCX-36……………………………….. 62

Table 5. Allelic frequencies of OCX-36 SNPs in Pedigree White Leghorn birds……………………………………………………………... 65

Chapter 3

Table 1. Differentially expressed genes in RAW 264.7 cells treated with OCX-36, dOCX-36, LPS, or LPS+dOCX-36……………………………… 119

Chapter 4

Table 1. Yield of soluble proteins extracted from eggshell membranes……………………………………………………………….. 152

Table 2. List of eggshell membrane proteins identified for the first time in our study…………………………………………………………….... 156

Table 3. Functional annotation analysis for abundant eggshell membrane proteins from fertilized eggs during embryonic development………………………………………………………………… 167

Table 4. Presence and absence of proteins identified in the blood, chorioallantoic membrane (CAM) and eggshell membrane (ESM) from fertilized eggs at day 19……………………………………………............ 169

Table S1. Solubilization of eggshell membranes……………………….. 195
Table S2. List of proteins identified in the eggshell membranes from fertilized eggs at day 0…………………………………………………… 196
Table S3. List of proteins identified in the eggshell membranes from fertilized eggs at day 3…………………………………………………… 198
Table S4. List of proteins identified in the eggshell membranes from fertilized eggs at day 7…………………………………………………… 201
Table S5. List of proteins identified in the eggshell membranes from fertilized eggs at day 11………………………………………………….. 203
Table S6. List of proteins identified in the eggshell membranes from fertilized eggs at day 15………………………………………………….. 206
Table S7. List of proteins identified in the eggshell membranes from fertilized eggs at day 19………………………………………………….. 209
Table S8. List of proteins identified in the eggshell membranes from unfertilized eggs at day 0………………………………………………… 211
Table S9. List of proteins identified in the eggshell membranes from unfertilized eggs at day 3……………………………………………… 213
Table S10. List of proteins identified in the eggshell membranes from unfertilized eggs at day 7……………………………………………… 215
Table S11. List of proteins identified in the eggshell membranes from unfertilized eggs at day 11……………………………………………….. 217
Table S12. List of proteins identified in the eggshell membranes from unfertilized eggs at day 15……………………………………………….. 219
Table S13. List of proteins identified in the eggshell membranes from unfertilized eggs at day 19……………………………………………….. 220

List of Figures

Chapter 1

Fig. 1. Longitudinal section to depict the interior contents of a chicken egg…………………………………………………………………………… 3

Fig. 2. Stylized depiction of the reproductive system of the hen, containing an incomplete egg in the uterus……………………………… 5

Fig.3. The three-tissue layers of the chick mature CAM……………….. 16

Fig.4. Scanning electron micrograph illustrating the morphology of the eggshell and eggshell membranes (ESM)……………………………….. 23

Fig 5. Immunofluorescence of OCX-36 in eggshell cross section…….. 31

Chapter 2

Fig.1. Western blot analysis for OCX-36 extracted using different extraction buffers…………………………………………………………. 54

Fig.2. SDS-PAGE and Western blot analysis during OCX-36 purification………………………………………………………………… 55

Fig.3. Proteomic analysis of purified OCX-36 samples (preparations 90 and 120)……………………………………………………………………… 58

Fig.4. OCX-36 enrichment during extraction and purification…………… 60

Fig.5. Antimicrobial activity of OCX-36 against Gram-positive (*L. monocytogenes, E. faecalis* and *S. aureus*) and Gram-negative bacterial strains (*P. aeruginosa, E.coli and S. typhimurium*)………… 67

Fig.6. Bacteriostatic activity of OCX-36 against *S. aureus* ATCC 6538………………………………………………………………………. 68

Fig.7. Antimicrobial activity of OCX-36 polymorphic forms from genotyped eggs (Pro-71 and Ser-71), and the OCX-36 mixture from standard eggs against *S. aureus* ATCC 6538………………………….. 70
Fig.8. LPS binding activity of purified OCX-36 and rhLBP towards biotinylated *E.coli* O111: B4 LPS……………………………………….. 72
Fig.9. LPS and LTA binding activity of OCX-36 (mixture) and OCX-36 individual forms (Pro-71 and Ser-71)……………………………………… 75

Chapter 3

Fig.1. Effect of OCX-36 on TNF-α and NO secretion in LPS-stimulated RAW 264.7 cells……………………………………………………….. 110
Fig.2. Effect of OCX-36 on TNF-α and NO production in LPS-stimulated RAW 264.7 cells in the absence of FBS………………………………... 112
Fig.3. SDS-PAGE analysis of OCX-36 digested with pepsin and thermolysin…………………………………………………………………... 115
Fig.4. Effect of OCX-36-derived peptides (dOCX-36) on TNF-α and NO secretion in LPS-stimulated RAW 264.7 cells……………………………. 116
Fig.5. Effect of OCX-36 and dOCX-36 on LPS-induced weight loss in mice…………………………………………………………………………... 122
Fig.6. Effect of OCX-36 and dOCX-36 on TNF-α and IL-6 concentrations in the (A) serum and (B) liver of LPS-treated mice…….. 123
Fig.7. (A) Cytokine and (B) MPO concentrations in the ileum of LPS-treated mice……………………………………………………………… 125

Chapter 4

Fig.1. Venn diagrams representing the specific and overlapping proteins identified in the fertilized and the unfertilized eggshell membrane conditions at combined days 0, 3, 7, 11, 15 and 19 of chick embryonic development…………………………………………………… 155

Fig.2. Heat-map showing an overview of comparative analysis of the eggshell membrane proteins with different profiles of abundance found in the fertilized and unfertilized eggs at days 0, 3, 7, 11, 15 and 19 of incubation………………………………………………………………… 160

Fig.3. Venn diagrams representing the specific and overlapping proteins identified in the fertilized eggshell membranes, blood and chorioallantoic membrane at day 19 of embryonic development……….. 171

Chapter 5

Fig.1. Recognition and neutralization of LPS in the human body……….. 225

List of Abbreviations

ACE	Angiotensin-I converting enzyme
AvBD-11	Avian beta defensin-11
ANSC	Adjusted normalized spectra counts
BCA	Bicinchoninic acid
BPI	Bactericidal permeability-increasing protein
BW	Body weight
CAM	Chorioallantoic membrane
CCL2	MCP-1, monocyte chemotactic protein-1
CREMP	Cysteine rich eggshell membrane protein
CXCR4	Chemokine receptor 4
Cybβ	Cytochrome b-245, beta polypeptide
DAVID	Database for Annotation, Visualization and Integrated Discovery
DMEM	Dulbecco's modified Eagle's Medium
dOCX-36	Digested Ovocalyxin-36
DTT	Dithiothreitol
ESM	Eggshell membrane
EDIL3	EGF-like repeats and discodin I-like domains 3
GO	Gene ontology
HRP	Horseradish peroxidase

HTAB	Hexadecyltrimethyl ammonium bromide
iNOS	Inducible nitric oxide
FDR	False discovery rate
LAL	Limulus Amebocyte Lysate
LC/MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LBP	Lipopolysaccharide-binding protein
LTA	Lipoteichoic acid
3-MPA	3-Mercaptopropionic acid
MPO	Myeloperoxidase
MWCO	Molecular weight cut-off
MyD88	Myeloid differentiation primary response gene 88
NO	Nitric oxide
NSC	Normalized spectra counts
OCX-32	Ovocalyxin-32
OCX-36	Ovocalyxin-36
OC-116	Ovocleidin-116
PAMPs	Pathogen-associated molecular patterns associated molecular patterns
Pglyrp1	Peptidoglycan recognition protein 1
PLUNC	Palate, lung, and nasal epithelium clone
PMSF	Phenylmethanesulfonyl fluoride

PRR	Pattern recognition molecule
PROC	Protein C
TENP	Transiently expressed in neural precursor
TCEP	Tris ((2-carboxyethyl) phosphine hydrochloride)
TMB	Tetramethylbenzidine
TLR	Toll-like receptor
VLDL	Very low-density lipoprotein

Acknowledgments

I would like to extend my sincere thanks and appreciation to Dr. Maxwell Hincke for being a great supervisor. I do appreciate his guidance, dedication and valuable advices during my Ph.D. thesis. He has given me a chance to gain knowledge and professional experiences through presenting my scientific work at international conferences and meetings. I am ever grateful to him for the opportunity to work in his lab.

Thank you also to the members of my thesis advisory committee, Dr. Illimar Altosaar, Dr. John T. Arnason and Dr. Simon Lemaire for valuable discussions and advice in my doctoral learning experience.

I am also grateful for the financial support I received from the Canadian Natural Sciences and Engineering Research Council (NSERC) Strategic and Discovery grants to Dr. Hincke.

Thanks to Hamed Esmaili for his technical support and to all labmates and colleagues for the encouragement, help and friendship.

Many thanks and love to my son Lucas for filling my life with happiness. Thanks for my parents Adelino and Lena for their love, support and for teaching me to always pursue my dreams.

CHAPTER 1

I. Introduction

General Introduction

Chicken eggs are a popular and inexpensive nutritive source. Eggs contain high-quality proteins, fatty acids, vitamins and minerals needed to maintain good health for humans (Seuss-Baum, 2007; Nys, Bain and Van Immerseel, 2011). Based on data from Statistics Canada, egg production in Canada was 600 million dozen (~7.2 billion eggs) in 2014. Agriculture and Agri-Food from Canada reported that about 70% of Canada's total egg production is directed to the table egg market whereas the remaining 30% is used for industrial egg-based products (liquid or frozen form). An increase in egg consumption has been observed from 1995 to 2013 with a per capita consumption of 21 dozens in 2013. Egg-borne diseases are a human health concern, which also have an economic impact on the poultry industry (EFSA, 2009). However, avian eggs have a complex system of physical and chemical innate immune protective mechanisms, including the shell and the eggshell membranes (ESM), to prevent pathogen invasion which can cause chick embryo mortality and human diseases (Board and Tranter, 1995; Hincke et al., 2011). My research program has sought to identify and explore the functions of selected ESM protein candidates that participate to protect the egg and the developing embryo from physical and microbial assaults, in order to improve our understanding about the egg protective mechanisms. Understanding these egg protective mechanisms will be an important assist to mitigate the risk of bacterial contamination of both table eggs and fertilized eggs. Consequently, this

information will lead to food safety improvements for the consumer and enhanced knowledge of the key factors at work in the protection of chick embryo development.

1. Avian Egg

Many physiological, biochemical, nutritional, structural and morphological studies have been conducted on avian eggs and most of these have utilized the egg of the domestic chicken, due to its availability and commercial importance as a nutritious food for human consumption (Hincke et al., 2012). I have adopted this convention in the course of this introduction. The avian egg is composed of four main compartments including the yolk, albumen (egg white), ESM and the calcified eggshell (Hincke et al., 2008) (Fig.1). Fertilized and unfertilized avian eggs share these egg compartments. However, some structures, such as the extra-embryonic membranes, only develop during incubation of the fertilized egg and development of the embryo (Sheng, 2010; Bellairs and Osmard, 2014).

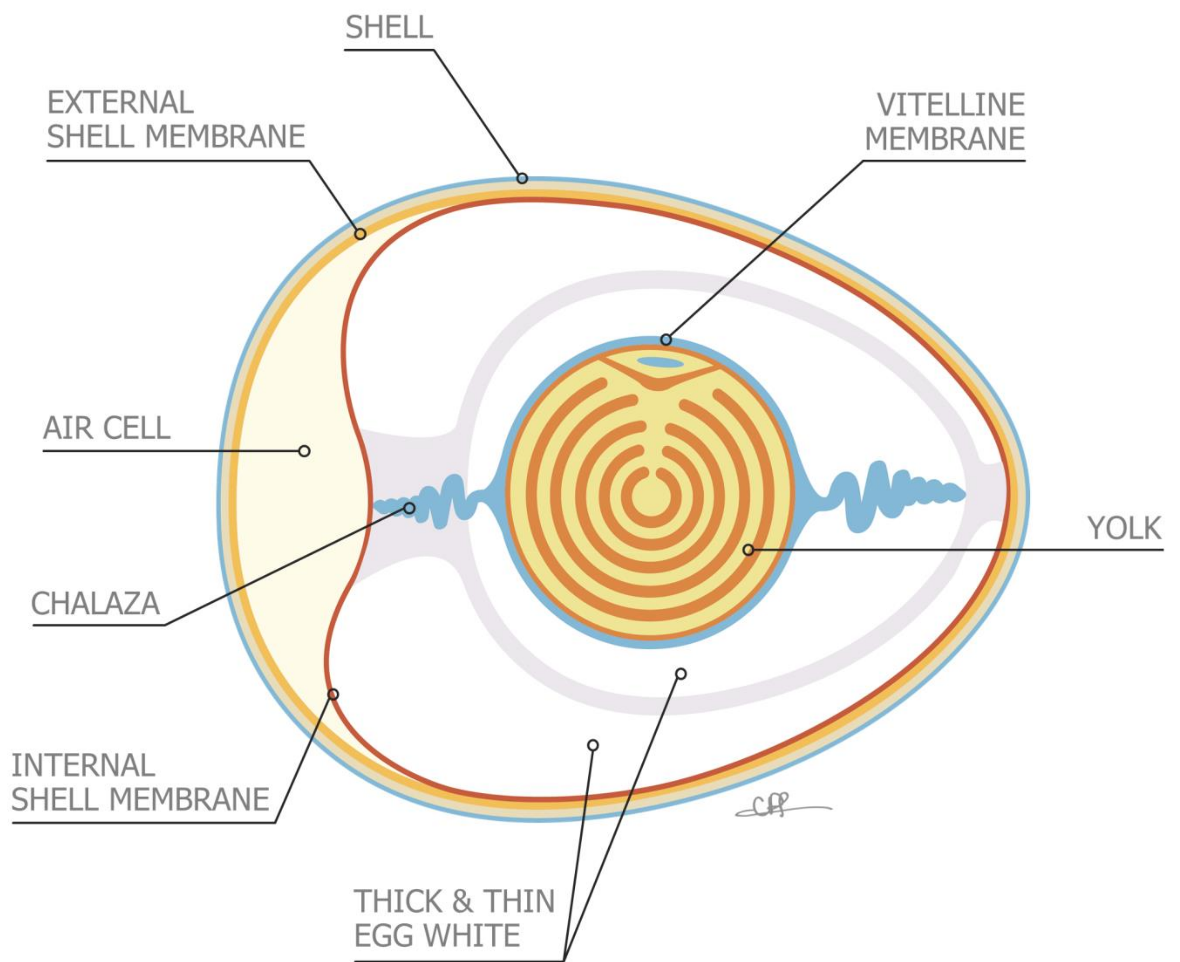


Fig. 1. Longitudinal section to depict the interior contents of a chicken egg. Source: Hincke et al., 2012. The eggshell: structure, composition and mineralization. *Frontiers in Bioscience.* 17:1266-80, with permission from Frontiers in Bioscience.

1.1. Formation and Structure of the Avian Egg

The hen ovary and oviduct are responsible for the process of egg formation. The ovary regulates the accumulation of egg yolk proteins and the maturation of the oocyte. The avian oviduct has specialized segments: infundibulum, magnum, white and red isthmus, uterus and vagina. The forming egg acquires its layers as it transverses this tubular organ (Hincke et al., 2012) (Fig.2).

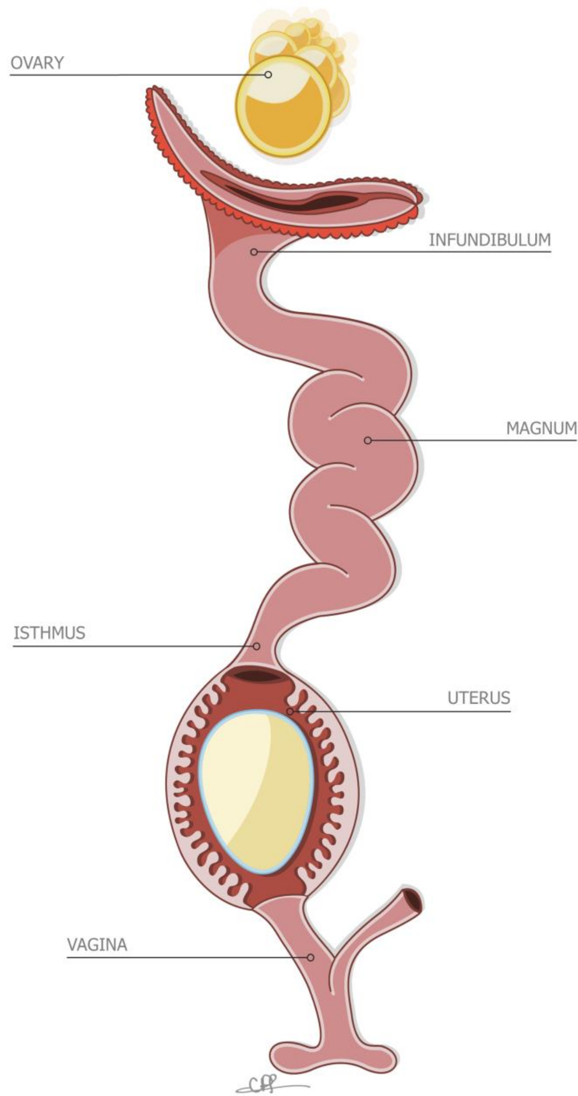


Fig. 2. Stylized depiction of the reproductive system of the hen, containing an incomplete egg in the uterus. Source: Hincke et al., 2012. The eggshell: structure, composition and mineralization. *Frontiers in Bioscience.* 17:1266-80, with permission from Frontiers in Bioscience

1.1.1. Egg yolk: Formation, Structure and Function

The synthesis of egg yolk precursors takes place in the liver of laying hens, which are secreted into the plasma and transferred via the bloodstream. The egg yolk is transferred to the oocyte by specific surface receptor-mediated endocytosis (Griffin et al., 1984; Retzek et al., 1992; Schneider et al., 2009).

The composition of egg yolk consists of water, vitamins (e.g., A, D, E and K), ions (calcium, phosphate, iron and zinc, etc.), unsaturated fatty acids (e.g., oleic acid, linoleic acid and palmitoleic acid) and saturated fatty acids (e.g., palmitic acids, stearic acid and myristic acid) and proteins (e.g., serum albumin, vitellogenin-derived products and apovitellenins) (Shenstone, 1968; Burley and Vadehra, 1989; Tokarski et al., 2006; Mann and Mann, 2008; Sunwoo and Gujral, 2014). Egg yolk is an extremely bioavailable source of lecithin, lutein and zeaxanthin due to their association with the lipid matrix. The yellow color of the chicken yolk is caused by lutein and zeaxanthin carotenoids known as xanthophylls (Handelman et al., 1999; Farinazzo et al., 2009). Hen egg yolk is formed by mainly low-density lipoproteins (LDL) such as apolipoprotein B (apo B), high-density lipoproteins (HDL), and livetins such as immunoglobulins (γ-livetin), serum albumin (α-livetin), and glycoproteins (β-livetin), and phosvitins (McCully et al., 1962; Williams, 1962).

Among the egg proteins, chicken serum albumin (α-livetin) is present in the egg yolk, and its primary function is the transport of metal ions in the bloodstream (Predki et al., 1992). Chicken immunoglobulins (γ-livetin) are found in hen blood serum, and these maternal hen antibodies (IgY) are accumulated in egg yolk to

provide acquired immunity to the chick (Schade et al., 2005). Chicken vitellogenins (I, II and III) are the main yolk precursor proteins synthesized in the liver, and their synthesis/secretion is dependent on estrogen stimulation (Byrne et al., 1989). Vitellogenin II is abundant in the egg yolk, and it can be proteolytically cleaved into a small number of fragments such as lipovitellin and phosvitin (Deeley et al., 1975). Apolipoproteins, involved in the recruitment of lipids for the growing embryo, are associated with very low-density lipoprotein (VLDL) of egg yolk and protect its breakdown during transport from the liver to oocytes (Romanoff, 1967; De Oliveira et al., 2008).

During ovulation, the ovarian follicle releases a mature oocyte (the yolk) that passes into the oviduct; the yolk acquires the egg layers as it passes through particular regions of the oviduct (Nys et al., 2004). The yolk is released into the infundibulum of the oviduct, where the vitelline membrane (VM) is secreted and surrounds the yolk (Nys et al., 2004). The VM is formed by proteinaceous layers that surround the yolk-filled oocyte (Jensen, 1969; Burley and Vadehra, 1989). The inner layer of VM (perivitelline layer) faces the oocyte, whereas the outer layer that faces the egg white is produced in the infundibulum. The VM proteinaceous extracellular matrix separates the yolk from egg white proteins, and is a barrier against pathogen invasion (Bellairs and Boyde, 1963; Bain and Hall, 1969; Mann, 2008).

Most of the proteins of the VM are also found in other egg compartments, such as the egg white proteins lysozyme C, ovalbumin, ovotransferrin, and ovomucin. However, specific VM proteins include zona pellucida (ZP) proteins

including ZP1, ZP3, ZPC, ZPD and ZP, oviductin protease, and two ATPases (Waclawek et al., 1998; Takeuchi et al., 1999; Bausek et al., 2000, Mann, 2008). The outer layer contains some egg white proteins (i.e. ovomucin and lysozyme) and vitelline outer membrane proteins (VMO I and VMO II) (Back et al., 1982; Debruyne et al., 1982; DeBoeck et al., 1986; Kido et al., 1992). Previous work showed that VMO II corresponds to avian beta-defensin 11 (AvBD-11) (Mann, 2008). In addition, some egg yolk proteins (serum albumin, immunoglobulins, apovitellin, apolipoprotein B) and other proteins (mucins, additional ZP proteins), serine proteases (similar to oviductin protease, similar to transmembrane serine protease 9) Na-K ATPase, ecto-ATP-diphosphohydrolase, ovocalyxin-32 and ovocalyxin-36, ovocleidin-17 and ovocleidin-116, olfactomedin I, semaphorin C3, actin and filamin) are also detected in the VM (Mann, 2008).

In the mature egg, the egg yolk is a single cell, suspended by spiral structures extending from the yolk into egg white at each end, which are known as chalazae (Fromm, 1966; Itoh et al., 1990). The chalazae are a structure of the avian egg which suspend the yolk in the albumen and prevent the yolk from being damaged during incubation, and thus allow healthy embryonic development (Fromm, 1966; Itoh et al., 1990). The yolk has high nutritive value for humans and the developing chick embryo (Farinazzo et al., 2009; Réhault-Godbert et al., 2014).

1.1.2. Egg white: Formation, Structure and Function

After acquiring its VM layer, the yolk passes into the magnum section of the oviduct, which secretes the egg white during its 4hr passage (Fernandez et al., 1997; Nys et al., 2004; Nys and Guyot, 2011; Hincke et al., 2012). The egg white (albumen) is 60% of total egg weight, and its main constituents are water, ions and proteins (Sugino et al., 1997). The most abundant egg white proteins are ovalbumin, ovotransferrin, lysozyme, and ovomucoid (Guérin-Dubiard et al., 2006). However, avidin, cystatin, and ovoinhibitor are also proteins present in the chicken albumen (Sugino et al., 1997; Tankrathok et al., 2009). Recent proteomic studies identified more than 148 proteins in egg white, including ovalbumin-related X, ovalbumin-related Y, chondrogenesis-associated lipocalin (prostaglandin H2 D-isomerase), ovoglycoprotein, transiently expressed in neural precursor (TENP), clusterin, Hep 21 and riboflavin-binding protein (Guérin-Dubiard et al., 2006; Mann, 2007; D'Ambrosio et al., 2008).

The egg white is a source of nutrients for the developing chick and contains antimicrobial proteins to protect the embryo against pathogens (Muramatsu et al., 1990; Cook et al., 2005; Alabdeh et al., 2011). The most abundant antimicrobial proteins found in the egg white are lysozyme and ovotransferrin (Hincke et al., 2000). Lysozyme is 3% of egg white and exhibits a strong antimicrobial activity against Gram-positive bacteria (Hughey and Johnson, 1987). Both lysozyme and its derived peptides have antimicrobial activity against Gram-positive bacteria such as *Bacillus stearothermophilus*, *Clostridium tyrobutyricum*, *Escherichia coli* and *Staphylococcus aureus* (Losso et al., 2000, Pellegrini et al., 2000; Mine et al.,

2004). In addition, an antimicrobial activity of lysozyme against Gram-negative bacteria in combination with EDTA, organic acid or in association with a hydrophobic carrier was observed to cause damage to the bacterial membrane (Losso et al., 2000, Ibrahim et al., 2001). Oral and topical applications of lysozyme are effective in preventing and controlling viral infections (Sava, 1996; Lee-Huang et al., 1999).

Ovotransferrin is an iron-binding glycoprotein which represents 12% of the chicken egg white. Ovotransferrin has antimicrobial activity against a variety of Gram-positive and Gram-negative bacteria as well as antiviral activity (Tranter and Board, 1982; Oratore et al.,1990; Giansanti et al., 2005). The antimicrobial activity mechanism of ovotransferrin relies on the iron chelation, which leads to the deprivation of iron which is necessary for bacterial growth (Garibaldi, 1970).

Ovalbumin is about 54% of egg white and a potential egg white antimicrobial protein, since antimicrobial peptides derived from digestion of ovalbumin by chymotrypsin possess antimicrobial activity against *Bacillus subtilis* (Pellegrini et al., 2004). In addition, ovalbumin-related protein X and ovalbumin-related protein Y are members of this serpin family, and exhibit protease inhibitory activity. Ovalbumin related protein X also shows antimicrobial activity against Gram-positive and Gram-negative bacteria (Benarafa et al., 2005; Réhault-Godbert et al., 2013). In addition, some egg white proteins with protease inhibitory activity including ovoinhibitor, ovomucoid, ovostatin, and cystatin possess antimicrobial activity (Molla et al., 1987; Miyagawa et al., 1991; Miyake et al., 2000; Wesierska et al., 2005). Natural and synthetic peptides derived from ovomucoid also have

immunomodulator properties. Synthetic ovomucoid peptides induce T-cell secretion of cytokines interleukin- (IL) 4, IL 10, IL-13, interferon- (IFN) gamma, and IL-6 (Holen et al., 2001). Peptides derived from ovomucin stimulate macrophage activity *in vitro* (Tanizaki et al., 1997).

1.1.3. Eggshell Membranes: Formation, Structure and Function

Eggshell membranes (ESM) are a meshwork of interlaced fibers that are organized into inner and outer layers that surround the egg albumen (Wong et al., 1984; Arias et al., 1991; Hincke et al., 2012) (Fig.4C) The ESM are formed in about 1 h while the forming egg remains in the white isthmus portion of the oviduct (Fernandez et al., 1997; Nys et al., 2004; Hincke et al., 2012). The inner ESM does not become calcified whereas the fibers of the outer ESM will become mineralized at discrete sites (Arias et al., 1993; Nys et al., 2004).

The ESMs are imperative for eggshell mineralization since they provide a fibrous support upon which shell biomineralization takes place. The ESM and associated eggshell are essential for embryonic development by providing physical and chemical protection against pathogen invasion (Nys et al., 2004). More information about the ESM are provided in section 2.1.2.

1.1.4. Eggshell: Formation, Structure and Function

The eggshell is assembled as the egg transverses the red isthmus (tubular shell gland) and subsequently the uterus (pouch shell gland) where it remains for up to 17h (Nys et al., 1999; Fernandez et al., 2001). The chicken eggshell is a

highly specialized mineralized structure resulting from the secretion of the organic matrix (eggshell matrix) which regulates the deposition of calcium carbonate in the form of calcite (Hincke et al., 2010; Hincke et al., 2012). Overall the eggshell is composed by 95% calcium carbonate (calcite polymorph), and 3.5% proteins and proteoglycans that constitute the shell organic matrix (Nys et al., 2004; Hincke and Gautron, 2010). The structure of eggshell is formed by four calcified layers (Solomon, 1991; Nys et al., 1999) (Fig. 4A). This porous bioceramic results from the sequential deposition of its layers while it remains within the uterus over an extended period of time (Nys and Gautron, 2007). These layers are the mammillary layer, palisade layer, vertical crystal layer and cuticle (Parsons, 1982; Solomon, 1991) (Fig. 4A).The inner layer of the calcified shell is composed of the mammillary cones. The mammillary layer consists of randomly orientated calcite ($CaCO_3$) crystals in cones-like structures. The tips of the mammillary cones are penetrated by the outer ESM fibers at specific sites (Cain and Heyn, 1964; Mann et al., 2006). The main calcified portion of the shell is the palisade layer that consists of roughly parallel and elongated calcite crystals. The palisade layer extends from the bases of the mammillary cones and ends in the vertical crystal layer. The vertical layer is a thin layer of vertically oriented crystallites underneath the cuticle (Parsons, 1982; Nys and Gautron, 2007). The outer layer of the avian eggshell is the cuticle that is an organic layer deposited on the surface of the egg at the final phase of shell calcification (termination) (Dennis et al., 1996; Hincke et al., 2008; Rose-Martel et al., 2012). The eggshell cuticle is a thin noncalcified layer that is secreted on the mineral surface of the shell. The inner zone of cuticle contains a thin film of

hydroxyapatite crystals and eggshell pigments whereas the outer zone remains non-mineralized (Dennis et al., 1996; Hincke et al., 2008). The cuticle is composed of glycoprotein, polysaccharides, lipids and inorganic phosphorus including hydroxyapatite crystals (Dennis et al., 1996; Whittow, 1999; Fernandez et al., 2001). The cuticle is a porous structure and it permits gaseous exchange through the pores of the eggshells (Guru and Dash, 2014). The cuticle covers the outer surface of eggs and fills its pores, forming a barrier. This barrier reduces the flux of water across the shell, preventing dehydration of the egg interior, and inhibits bacterial shell invasion through the pores (Lunam and Ruiz 2000; De Reu et al., 2006).

The biomineralization process to form the eggshell is divided into three stages: initiation of crystal growth (crystal deposition of crystal to form the mammillary cones), linear crystal growth (palisade layer) and termination of mineralization with the deposition of cuticle layer (Nys et al., 1991).These stages are important to the eggshell texture and microstructure as well as its mechanical properties (Gautron, Hincke and Nys, 1997; Hincke et al., 2010). At the initiation stage, in the red isthmus, organic aggregates are deposited on the surface of the outer ESM (approximately 4.5 h postovulation) of the forming egg at a quasi-periodical, but randomly located sites where heterogeneous nucleation of calcium carbonate occurs in the form of polycrystalline aggregation. During the next phase, linear growth stage (between 10 and 22 h postovulation), the forming egg is fully inflated and rotates in the uterus. The eggshell is mineralized until shell deposition

is arrested at 22 h postovulation, and two hours before oviposition (expulsion) (Nys et al., 1999; Nys et al., 2004; Hincke et al., 2012).

The calcified eggshell is the first physical barrier of the egg that resists microbial invasion. It also protects the embryo from physical damage, allows the exchange of metabolic gases and water and provides calcium to the chick embryo (Nys et al., 2004; Hincke et al., 2011; Hincke et al., 2012).

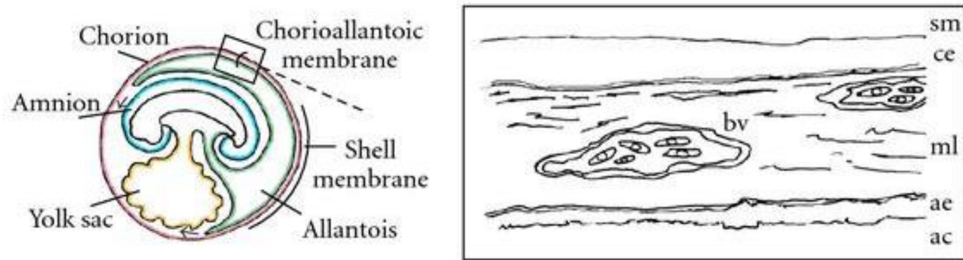
1.1.5. Embryo: Formation, Structure and Function

The forming egg transverses the infundibulum in about 15 min; it may become fertilized if a spermatozoa penetrates the oocyte at the germinal disc at this point (Olsen and Fraps, 1944; Howarth and Digby, 1973; Birkhead et al., 1994)(Fig.3). In fertilized eggs, the growth and differentiation of the chick embryo occurs during incubation following oviposition. As it develops, the embryo becomes surrounded by four extra-embryonic membranes that include the yolk sac, amnion, chorion and allantois (Sheng, 2010; Bellairs and Osmard, 2014). These extra-embryonic membranes develop at the same time as the embryonic tissues according to a precise temporal sequence, but they are discarded at hatching (Sheng, 2010; Bellairs and Osmard, 2014). Intrauterine chick embryonic development starts after fertilization in the infundibulum and continues during the next ~ 22 h until the deposition of the calcified shell is completed within the uterus and oviposition occurs (Romanoff, 1960). The development of the chick embryo then continues when incubated during the extrauterine phase; in chickens an incubation period of 21 days is necessary before hatching; this process has been

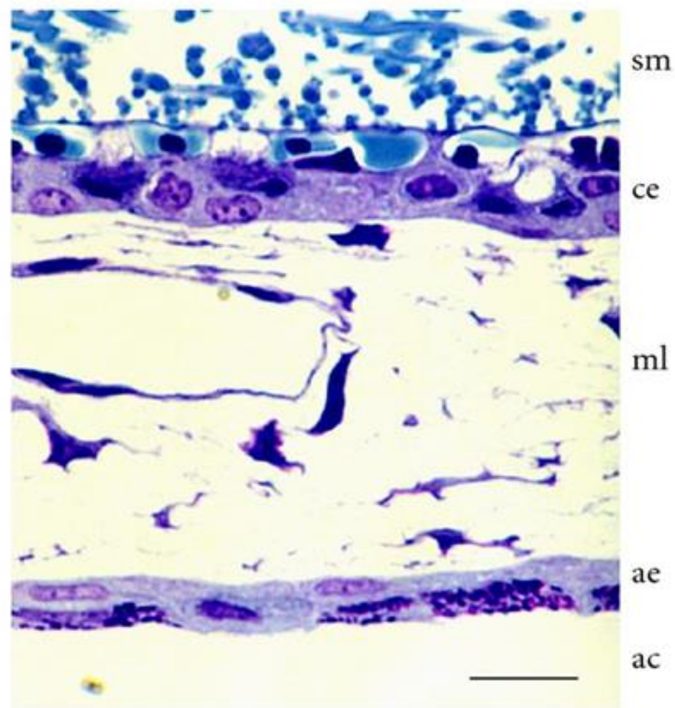
divided into three phases by Moran (2007): establishment of germ (days 0 to 7) embryo completion (days 8 to 18) and emergence (days 19, 20 and 21) (Romanoff, 1960; Moran et al., 2007). The chick embryo is structurally completed around 14 days of incubation, and the embryo then starts to move into position for hatching by turning its head toward the large end of the egg (Tong et al., 2013).

The extra-embryonic membranes protect the chick embryo from pathogens, regulate the extraembryonic fluid retention and aid in the transfer of nutrients to nourish the embryo (Schmidt, 1992; Sheng, 2010; Bellairs and Osmard, 2014). The yolk sac aids the transfer of partially digested yolk contents into the embryonic blood (Noble and Cocchi, 1990; Moran, 2007; Bellairs and Osmond, 2014). The yolk sac is also a source of calcium for chick embryo until around day 10 of development (Simkiss, 1961; Terepka et al., 1976). The amnion encloses the growing embryo at day 3 of development and secretes fluid into the amniotic cavity around the embryo. The amniotic fluid protects the embryo from physical damage and dehydration (Wu et al., 2001; Sheng, 2010). The chorion fuses with the allantois to form the chorioallantoic membrane (CAM) around day 3 and of 4 of incubation (Romanoff, 1960). The vascularized CAM is mature at day 16 and firmly attached to the ESM, allowing gas exchange through the pores of the shell (Romanoff, 1960).

The CAM participates in the transport and mobilization of calcium from the shell into the embryo, gas exchange and electrolyte reabsorption (sodium and chloride ions) from the allantoic cavity where urinary waste products are discharged (Ribatti et al., 2001; Gabrielli and Accili, 2010).



(a)



(b)

Fig. 3. The three-tissue layers of the chick mature CAM are indicated in the schematic representation in (a) and clearly recognizable at day 16 of incubation, after Toluidine blue staining (b): the chorionic epithelium (ce) adheres to the shell membrane (sm); the intermediate mesodermal layer (ml) contains blood vessels; the allantoic epithelium (ae) lines the allantoic cavity (ac). (a) Modified from Gabrielli et al., 2004 (b) Original magnification: 40x; scale bar: 8 μm. Source: Gabrielli and Accili, 2010. The chick chorioallantoic membrane: a model of molecular, structural, and functional adaptation to transepithelial ion transport and barrier function during embryonic development. *Journal of BioMed Research,* with permission from Hindawi Publishing Corporation.

2. Defenses of the Avian Egg

The innate immune system is the first line of defense against pathogen microorganisms and is formed by physical barriers such as skin or, in the egg – the eggshell, as well as chemical innate immune protective mechanisms, to resist pathogen invasion from a contaminated environment.

Therefore, physical protection of the avian egg is constituted by the ESMs and associated eggshell and cuticle (Hincke et al., 2012). Chemical defense consists of antimicrobial proteins found in all egg compartments (Réhault-Godbert et al., 2011). These defense mechanisms are extremely necessary to protect unfertilized eggs against food-borne pathogens especially *Salmonella enterica serovar enteritidis* (*S. enteritidis*), as well as fertilized eggs against bacteria that can cause embryonic death. The potential routes for bacterial contamination of eggs are divided into vertical and horizontal transmission.

Vertical transmission occurs by the bacterial contamination of the hen egg during egg formation from the chicken reproductive organs (Keller et al., 1995; Miyamoto et al., 1997). Horizontal transmission is the penetration of bacteria through pores or microcracks in the shell after laying, when the shell is exposed to a contaminated environment from hen faecal pathogens or other pathogens present in the environment (De Reu et al., 2006; Gantois et al., 2009; Baron et al., 2011). This exogenic contamination of chicken eggs (horizontal contamination) by bacteria is facilitated in eggs with absent or incomplete cuticle that allows the invasion of bacteria through the shell pores (Mayes and Takeballi,1983; Sparks and Board, 1984; Bain et al., 2013).

Salmonella contaminates eggs through ovarian transmission or invades the shell after the egg has been laid (Cox et al., 2000). Once Salmonella traverses the ESM, it becomes very difficult to destroy or prevent further invasion of the egg interior and developing embryo (Cason et al., 1994). The incidence of *S. enteritidis* egg-associated outbreaks cases in the UK, Europe, and North America has increased from 1986 to 2009 (Threlfall et al., 2014). Pathogenic bacteria include *Campylobacter jejuni*, *Listeria monocytogenes, Escherichia coli*, *Yersinia enterocolitica, Pseudomonas aeruginosa* and *Staphylococcus*, which have been associated with the contamination and spoilage of hen eggs (Board and Tranter, 1995; Ricke et al., 2001; Shebuski and Freier, 2010; Abdullah, 2010; Chousalkar et al., 2010; Moffatt and Musto, 2013; Faostat, 2014).

2.1. Physical Innate Immune Defenses

2.1.1. Eggshell

The avian calcified eggshell is a structured porous bioceramic with exceptional mechanical properties (Romanoff and Romanoff, 1949; Tyler, 1961; Hincke et al., 2011). The chicken eggshell is the greatest physical barrier against mechanical damage and microbial ingress from the environment. It also regulates gas and water exchange through its pores, as well as providing calcium during chick embryonic development (Nys et al., 2004; Hincke et al., 2012). Proteomics analysis of the eggshell matrix has identified 520 proteins including eggshell specific proteins (ovocleidin-17 ovocleidin-116, ovocalyxin-32 and ovocalyxin-36), proteins found in other egg compartments (egg white proteins such as ovalbumin,

lysozyme, ovotransferrin, ovoinhibitor and cystatin), lipid-binding proteins (extracellular fatty acid-binding protein, prosaposin and apolipoprotein D), micellaneous proteins (serum albumin, vitamin D-binding protein,calcyclin and dickkopf-related protein 3) and extracellular signaling molecules (pleiotrophin, IGF-binding proteins, calcium-binding protein nucleobindin) and immune system-related and antimicrobial proteins (immunoglobulins, mucin, avidin, histones and β-defensins) (Mann et al., 2006; Mikšík et al., 2010).

2.1.2. Eggshell Membranes (ESMs)

The ESMs are light pink double-layered meshworks of fibres found between the egg white and the inner surface of the eggshell. The ESMs are constituted by two layers: the thin inner and thick outer ESM; in addition, a limiting membrane surrounds the surface of the egg white (Hincke et al., 2000; Nakano et al., 2003; Tsai et al., 2006) (Fig.4). The thickness of fibers of the inner ESM ranges from 0.1 to 3 μm whereas the fibers of outer ESM ranges between 1 and 7 μm (Bellairs and Boyde, 1969). The fibers of the inner ESM are interlaced with the outer ESM whereas the outer ESM fibers are inserted in the mammillary cones of the shell (Bellairs and Boyde, 1969; Arias et al., 1993; Liong et al., 1997). The ESM fibers are essential elements of calcified eggshell that prevent the entry of invading pathogenic bacteria and form a platform for mineralization (Hincke et al., 2000, Cordeiro and Hincke, 2011).

The biopolymeric fibrous ESM functions as a support for biomineralization of the eggshell (Arias et al., 1993; Nys et al., 2004). Briefly, organic aggregates

are deposited on the surface of the outer ESMs in a quasi-periodic pattern. At these sites, nucleation of calcium carbonate mineral begins with progressive deposition of amorphous calcium carbonate giving rise to the mammillary cones and subsequently the palisade layer (Nys et al., 2004; Rodriguez-Navarro et al., 2015). The spacing and dimensions of the mammillary cones determines the biomechanical properties of the completed eggshell and, therefore, its physical strength (Bain, 1992; Rodriguez-Navarro et al., 2002).

The highly crosslinked fibers of the ESMs are constituted by proteins that include collagens (types I, V and mainly X), the cysteine-rich eggshell membrane protein (CREMP) with multiple disulfide crosslinks, and glycoproteins; a variety of lysine-derived desmosine and isodesmosine linkages stabilize the membranes (Harris et al., 1980; Leach, 1982; Wong et al., 1984; Arias et al., 1991; Zhao and Chi, 2009; Kodali et al., 2011). The detailed identification of protein constituents of the ESMs is challenging due to the natural insolubility of ESM, due to the presence of cross-linked fibers with the lysine-derived desmosine and isodesmosine and high levels of disulfide bonds (Arias et al., 1991; Takahashi et al., 1996). Some studies have reported the use of different treatments to extract proteins from ESM for proteomics, but they were not able to dissolve the ESM fibers (Kodali et al., 2011; Cordeiro et al., 2013; Kaweewong et al., 2013; Rose-Martel et al., 2015).

In the egg producing industry, a high percentage of eggs are diverted to breaker operations to generate liquid egg products and are not consumed as shell eggs. The by-products of these operations are the eggshell and associated membranes, which constitute approximately 6g / egg; this represents a significant

waste from the egg-derived products processor plant (Stadelman, 2000). The shell and ESM are an inexpensive and abundant waste material and a potential source of various inorganic and bioorganic materials. The eggshell residue generated by these operations has been considered useless and is commonly disposed of in landfill without any pre-treatment. However, this waste management is not a desirable practice in view of the environmental odor from biodegradation. Research is necessary to develop processes to extract products with potential commercial application from this material (Cordeiro and Hincke, 2011).

The ESM waste is a source of active compounds that are naturally concentrated and can be extracted using ecofriendly and cost effective processes. Some processes for separating ESM from the shell the application potential of eggshell membrane waste have been patented (Thoroski, 2004; MacNeil, 2005; MacNeil, 2006; summarized in Cordeiro and Hincke, 2011). Suitable processing may be developed for using ESM in different applications, and better knowledge of the ESM proteome will allow the rational design for applications of ESM as a biomaterial. Some applications include the utilization of ESM as a biotemplate to produce nanoparticles, the sorbent of heavy metals and dyes, biosensors and nutraceuticals (Cordeiro and Hincke, 2011; Balaz, 2014).

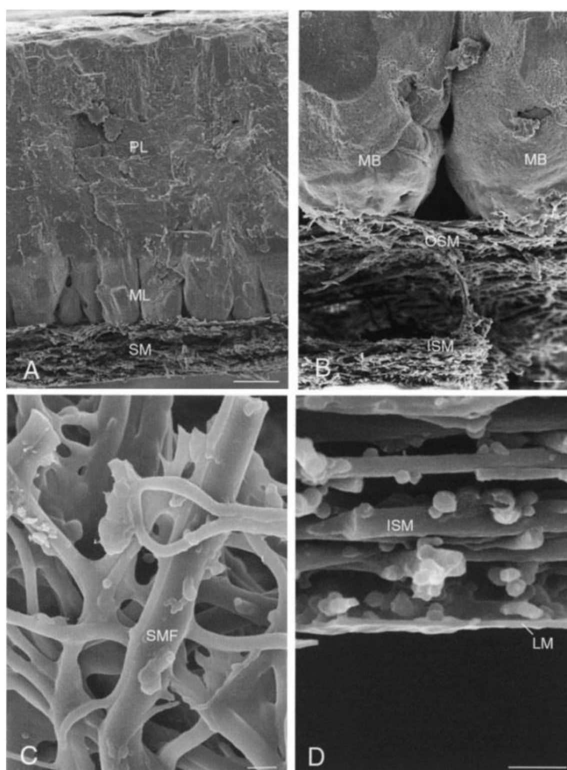


Fig. 4. Scanning electron micrographs illustrating the morphology of the eggshell and eggshell membranes (ESM). (A) Eggshell cross-fractured to reveal the shell membrane (SM), mammillary layer (ML) and palisade layer (PL). (B) Higher magnification of the membrane–mammillary body interface. Outer shell membrane fibers (OSM) insert into the tips of the mammillary bodies (MB). Inner shell membranes (ISM). (C) Enlargement of the shell membrane fibers (SMF) to reveal their interwoven and coalescing nature. (D) The inner aspect of the inner shell membrane (ISM), demonstrating the limiting membrane (LM) that surrounds the egg white (here removed during sample preparation). Scale bars (A) 50 μm; (B)

20 μm; (C, D) 2 μm. Source: Hincke *et al*., 2000. M*atrix Biology* 19(5), with permission from Elsevier.

2.2. Chemical Innate Immune Defenses

The egg is susceptible to surface contamination by pathogens that can occur during oviposition as it exits the hen reproductive tract or from bacteria present in the environment; either may subsequently cross the eggshell (Kelller et al., 1995; De Buck et al., 2004). The avian innate immune system is formed by chemical defense mechanisms against invading pathogens that includes pattern-recognition receptors (PRRs), immune effector cells, enzymes, proteins and peptides that are important players in the first line of defense (Cuperus et al., 2013).

The chemical innate immune defense of chicken eggs is constituted of various immune defensive mechanisms present in eggs – antimicrobial peptides (AMPs) in eggshell, iron depleted and lysozyme-rich egg white (O'Farrelly et al., 1992; Guard-Petter, 2001; Deignan et al., 2001; Wellman-Labadie et al., 2008). These proteins possess antimicrobial activity that is important for the protection of the embryo. Some examples of proteins that play a role in the antimicrobial protection of chicken egg are lysozyme, ovotransferrin, transiently expressed in neural precursor (TENP), avian beta-defensins (AvBDs) and ovocalyxin-36 (OCX-36) (Réhault-Godbert et al., 2011). These proteins are found distributed in different egg compartments, and most of them are mainly concentrated in the egg white (Guérin-Dubiard et al., 2006; Man, 2007).

2.2.1. Lysozyme

Lysozyme is one of the main proteins in the egg white protein and it is present in eggshell, egg yolk and VM (Hincke et al., 2000, Mann et al., 2006; Mann, 2007; D'Ambrosio et al., 2008; Mann and Mann, 2008; Mann, 2008; Farinazzo et al., 2009). High levels of lysozyme are found in the ESM and the limiting membrane. Lysozyme is a bacteriolytic protein that plays an important defensive role in chick embryos and conservation of other egg components (Hincke et al., 2000). Lysozyme is an N-acetyl-muramidase (14 kDa protein) that displays antimicrobial activity against Gram-positive bacterial strains. The antimicrobial effect of lysozyme is based on the hydrolysis of the bacterial peptidoglycan that results in cell wall lysis, as well as its cationicity (Nash et al., 2006).

2.2.2. Ovotransferrin

Ovotransferrin is a monomeric 78 kDa glycoprotein that is abundantly found in the egg white (Guérin-Dubiard et al., 2005; Guérin-Dubiard et al., 2006; Mann, 2007). The presence of ovotransferrin was detected by immunofluorescence in ESM and by Western blot analysis (Gautron et al., 2000; Varon et al., 2013). The expression of ovotransferrin was detected in the proximal oviduct (magnum and white isthmus) and distal oviduct (red isthmus and uterus) by Northern blotting and RT-PCR analysis (Gautron et al., 2001). Ovotransferrin is also found in the calcified mammillae and particularly in the ESM, where it functions as a bacteriostatic filter to reinforce inhibition of *Salmonella* growth in egg white (Gautron et al., 2001). Chicken ovotransferrin shows antibacterial properties against different bacteria including foodborne pathogens such as *E. coli* O157: H7

and S. aureus (Tranter and Board, 1982; Oratore et al., 1990). Ovotransferrin can bind two Fe^{2+} ions per molecule in the presence of bicarbonate (Schalabach and Bates, 1975). The ability of ovotransferrin to bind to iron allows inhibition of bacterial growth by restricting iron availability through chelation and forming iron-saturated ovotransferrin (Alderton, Ward, and Fevold 1946; Oratore et al., 1990; Gautron et al., 2001). Iron is essential for bacterial growth in strains that include *Pseudomonas spp*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus* and *Streptococcus mutants* (Valenti et al., 1982). In addition, ovotransferrin was reported to have an antimicrobial effect against *S. enteritidis*, based upon its ability to chelate iron (Baron et al., 1999). However, some studies have reported another antimicrobial mechanism of ovotransferrin and its derived peptides through bacterial membrane permeabilization or membrane rupture (Valenti et al., 1987; Ibrahim et al., 1998; Ibrahim, Sugimoto and Aoki, 2000; Aguilera et al., 2003).

2.2.3. Avian Beta-Defensins (AvBDs)

Avian β-defensins (AvBDs) are found in avian species and they are approximately 25 according to the analysis in the chicken genome (Higgs et al., 2005; Lynn et al., 2007; van Dijk et al., 2008; Hellgren and Ekblom, 2010). The AvBDs are a family of small cationic peptides with a three-stranded ß-sheet structure connected with a loop of ß-hairpin turn (Evans et al., 1994; Sugiarto and Yu, 2004; Cuperus et al., 2013). The AvBDs form disulfide bridges between Cys^1–Cys^5, Cys^1– Cys^5 and Cys^3– Cys^6 (Lehrer and Ganz, 2002; Klotman and Chang, 2006). The presence of AvBDs was mainly detected in the hen reproductive tract

but they are also found in other egg compartments (Mann, 2008; Cuperus et al., 2013). AvBD11 is abundantly found in the VM and eggshell membrane of chicken eggs (Mann, 2008; Mageed et al., 2009).

Avian beta-defensins (AvBDs) are AMPs of the chicken innate immune defenses which protect the embryo during its development and contribute to the production of pathogen-free eggs. The antimicrobial mechanism of AvBDs is suggested to be based on the electrostatic interaction of its cationic amino acids chain with negatively charged membrane components, such as lipopolysaccharide (LPS) and lipoteichoic acidic (LTA) (Hancock, 1997). This interaction promotes the accumulation of peptides, parallel to the membrane surface, and the formation of dimers and multimers that facilitate the formation of pores in the bacterial membrane (Wellman-Labadie et al., 2007).

2.2.4. Bactericidal permeability-increasing protein (BPI) / lipopolysaccharide-binding protein (LBP) / palate, lung and nasal epithelial clone (PLUNCs) protein family

The BPI/LBP/PLUNC protein family is well known for their participation in the host defense against pathogen invasion and function as PRRs. The most important PRRs molecules are Toll-like receptors (TLRs). TLRs are expressed on the host cell membrane and crucial players in the host response against microbial invasion. For instance, TLR-4 detects LPS and TLR-5 recognizes flagellin (Higgs et al., 2006). BPI/LBP/PLUNC proteins are important PRRs molecules of the innate

immune system, which are involved in the host response to bacterial components such as LPS and LTA (Bingle and Craven, 2004; Zweigner et al., 2006).

BPI and LBP proteins members bind LPS. However, they play antagonistic LPS mediated cellular signaling. LPB is one of the molecules that recognize LPS after the release of LPS in the bloodstream (Gioannini et al., 2003; Weiss, 2003). LBP upregulates the inflammatory response by catalysing binding of LPS monomers to CD14 present on the cell surface. CD14 associated with TLR-4 modulate the activation of immune cells with the production of pro-inflammatory mediators (nitric oxide (NO), tumor necrosis factor (TNF)-α and interleukins (ILs)) in order to promote the clearance of LPS from the host (Tobias et al., 1988; Medzhitov and Janeway, 2000; Kumar et al., 2009; Krasity et al., 2011).

BPI possesses a direct antibacterial activity against bacteria. BPI binds to LPS and penetrates the inner membrane of bacteria to causes depolarization of its membrane and cell death (Shai, 1999; Levy et al., 2003). The interaction of N-terminal domain of BPI to LPS is important not only for its antimicrobial but also to its anti-inflammatory activities of BPI (Mannion et al., 1990; Ooi et al., 1991; Gazzano-Santoro et al., 1992). The C-terminal domain of BPI is associated with opsonic effects thus increasing phagocytosis (Schultz et al., 2001).

The PLUNC proteins are structural homologs of BPI and LBP proteins and are divided into two groups; short (SPLUNC) and long (LPLUNC) proteins (Chiang et al., 2011). SPLUNCs show similarities with the BPI N-terminal domain whereas LPLUNCs show homology with domains similar to both the BPI domains (Bingle and Craven, 2002). The structural homology of PLUNCs with BPI was confirmed

by its antimicrobial and anti-inflammatory activity (Gorr et al., 2011; Lukinskiene et al., 2011). For instance, SPLUNC1 protein shows bactericidal activity and reduces biofilm formation by *Pseudomonas aeruginosa* (Gally et al., 2011; Lukinskiene et al., 2011; Tsou et al., 2013).

Another LBP/BPI protein found in chicken egg compartments is Transiently Expressed in Neural Precursors (TENP). Chicken TENP was first identified as a gene transiently expressed during neurogenesis (Yan and Wang, 1998 from Golbert). However, this protein was also detected in chicken egg white, yolk, VM and ESM (D'Ambrosio et al., 2008; Mann and Mann, 2008, Cordeiro et al., 2015; Rose-Martel et al., 2015), vitelline membrane and eggshell matrix (Mann, 2008). In Emu, RT-PCR analysis showed that the TENP gene was highly expressed in the magnum of the oviduct, suggesting that TENP is a major egg white component in this bird species (Maehashi et al., 2014). The LPS binding domains of TENP suggests an innate immune protection of eggs against pathogens (Whenham et al., 2014). Purified emu TENP displayed antibacterial activity specificity against Gram-positive bacteria including *Micrococcus luteus* and *Bacillus subtilis* (Maehashi et al., 2014).

OCX-36 is an eggshell matrix protein , which is found in the calcified shell and vitelline membrane, but predominantly enriched in the ESM (Gautron et al., 2007; Mann, 2008) (Fig.5). OCX-36 expression was detected in the regions of the oviduct where eggshell formation takes place (isthmus and uterus), and upregulated levels of OCX-36 were observed specifically in the uterus during shell calcification and quantified by real-time RT-PCR (Gautron et al., 2007). Blast N

database searching analysis showed that OCX-36 share similar intron-exon gene organization and 20-25% protein sequence homology to mammalian proteins LBP/LBP/PLUNC (Gautron et al., 2007). The OCX-36 gene is located in the BPI/LBP/PLUNC gene cluster on chromosome 20. These findings suggest that OCX-36 is a member of BPI/LBP/PLUNC protein family.

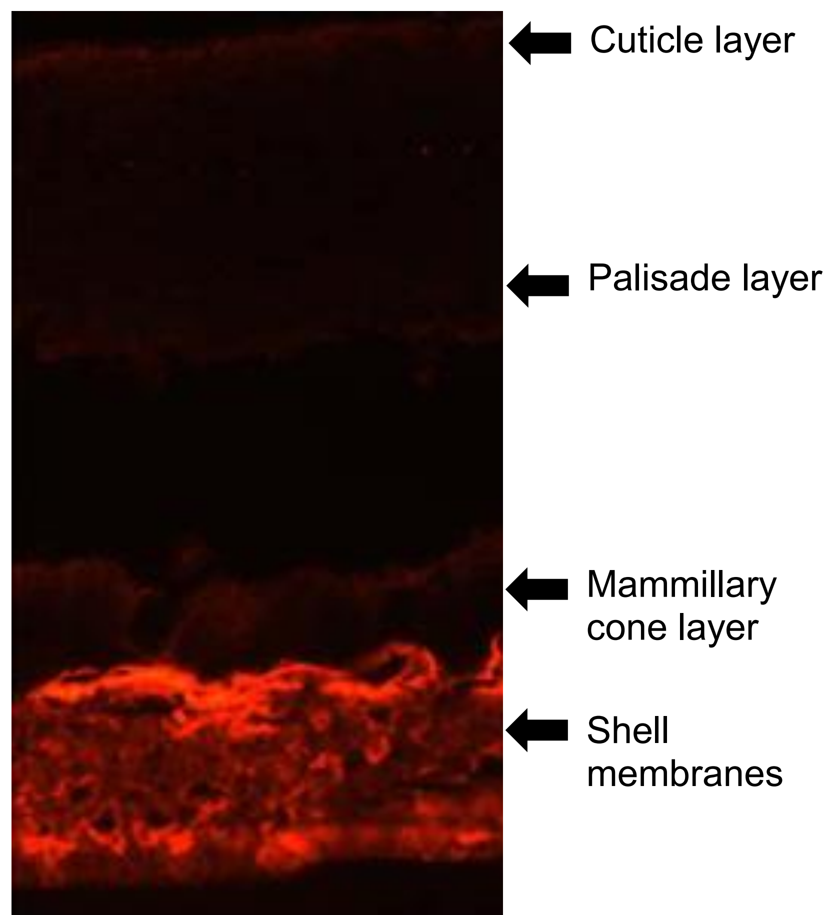


Fig.5. Immunofluorescence of OCX-36 in the eggshell cross section. Chicken eggshell was fixed, decalcified, and paraffin-embedded prior to sectioning Immunofluorescence was performed using a primary antibody raised against a synthetic OCX-36 peptide. Source: Rose-Martel and Hincke., 2013. Eggshell as Source of Novel Bioactive Molecules. *Journal of Food Science and Engineering* 5: 219, with permission from David Publishing.

II. Hypotheses

Chicken OCX-36 protein possesses a protein sequence homology to BPI, LBP and PLUNC proteins that have major roles in the innate immune response (Gautron *et al*., 1997). This observation led to my first hypothesis, which is that OCX-36 protein has a role in the innate immune protection and is involved in the endogenous defenses of chicken eggs against pathogens. To test this hypothesis, OCX-36 protein was extracted from chicken ESM and purified in order to characterize its biological activity.

The second hypothesis is that OCX-36 and OCX-36 derived peptides have immune stimulating and anti-endotoxin effects. The second hypothesis was tested with purified OCX-36 by investigation *in vitro* and *in vivo.*

The third hypothesis was that insights into the role of ESM proteins in innate protection during chick embryonic development could be obtained with a proteomics and bioinformatics approach, by identifying ESM proteins and assessing changes in their abundance during incubation and chick embryonic development.

III. Thesis Objectives

To test the three hypotheses, I performed the studies described in chapters 2, 3 and 4.

1) Chapter 2: OCX-36 protein was extracted and purified from ESM using a novel method developed in our laboratory, in order to characterize its antimicrobial activity *in vitro*, evaluate its LPS and LTA binding activities and determine the

impact of the OCX-36 gene polymorphism on the biological functions of OCX-36.

2) Chapter 3: The potential immune-stimulating and anti-endotoxin properties of purified native and digested OCX-36 were studied using mouse macrophages and in a whole mouse model of LPS challenge.

3) Chapter 4: ESM proteins were solubilized from both fertilized and unfertilized eggs, using a novel chemical approach, in order to determine the ESM proteome, and to assess their biological functions by bioinformatics, using a fertilized model of chick embryogenesis during incubation.

IV. Thesis outline

Chapter 1 provides a literature review of avian egg compartments and its function and an overview of chicken embryonic development. Egg physical and chemical defenses against microorganism invasion were highlighted. Chapter 2 reports my publication (Cordeiro et al., 2013), in which we describe optimal methods to extract OCX-36 from ESM, followed by purification to characterize its properties. This study suggests that OCX-36 is a pattern recognition molecule that recognizes bacterial endotoxins (PAMPs) such as the bacterial cell wall constituents LPS and LTA. In chapter 3 (Kovacs-Nolan et al., 2014), I describe *in vivo* and *in vitro* studies to investigate the immune-stimulating and anti-endotoxin properties of purified native and digested OCX-36 in murine macrophages and a mouse model of LPS challenge. Chapter 4 (Cordeiro et al., 2015) reports proteomics identification and bioinformatics characterization of the function of ESM proteins extracted from fertilized and unfertilized eggs using novel methods of

extraction that I developed. This analysis provides some insight into the role of proteins related to the ESM that are involved in chick embryonic development and also may be important in unfertilized eggs. In Chapter 5, a general discussion summarizes the novelty and findings of this body of work. This last chapter addresses the importance of understanding the pivotal roles of these ESM proteins in order to guide future development of a novel antimicrobials or nutraceuticals to mitigate the risk of food-borne disease, to improve food safety for the consumer and to enhance innate immune protection for humans.

V. CHAPTER 2. Ovocalyxin-36 is a pattern recognition protein in chicken eggshell membranes.

Cordeiro CMM, Esmaili H, Ansah G and Hincke MT (2013) *PloS one*, *8* (12): e84112.

This chapter addresses the importance of extracting and purifying OCX-36 to characterize its biological function. It was previously recognized that OCX-36 shares protein sequence homology, and has similar exon and intron gene organization, with mammalian BPI / LBP / PLUNC proteins that have a significant role in the innate immune response. My characterization of the biological function of purified OCX-36 protein provides new insight into the role of PLUNC family members in innate immunity and antimicrobial protection in non-mammalian organisms. Our results show OCX-36 acts as pattern recognition molecule that recognizes bacterial endotoxins, which could be a first step to eliminating pathogens. This study addresses the hypothesis that OCX-36 is a promising candidate in the innate immune protection of avian eggs against pathogen invasion.

Author contributions:

Experiments were conducted by Cristianne Cordeiro and Hamed Esmaili. George Ansah provided biological samples and contributed to the design, implementation and analysis of results for the OCX-36 single nucleotide polymorphisms studies. Data interpretation and manuscript preparation was done by Cristianne Cordeiro. Experimental procedures, data analysis, and manuscript preparation were supervised by Dr. Maxwell T. Hincke.

Ovocalyxin-36 is a pattern recognition protein in chicken eggshell membranes.

Cristianne M. M. Cordeiro[1], Hamed Esmaili[1], George Ansah[2], Maxwell T. Hincke[1]

1 Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada.

2 ISA North America, Division of Hendrix Genetics, Kitchener, Ontario, Canada.

To whom correspondence should be addressed: Maxwell T. Hincke, Department of Cellular and Molecular Medicine, University of Ottawa, 451 Smyth Road, Ottawa, K1H8M5, Canada, Tel.:(1) 613-562-5800, x8193; Fax: (1) 613- 562- 5687; E-mail: mhincke@uottawa.ca.

Abstract

The avian eggshell membranes are essential elements in the fabrication of the calcified shell as a defense against bacterial penetration. Ovocalyxin-36 (OCX-36) is an abundant avian eggshell membrane protein which shares protein sequence homology to bactericidal permeability-increasing protein (BPI), lipopolysaccharide-binding protein (LBP) and palate, lung and nasal epithelium clone (PLUNC) proteins. We have developed an efficient method to extract OCX-36 from chicken eggshell membranes for purification with cation and anion exchange chromatographies. Purified OCX-36 protein exhibited lipopolysaccharide (LPS) binding activity and bound lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 in a dose-dependent manner. OCX-36 showed inhibitory activity against growth of *Staphylococcus aureus* ATCC 6538. OCX-36 single nucleotide polymorphisms (SNPs) were verified at cDNA 211 position and the corresponding proteins proline-71 (Pro-71) or serine-71 (Ser-71) were purified from eggs collected from genotyped hens. A significant difference between Pro-71 and Ser-71 OCX-36 for *S. aureus* lipoteichoic acid (LTA) binding activity was detected. The current study is a starting point to understand the innate immune role that OCX-36 may play in protection against bacterial invasion of both embryonated eggs (relevant to avian reproductive success) and unfertilized table eggs (relevant to food safety).

Introduction

Multilevel, interactive defense strategies that function across biomineralized barriers are a hallmark of sophisticated biological structures. The calcareous avian egg, the hallmark of reproduction in birds, is a complex multilayered structure [1]. The eggshell resists physical and pathogen challenges from the external environment, while satisfying a variety of metabolic and nutritional needs of the developing embryo. Following ovulation, the forming egg traverses specialized regions of the oviduct where the egg white, eggshell membranes and eggshell are sequentially deposited in the magnum, white isthmus and uterine segments, respectively [2]. The innermost layer of the shell is the eggshell membranes that are deposited as a highly crossed-linked extracellular fibrous meshwork during ≤1.5 h passage through the white isthmus [1-3]. The fibres are organized into inner and outer membranes and are essential elements of a normal eggshell which will resist bacterial contamination [4, 5].

The eggshell membranes fibres are composed of highly cross-linked proteins such as collagens and cysteine-rich eggshell membrane protein (CREMP) [3, 6, 7]. These fibres serve as a structural support for enzymes and proteins that protect against invading microorganisms [1, 2]. Several studies have identified proteins in the eggshell membranes that possess antimicrobial activity, such as lysozyme and ovotransferrin [4, 8].

Ovocalyxin-36 (OCX-36) is a protein present in the uterine fluid collected during the active calcification stage of shell mineralization. It is present in the calcified shell, but particularly abundant in the eggshell membranes [9]. A polyclonal

antibody against OCX-36 was used to expression-screen a hen uterine library, and a positive clone was sequenced and used for further hybridization screening. The resulting consensus sequence was subsequently assembled with ESTs to obtain a complete full-length cDNA [9]. The uterine OCX-36 message is strongly upregulated during eggshell calcification. OCX-36 expression occurs in the regions of the oviduct where eggshell formation takes place (isthmus and uterus), and also in the digestive tract [9, 10].

OCX-36 shares protein sequence homology, and similar exon and intron gene organization, with mammalian BPI and LBP proteins that have a major role in the innate immune response [9]. According to the new BPIFAn/BPIFBn systematic nomenclature for PLUNC proteins, the SPLUNC root has been replaced by BPIFA and the LPLUNC root was replaced by BPIFB. OCX-36 protein is a new member of BPIFB8 protein family [11]. The OCX-36 gene is nested with in the BPI/LBP/PLUNC gene cluster on chromosome 20. However, the OCX-36 gene is highly specific to birds and is thought to have arisen by tandem duplication of an ancestral BPI/LBP/PLUNC gene cluster after the divergence of birds and mammals [10, 12]. LBP and BPI were the original members of the PLUNC protein family. These two protein members bind to LPS and play antagonistic functions in LPS mediated cellular signaling. Human LPB increases the inflammatory response induced by LPS whereas BPI shows antibacterial and anti-inflammatory functions [13, 14]. In addition to its well known functions, BPI has anti-angiogenic activity, inhibits human endothelial cell growth and induces apoptosis [15, 16]. The functional human PLUNCs are classified as short PLUNCs (SPLUNCs 1, 2 and 3)

and long PLUNCs (LPLUNCs 1, 2, 3, 4 and 6) proteins. SPLUNCs have homology to the LPS- binding N-terminal domain of BPI, whereas LPLUNCs have overall homology to both the N-terminal and C-terminal domains of BPI. The N-terminal domain of BPI is responsible for its endotoxin neutralization and antibacterial activities while opsonic activity is associated with its C-terminal domain [17].

PLUNC and BPI proteins share similar functions. PLUNC proteins bind LPS, have bacteriostatic activity, induce bacteria agglutination and participate in cytokine production [18]. The common structural features that OCX-36 shares with BPI/LBP/PLUNC proteins are the basis for our hypothesis that OCX-36 participates in the innate immune response to pathogens as a pattern recognition protein [19].

Characterizing the biological function of OCX-36 protein will provide new insight into the natural defences of eggs which could mitigate the risk of food-borne disease for egg consumers. In this study, we have extracted, purified and characterized OCX-36 from eggshell membranes, as a first step to understand its functional role.

Material and Methods

1. Materials

Staphylococcus aureus ATCC 6538, *Listeria monocytogenes* ATCC 19112 and *Enterococcus faecalis* (clinical isolate), *P. aeruginosa* ATCC 15442, *Salmonella typhimurium* and *Escherichia coli* O111:B4 were obtained from Dr. Syed A. Sattar (Centre for Research on Environmental Microbiology, University of Ottawa). Luria-Bertani (LB) broth, bovine serum albumin (BSA) and casein were purchased from Bioshop Canada Inc. CM-Sepharose Fast Flow and DEAE-Sepharose Fast Flow resins were from Amersham Biosciences. Dialysis tubing (MWCO 12,000-14,000) was purchased from Fisher. Butyl, Pentyl, Hexyl, Octyl Agaroses and Concanavalin A Sepharose, phenol/chloroform/isoamylalcohol and Proteinase K were purchased from Sigma-Aldrich. Ni-NTA agarose was from Qiagen. Bio-Gel Hydroxyapatite (HTP), alkaline Phosphatase Substrate Kit and protein molecular weight marker (Blue Standards161-0373) were purchased from Bio-Rad. BCA protein assay reagent (bicinchoninic acid), 1-Step ABTS (1- 2'-azinobis-3-ethylbenzthiazoline-6-sulfonic acid) and protein molecular weight markers (SM0431) were purchased from Thermo Scientific. Anti-Rabbit IgG–horseradish peroxidase conjugate (HRP) conjugate was purchased from Promega. Western lightning Plus-ECl was purchased from Perkin Elmer. Biotinylated LPS and LPS from *E. coli* O111:B4, and LTA from *S. aureus* ATCC 6538 were purchased from InvivoGen. Streptavidin - alkaline phosphatase (SAP) was purchased from Invitrogen. Recombinant hLBP

(rhLBP) was purchased from R&D Systems. The 96-well medium binding microplates were purchased from Costar, and 100-well honeycomb plates were purchased from Oy Growth curves. Amplitaq gold PCR master mix was purchased from Applied Biosystems.

2. Extraction of OCX-36

After extensive preliminary trials, an optimized protocol to extract OCX-36 from unfertilized chicken eggs was developed. “Standards eggs” used throughout method development and purification of OCX-36 were the same brand: BurnBrae Farms Super Bon-EE (oversized eggs that are 25% larger than regular large eggs), purchased from local groceries stores. The eggs (in batches of 5 to 10 dozen eggs) were broken and their contents were discarded. Eggshell interiors were rinsed under running demineralized water (DM). Eggshell membranes were manually stripped from the interior of the calcified eggshell and collected in DM. The wet membranes were sliced into smaller sizes for extensive rinsing with DM. Eggshell membranes were placed in 5L of 1M NaCl and stirred using an overhead mixer (IKA RW 20 digital, Cole-Parmer Canada) for 1h at 4 °C. The eggshell membranes were again rinsed using DM to remove any residual NaCl, followed by their collection on a Whatman No. 2 filter paper under vacuum suction. The moist membranes were weighed and added to the extraction buffer: 50 mM Tris-HCl, pH 8.5 containing 10 mM dithiothreitol (DTT) (30 mL of extraction buffer per gram of membrane). This extraction mixture was stirred (overhead mixer)

to extract OCX-36 overnight at room temperature. The next day, the suspension was filtered (Whatman No. 2 filter paper) to remove large particles of membrane. This turbid solution containing extracted OCX-36 was centrifuged (3,500 x g, 20 minutes, 4°C) to clarify and remove finer membrane particles.

3. Purification of OCX-36

The supernatant from the OCX-36 extraction was passed through two columns (CM-Sepharose Fast Flow and DEAE-Sepharose Fast Flow) connected in series. Both columns were pre-equilibrated with 50 mM Tris-HCl, pH 8.5 at a flow rate of 1ml/min. After loading the supernatant onto the columns, the beads were washed with 50 ml of Tris-HCl, pH 8.5, 2 mM of DTT (all at 1ml/min). After this washing, the CM- Sepharose column was disconnected and bound proteins were eluted from the DEAE column with 25 ml of 50 mM NaHepes, pH 7.0, 2 mM DTT, 350 mM NaCl at a flow rate of 1 ml/min. Eluted fractions (1ml) containing OCX-36 were dialyzed and freeze dried.

4. Electrophoresis and Densitometry analysis of OCX-36

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously [20], followed by staining with Coomassie Blue and destaining. Quantitative densitometric analysis of the stained bands was performed with Image Quant 300 and TL (GE Healthcare).

5. Western Blotting

OCX-36 samples were separated on 12% SDS–PAGE gels and electrotransferred to nitrocellulose membrane. The nitrocellulose membrane was washed in PBS-Tween 0.1% (10 mM sodium phosphate buffer, 0.154 M sodium chloride, pH 7.4, 0.1%Tween 20). The membrane was blocked with 3% BSA for 1 h, and then the membrane was washed in PBS-Tween 0.1% (2 x 5 min) and then incubated in PBS-Tween 0.1% for 1 h with primary antibody prepared against a 15-amino acid synthetic peptide corresponding to residues 51– 65 of the mature OCX-36 protein, KHLQGMALPNIMSDR [9], diluted 1:50,000 in PBS-Tween. After three washes in the same buffer, the membrane was incubated for 30 min with anti-rabbit IgG–HRP (1:10,000), followed by washing (4 x PBS-Tween 0.1%, 2 x 0.1 M sodium phosphate buffer, pH 7.4). The membrane was then incubated with Western lightning Plus-ECl reagent. Immunoreactive bands were visualized with X-ray film for different exposure times.

6. Sample Preparation for Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS) analysis

Two different OCX-36 samples (preps 90 and 120) were resolved by 12% SDS-PAGE gels and lightly stained with Coomassie Blue following the recommended protocol. The gel pieces were sent to the Proteomics Platform of the Eastern Quebec Genomics Centre (Laval, QC) for LC/MS/MS analysis (include in-gel digestion, mass spectrometry analysis and Mascot data-base

searching). The procedures for all these analyses were performed as previously described [21].

7. Data base searching and criteria for protein identification

MS/MS data were analyzed using Mascot (Matrix Science, London, UK; version 2.2.0), searching the uniref 100.2010.06.Gallus.gallus.9031 database, with trypsin digestion. Validation of MS/MS based peptide and protein identification were performed using Scaffold (version Scaffold-3_00_08, Proteome Software Inc, Portland, OR). Protein identification was accepted at $p = 0.05$ probability, as specified by the Protein Prophet algorithm [22, 23], with at least 2 unique peptides (> 95% confidence).

8. Bioinformatics analysis

The relative quantification of the identified proteins was calculated using the exponentially modified protein abundance index (emPAI) defined as 10^{PAI}-1 [24].

9. Single Nucleotide polymorphisms (SNPs) in the OCX-36 gene

9.1 Genomic DNA Purification

Chicken blood samples from pedigree White Leghorn laying hens (Hendrix Genetics) were assessed to identify birds possessing specific OCX-36 SNPs. Chicken blood samples were diluted in PBS, and then 50 to 100 uL of chicken blood cells were lysed by adding 500 uL of lysis buffer (10 mM

Tris-HCl, 10 mM EDTA, 100 mM NaCl, 0.5% SDS and 20 μL/mL of mercaptoethanol) and vortexed for 30 min at 50 °C. After 30 min of incubation, 5 μL of proteinase K (10 mg/mL) was added to the cell suspension and incubated overnight at 50^0C. Genomic DNA was extracted by adding 600 μL of phenol/chloroform/isoamylalcohol, mixed by inversion 10 times followed by centrifugation at 14,300 x g for 10 min at room temperature. After centrifugation, 500 uL of ethanol (100%) was added to the suspension containing DNA and centrifuged again at 14,300 x g for 10 min at room temperature. DNA pellets were washed with 1 mL of cold ethanol and centrifuged again at 14,300 x g for 10 min at 4 °C. DNA pellets were dried overnight and then dissolved in 50 uL of sterile distilled water with incubation at 65 °C for 30 min.

9.2 SNP fine mapping analysis

SNP fine mapping analysis of genomic DNA prepared from pedigree White Leghorn laying hens was performed by Polymorphic DNA Technologies (Alameda, CA) using a ‘‘boost/nest’’ PCR. Boost and Nested primers were used for two step nested PCR (Table 1). The boost PCR reaction generated a larger fragment that was used as a template for the nested reaction. DNA (10 ng) was used for boost PCR reaction and 1 μL of boost product was a template for the nested PCR reaction. The PCR reactions were carried out using Amplitaq gold PCR master mix (Applied Biosystems). PCR conditions were: denaturation for 4 min at 94°C, annealing

25 min at 55°C, and extension 1 min at 72°C. DNA sequencing was done using the 3730/3730xl DNA analyzer machine (Applied Biosystems).

Table 1. Primer pairs used for boost/nested PCR

Primers	Direction	cDNA region (5' to 3')	Size (bp)
Boost	Forward	ATCACCCCCTCTATTTG	302
	Reverse	GACGACCAACTGCATC	
Nested	Forward	CGTGGGTGCTGGAAA	347
	Reverse	CGGCAGCAGTGCTAT	

10. Antimicrobial Assays

10.1 Viability assay

Overnight cultures were inoculated into LB broth and incubated at 37ºC until an optical density of 0.2 at 600 nm was obtained. This bacterial suspension was centrifuged at 3000 x g, 4°C for 10 min. The bacterial pellet was resuspended in PBS, to pH 7.4 to obtain 10^5 CFU/mL. Bacteria were incubated with OCX-36 at 100 µg/mL in PBS for 1h at 37ºC. After incubation, the bacteria were serially diluted, plated on LB agar and then incubated for 24h at 37ºC to determine CFU's of surviving bacteria. Every dilution was performed in triplicate.

10.2 Monitoring bacterial growth via the Bioscreen assay

This assay was performed in 100-well honeycomb plates containing 10^5 CFU/mL and OCX-36 at 100 µg/mL in PBS, with incubation for 1 h at 37°C. After incubation, LB broth was added to each wells and the bacterial growth was monitored by optical density measurements at dual wavelength (420 nm-580 nm) every 15 min for 10 h using a Bioscreen C microplate reader. Results are shown as the average of at least three independent experiments.

11. LPS and LTA binding assays

11.1 LPS binding assay using biotinylated LPS

The ability of OCX-36 to bind to lipopolysaccharide was measured using a modified plate-binding assay [25]. OCX-36 samples (100 uL) were incubated in 0.01% casein in PBS in a 96-well plate, overnight at 37°C. The wells were blocked with 300 uL of 0.1% casein in PBS and washed before adding the biotinylated *E. coli* O111:B4 LPS in 0.1% casein and PBS. Streptavidin- alkaline phosphatase in PBS was added for 30 minutes followed by washing and the addition of substrate. The rate of color development was monitored for 60 minutes by optical density measurements every 10 minutes at 405 nm-630 nm (dual wavelength mode). Recombinant hLBP was used as a positive control.

11.2 ELISA-based LPS and LTA binding assays

The ability of OCX-36 to bind unmodified LPS and LTA was tested using a modification of a published protocol [26]. Briefly, 96 wells medium-binding microplates were coated with 100 µL of LPS from *E. coli* O111:B4 at 50 µg/mL or LTA from *S. aureus* ATCC 6538 at 0.5 µg/mL in PBS for overnight incubation at 4°C. The coated wells were blocked with 300 µL of PBS, 5% BSA (endotoxin :< 2EU/mg) for 1 h at 37°C. After washing the wells (three times) with PBS, 0.05% Tween-20 (PBS-T 0.05%), 100 µL of OCX-36 at 1.5; 5; 15 and 50 µg/mL in PBS were added to wells in triplicate and incubated at 37°C for 1h. The wells were washed three times with PBS-T 0.05% and 100

μL of primary rabbit anti-OCX-36 antibody (1:20,000 in PBS, 5% BSA) was added to wells and incubated at 37°C for 1h. Following three washes, anti-rabbit IgG– HRP (1:10,000 in PBS, 5% BSA) was added to wells and incubated at 37°C for 1h. The wells were washed with PBS-T 0.05% before adding 150 μL of 2, 2'-azinobis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) substrate solution to each well. The rate of color development was monitored for 60 minutes by optical density measurements every 10 minutes at 405 nm-630 nm (dual wavelength, BIO-TEK model EL 311SL microplate reader), and is expressed as Abs / min.

12. Statistical Analysis

All experiments were carried out in triplicate and statistical significance was determined by Student's t-test with $p < 0.05$ taken as significant. Results are reported as mean ± SEM.

Results

1. OCX-36 extraction and purification.

Different extraction buffers were tested to extract OCX-36 from eggshell membranes. The amount of extracted OCX-36 protein from eggshell membranes is dependent on the pH and DTT concentration used for the extraction buffer. Among all the tested buffers, buffer D and E yielded a detectable OCX-36 protein from eggshell membranes (Figure 1D and Figure 2B). Buffer E was selected as the buffer for further OCX-36 extraction to avoid harsh alkaline conditions. OCX-

36 was characterized by SDS-PAGE and Western Blotting analysis which identified an approximately 33 KDa band (Figures 1 and 2).

The purification of extracted OCX-36 was carried out using ion exchange chromatography after investigating a variety of purification methods which were unsuccessful since OCX-36 was not retained by the resin (Table 2). Successful purification of OCX-36 protein was accomplished using a two-step procedure. The first step was to remove fine membrane particles from the OCX-36 extraction supernatant and also retain positively charged egg white proteins (i.e. lysozyme), by using the CM-Sepharose column as a guard column. OCX-36 protein passed through the CM-Sepharose column and was retained by the DEAE-Sepharose resin, from which the relatively pure protein could be eluted with a step-gradient of 0.25 M NaCl. At this stage, eluted fractions from the DEAE-Sepharose column resulted in an OCX-36 immunoreactive band with an apparent molecular weight around 33 kDa (Figure 2A and 2B). LC/MS/MS analysis and Mascot database for protein identification confirmed the immunoreactive band as OCX-36 (Figure 3, Table 3). The comparison between OCX-36 contents during extraction and purification steps was performed by densitometry using Image Quant software. Most of the extractable proteins from the fresh eggshell membrane starting material were egg white proteins that include ovotransferrin, ovoalbumin and lysozyme whereas; these were almost completely removed from the purified OCX-36. Calculations indicated that OCX-36 in the purified material was approximately 98%, and that the degree of purification was about 1000-fold (Figure 4, Table 4).

The average weight of eggshell membranes stripped from 60 eggs was 27.5 ± 4.9g (0.44 g / egg) and the yield of purified OCX-36 protein was 0.33 ± 0.08mg OCX-36 / gram of eggshell membranes (0.13 mg / egg). The total amount of OCX-36 samples extracted and purified from standards eggs over all experiments (approximately 90 preparations) was 843 mg.

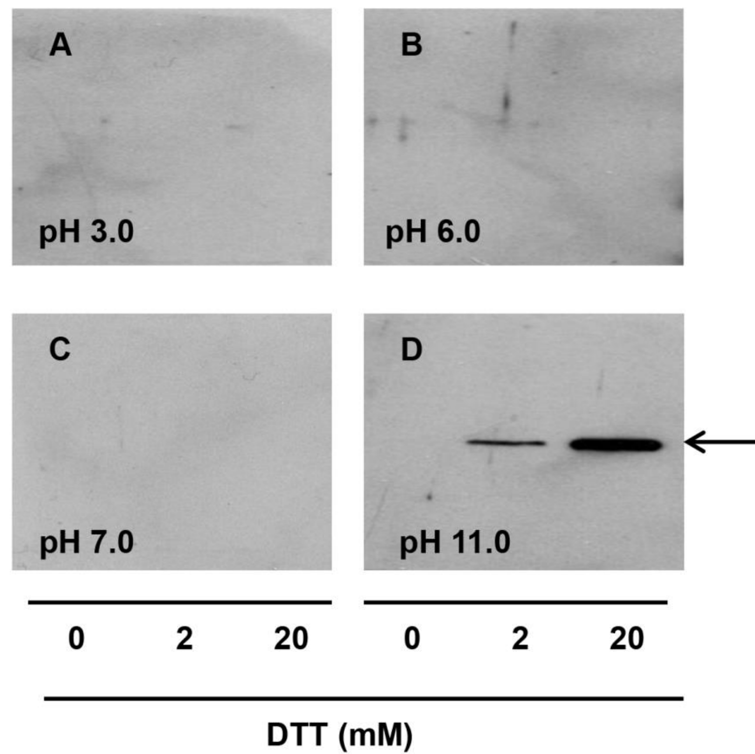
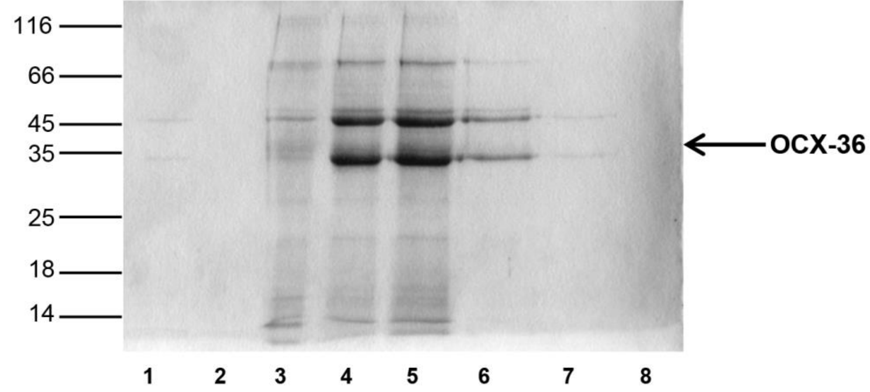


Figure 1. Western blot analysis for OCX-36 extracted using different extraction buffers. OCX-36 was extracted from eggshell membranes with different buffers at 0, 2 and 20 mM of DTT. (A) Buffer A (50 mM acetate, pH 3.0); (B) Buffer B (50 mM phosphate, pH 6.0); (C) Buffer C (50 mM phosphate, pH 7.0) and (D) Buffer D (50 mM Tris base, pH 11.0). Immunoreactive OCX-36 identified by Western Blotting (arrow).

A



B

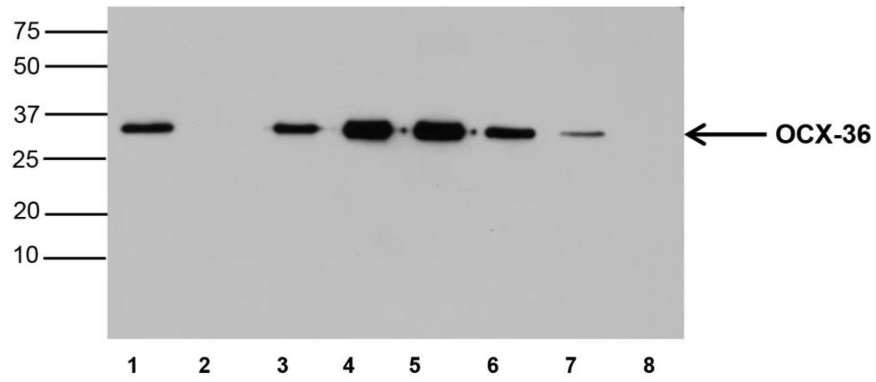


Figure 2. SDS-PAGE and Western blot analysis during OCX-36 purification. OCX-36 protein was extracted with buffer E (50 mM Tris-HCl, pH 8.5) and purified by CM-Sepharose Fast Flow and DEAE-Sepharose Fast Flow chromatographies. (A) 12.5% SDS-PAGE gel and (B) Western blot of OCX-36 fraction collected from each step of purification. The position of molecular weight standards (KDa) is indicated. Lane 1: Supernatant from overnight extraction prior to chromatography; Lane 2: Flow-through unbound to DEAE Sepharose; Lane 3: Sample prepared from DEAE beads before elution; Lanes 4 – 7: Fractions 1, 2, 3 and 4 eluted from the DEAE Sepharose with 50 mM Na Hepes, pH 7.0, 2 mM DTT, 350 mM NaCl; Lane 8: Sample prepared from DEAE beads after elution. Position of OCX-36 (Ovocalyxin-36) indicated by the arrow.

Table 2. Purification methods investigated for OCX-36 purification

Chromatography technique	Types
Hydrophobic interaction chromatography	Butyl, Pentyl, Hexyl and Octyl (Agarose)
Carbohydrate binding	Concanavalin A
Affinity Chromatography	Immobilized Nickel
Hydroxyapatite	

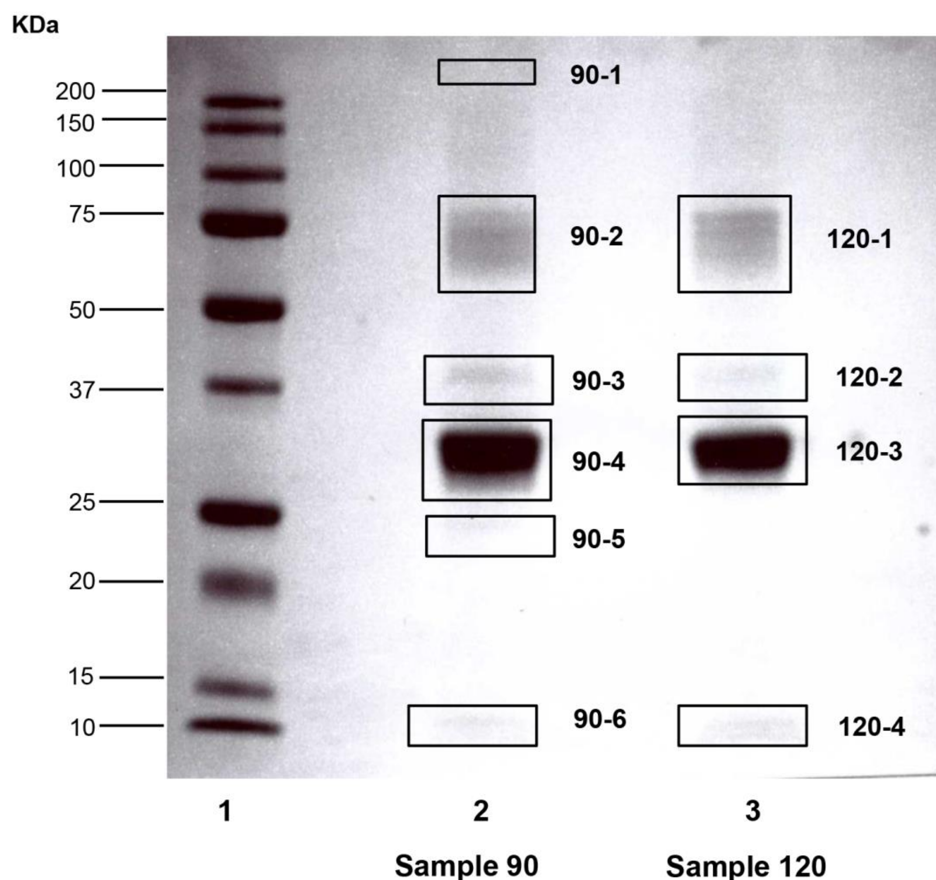


Figure 3. Proteomic analysis of purified OCX-36 samples (preparations 90 and 120). Boxes indicate the bands in the 12.5% Coomassie Blue - stained gel that were cut out. The excised bands were sent for LC/MS/MS sequencing analysis (methods). Samples 90 and 120 are two individual preparations of OCX-36. Lane 1: Molecular weight standards (KDa); Lane 2: Sample 90; Lane 3: Sample120.

Table 3. Merged proteomic results for purified OCX-36 samples using two independent samples

Identified Proteins	Accession Number	MW (kDa)	No. unique peptides [a] prep 90	emPAI [b]	No. unique peptides [a] prep 120	emPAI [b]
Ovocalyxin-36	Q53HW8	49	7	3.01	7	3.37
Ovalbumin	P01012	43	11	3.21	5	0.49
Ovalbumin-related protein Y	UPI0000E7FE38	44	4	0.37	-	-
Tiarin-like	Q25C35	56	7	0.54	3	0.2
Actin, cytoplasmic type 5	P53478	42	4	0.39	-	-
Hypothetical protein	UPI0000E806B4	76	-		2	0.1
BPI-like 2	UPI0000E7F8E6	44	2	0.17	-	-
Ig mu chain C region	P01875	49	2	0.15	-	-
IG heavy chain variable region	UPI0000ECBF72	13	2	0.67	-	-
Tenascin	F1N8F4	204	2	0.03	-	-

Proteomic analysis was performed on the bands identified in Figure 4, followed by merging the peptides (Mudpit analysis) to assess the total protein constituents and estimate their relative abundance (emPAI).

[a] Pro-71 (GLLSSPTIITGLHLER) and Ser-71 (GLLSSSTIITGLHLER) were combined

[b] Exponentially modified protein abundance index

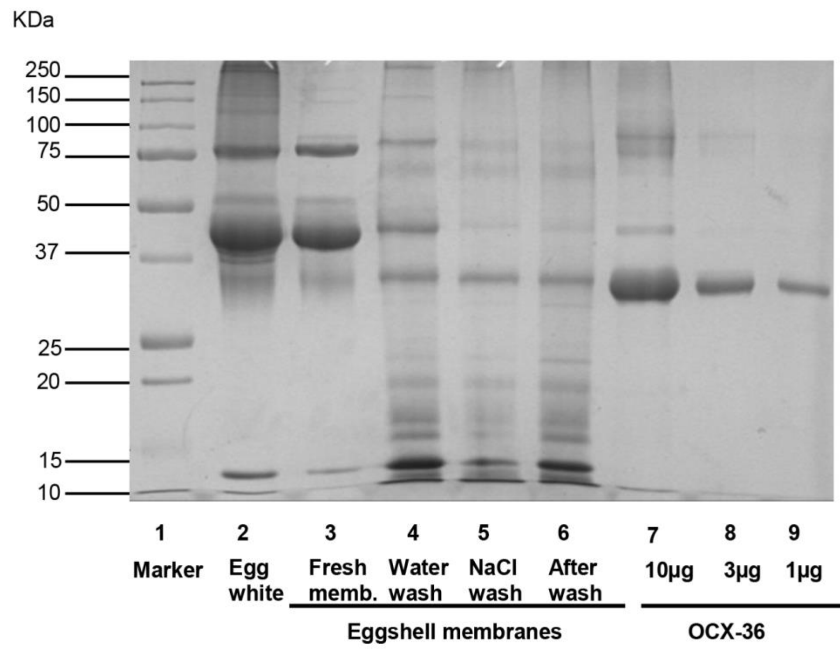

KDa
250
150
100
75
50
37
25
20
15
10
1
Marker
2
Egg white
3
Fresh memb.
4
Water wash
5
NaCl wash
6
After wash
7
10μg
8
3μg
9
1μg
Eggshell membranes
OCX-36

Figure 4. OCX-36 enrichment during extraction and purification. Samples from each stage of OCX-36 purification were prepared for SDS-PAGE by heating in Laemmli buffer, followed by determination of protein concentration for the extracted proteins (from membranes). All samples are 10 ug, except as indicated. Lane 1: Molecular weight standards (KDa) are indicated. Lane 2: Egg white; Lane 3: Fresh – sample from eggshell membranes prior to stripping from eggshell interior; Lane 4: Water wash - sample from eggshell membranes after extensive washing with water; Lane 5: NaCl wash – sample from eggshell membranes after washing with NaCl; Lane 6: After wash – membranes after OCX-36 extraction with buffer E, revealing unextracted proteins soluble in SDS; Lane 7-9: Purified OCX-36 (after sequential CM and DEAE chromatography). The purity of OCX-36 at the final purification step (1μg) was estimated as 98%.

Table 4. Densitometry analysis of OCX-36

Extraction and Purification steps	Estimated OCX-36 content (%)	Fold Purification
A. Stripped membranes	< 0.1%	1
B. Membranes: water wash	9%	90
C. Membranes: NaCl wash	15.6%	156
D. Purified OCX-36	98%	980

2. Polymorphisms in OCX-36 gene

Data base analysis of the NCBI chicken genome (www.ncbi.nlm.nih.gov/snp) identified rs15177583 predicting a non-synonymous SNP (conferring a C-T substitution leading to a change at amino acid position 71 (Proline-71 / Serine-71). This polymorphism was apparent in the proteomic sequencing data obtained with OCX-36, indicating that our typical purification from 5 – 10 dozen standard eggs had purified a mixture of the ser / pro versions (Table 3). This suggested that individual birds could be identified which laid eggs containing either the Pro-71 or Ser-71 polymorphic forms. SNP fine mapping of genomic DNA isolated from individual White Leghorn pedigree birds confirmed the predicted mixture of genotypes (Table 5). Two distinct populations of birds were examined. Their genetic background differed in that one was a fast feathering population and the other was a slow feathering population which had undergone many generations of selection for egg production and related traits. We observed the presence of homozygous C alleles coding for proline, homozygous T alleles coding for serine and heterozygous Y coding for either proline or serine. The homozygous C alleles coding for proline-71 was most represented in these animals (61.3%)(Table 5). However, significant differences in the abundance of the alleles were observed between the two (slow / fast feathering) populations. This trait is likely unrelated to OCX-36 polymorphism, but rather reflects a founder effect. Birds homozygous for each allele were identified and caged individually for egg collection. OCX-36 was extracted and purified from eggshell membranes harvested from eggs laid by birds

tested that were homozygous for the OCX-36 alleles coding for either Pro-71 or Ser-71.

Table 5. Allelic frequencies of OCX-36 SNPs in Pedigree White Leghorn birds

Bird i.d.	Alleles (polymorphism)	Number of birds	%
1-40 [a]	Heterozygous Y (OCX-36)	18	45
	Homozygous C (Pro-71)	10	25
	Homozygous T (Ser-71)	12	30
41-80 [b]	Heterozygous Y (OCX-36)	1	2.5
	Homozygous C (Pro-71)	39	97.5
	Homozygous T (Ser-71)	0	0

[a] Slow feathering group

[b] Fast feathering group

3. Antimicrobial activity of purified OCX-36 from standard eggs

Purified OCX-36 protein at 100 µg/mL was able to cause a significant (* $p < 0.001$) inhibition of *S. aureus* ATCC 6538 cell viability providing bactericidal effect during 24h of exposure compared to vehicle control (*S. aureus* 10^5 CFU/ mL, PBS) (Figure 5). OCX-36 at 30 µg/mL as well as 100 and 300 µg/mL (* $p < 0.05$) showed a significantly bacteriostatic activity against *S.aureus* for 10 h of incubation compared to vehicle control. OCX-36 at 300 ug/mL was significantly different from OCX-36 at 30 ug/mL and at 100 ug/mL (§ $p < 0.05$) (Figure 6).

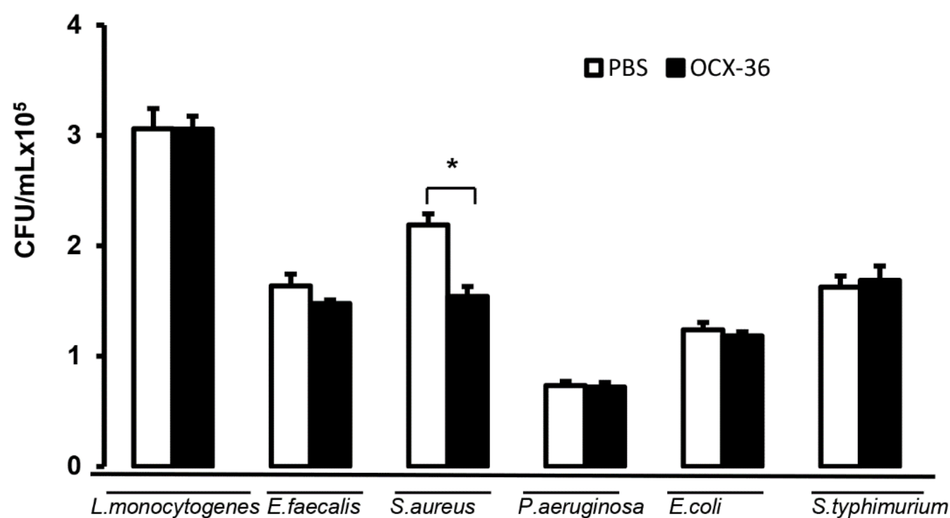


Figure 5. Antimicrobial activity of OCX-36 against Gram-positive (*L. monocytogenes, E. faecalis* and *S. aureus*) and Gram-negative bacterial strains (*P. aeruginosa, E.coli and S. typhimurium*). OCX-36 only showed antimicrobial activity against *S. aureus* ATCC 6538 which was significantly different from the vehicle control (PBS) (* $p < 0.001$). The results are the average of three independent experiments, each performed in triplicate.

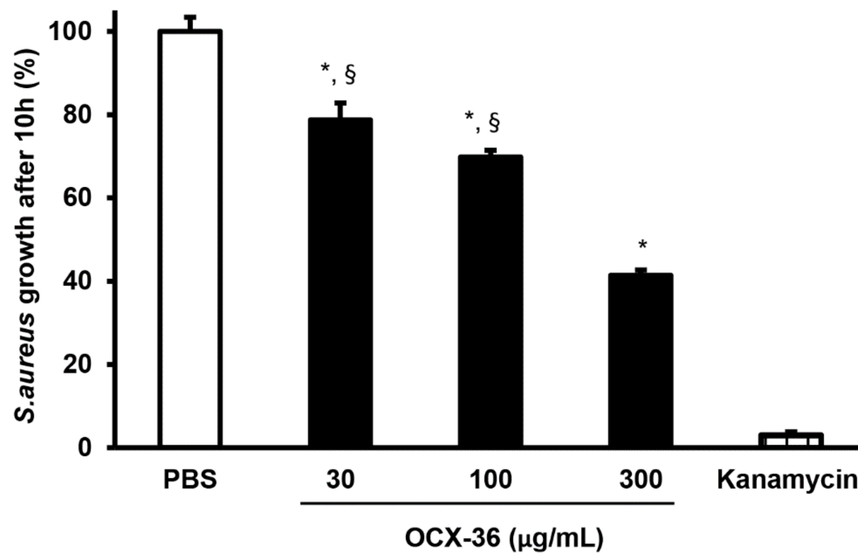


Figure 6. Bacteriostatic activity of OCX-36 against *S.aureus* ATCC 6538. Bacterial growth was analyzed using the Bioscreen C microplate reader over 10h of growth. The reduction of bacterial growth induced by OCX-36 at 30, 100 and 300 µg/mL was found to be significantly different from the vehicle control (PBS) (* $p < 0.05$). OCX-36 at 30 and 100 µg/mL was statistically different from OCX-36 at 300 µg/mL (§ $p < 0.05$). The results are the average of three individual experiments, each performed in triplicate.

4. Antimicrobial activity of OCX-36 from genotyped hens

Both versions of OCX-36 (Pro-71 and Ser-71) at 100 μg/mL showed significant bacteriostatic activity against *S. aureus* ATCC 6538 compared to vehicle control (10^5 *S. aureus* CFU/ mL, PBS). Both polymorphic forms of OCX-36 were also significantly bacteriostatic against *S. aureus* for 10h of incubation ($^*p < 0.05$) (Figure 7).

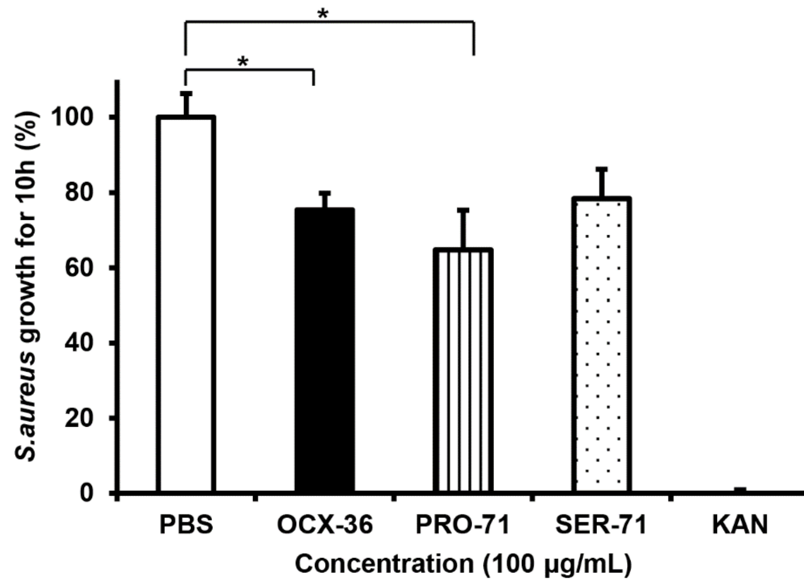
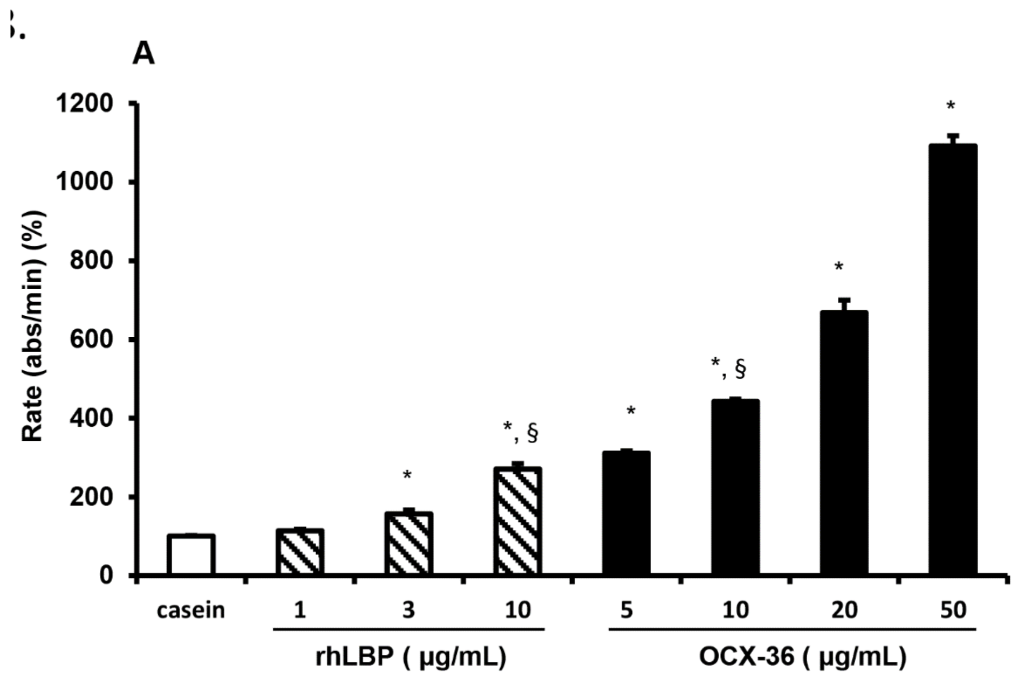


Figure 7. Antimicrobial activity of OCX-36 polymorphic forms from genotyped eggs (Pro-71 and Ser-71), and the OCX-36 mixture from standard eggs against *S. aureus* ATCC 6538. *S. aureus* growth was inhibited by OCX-36 (mixture of Pro-71 and Ser-71) and the individual forms at 100 µg/mL for 10h of incubation, as assessed with the Bioscreen C microplate reader (compared to vehicle control growth in PBS) (* $p < 0.05$).The results are averages of three individual experiments, with each experiment performed in triplicate.

5. LPS – binding activity of purified OCX-36

The ability of OCX-36 to bind LPS was investigated. OCX-36 protein at 100 µg/mL showed significant binding towards biotinylated *E. coli* O111:B4 LPS in a dose dependent manner, which was significantly higher than the positive control, rhLBP (Figure 8). Control tests of LPS binding to purified ovalbumin and ovotransferrin at 100 µg/mL were also tested (data not shown). Ovotransferrin exhibited LPS binding activity but this protein was not detected in purified OCX-36 contents by MS/MS analysis. Although ovalbumin was detected in purified OCX-36 preparations by MS/MS analysis, controls using purified ovalbumin found that it did not bind to biotinylated *E. coli* LPS O111:B4. The LPS binding activity of OCX-36 from standard eggs was also confirmed using non-biotinylated LPS binding activity assay (Figure 9A).

3.



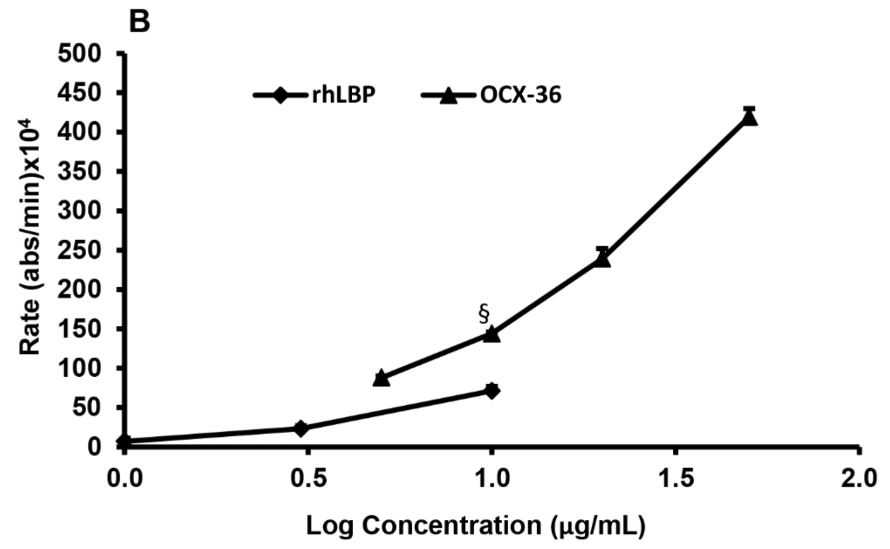
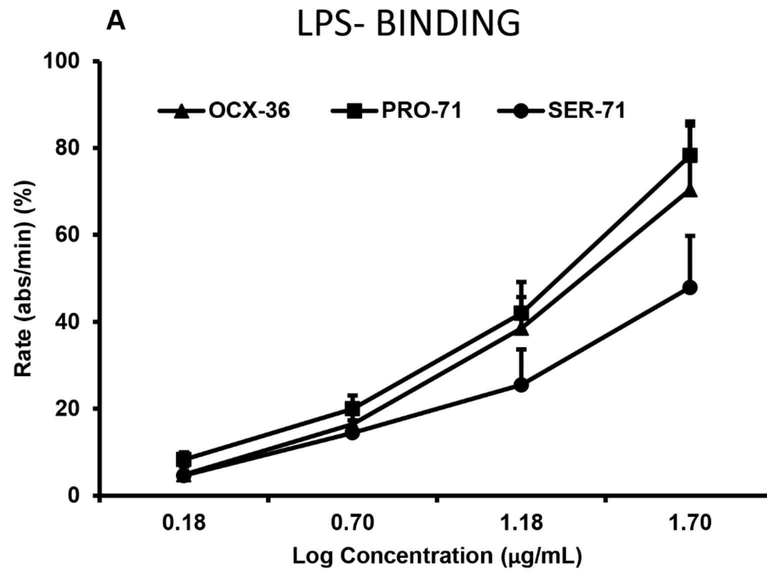


Figure 8. LPS binding activity of purified OCX-36 and rhLBP towards biotinylated *E.coli* O111:B4 LPS. The LPS binding activity of OCX-36 purified from standard eggs (5, 10, 20 and 50 µg/mL) and recombinant human Lipopolysaccharide binding protein (rhLBP) (1, 3 and 10 µg/mL) was tested using the biotinylated LPS plate-binding assay. (A) The LPS binding activity of rhLBP and OCX-36 were significantly different than the negative control, casein (* $p < 0.05$). (B) OCX-36 and rhLBP proteins showed a significant binding towards biotinylated *E. coli* O111:B4 LPS and OCX-36 was significantly higher than the positive control, rhLBP at 10µg/mL (§ $p < 0.05$). The LPS binding activity of both protein were normalized to casein. The results are three individual experiments and each experiment was performed in triplicate.

6. LPS and LTA activity of OCX-36 from standard and genotyped eggs

Purified OCX-36 (mixture of Pro-71 and Ser-71 forms) and individual OCX-36 polymorphic forms showed significant *E. coli* O111:B4 LPS binding, but there was no significant difference in binding between these proteins (Figure 9A). We also tested *S. aureus* LTA binding activity (Figure 9B). The Pro-71 form of OCX-36 showed significantly higher LTA binding activity than both mixed OCX-36 and Ser-71 protein at 15 ug/mL. Mixed OCX-36, containing both the Pro-71 and Ser-71 forms, showed an intermediate LTA binding activity (Figure 9B).


A
LPS- BINDING
OCX-36
PRO-71
SER-71
Rate (abs/min) (%)
100
80
60
40
20
0
0.18
0.70
1.18
1.70
Log Concentration (µg/mL)

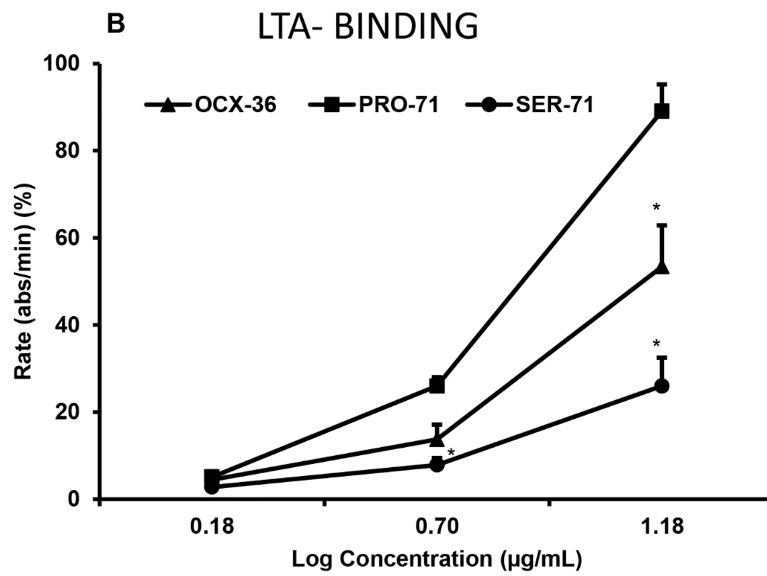

B
LTA- BINDING
OCX-36
PRO-71
SER-71
Rate (abs/min) (%)
100
80
60
40
20
0
0.18
0.70
1.18
Log Concentration (µg/mL)

Figure 9. LPS and LTA binding activity of OCX-36 (mixture) and OCX-36 individual forms (Pro-71 and Ser-71). The LPS and LTA binding to OCX-36 (mixture), OCX-36 (Pro-71) and OCX-36 (Ser-71) were tested with the ELISA binding assay. Binding activity was corrected for background BSA binding and individual experiments were normalized before merging. (A)LPS-BINDING: All proteins tested showed *E. coli* LPS binding activity at 1.5, 5, 15 and 50 µg/mL but a significant difference was not observed between them at any concentration. All concentrations of Pro-71 showed significant different LPS binding activity between them ($p < 0.05$). (B) LTA-BINDING: The LTA binding activity of OCX-36, Pro-71 and Ser-71 were tested at 1.5, 5 and 15 µg/mL. The LTA binding activity of OCX-36 (15 µg/mL) and Ser-71 (5 and 15 µg/mL) were significantly different than the Pro-71 (*$p< 0.05$). All concentrations of Pro-71 showed LTA binding activity significant difference between them ($p < 0.05$). The LTA binding activity of OCX-36 at 5 and 15 ug/mL were significant different between these concentrations ($p < 0.05$). The results are the average of three individual experiments, with each experiment performed in triplicate.

Discussion

1. OCX-36 identification and purification

OCX-36 is an abundant protein in eggshell membranes, where it is readily detected by immunofluorescence techniques, leading to speculation about its role in defence of the developing embryo and in unfertilized table eggs against pathogenic bacteria [9]. This previous work underscores the relevance of the current study to investigate the biological function of OCX-36 in chicken eggs. A key feature of the current study was to develop a suitable method for extraction and purification of OCX-36 protein from eggshell membranes. DTT was found to be an essential component of the extraction buffer. In previous studies on chicken egg proteins, reducing agents such as 2-mercaptoethanol and DTT, have been frequently used to break intra- and inter- molecular disulfide bonds [27, 28]. The efficient extraction of OCX-36 by DTT leads us to speculate that association of OCX-36 protein with chicken eggshell membranes is stabilized by disulfide bonds. After separation of extracted and purified proteins by SDS-PAGE, an immunoreactive OCX-36 band at approximately 33 kDa (confirmed by proteomic analysis) was detected (Figure 2A), which is slightly lower than the originally identified band at 36 kDa [9]. Differences in the molecular weight markers and electrophoretic conditions (i.e. % acrylamide) between these studies are likely at the origin of these differences in apparent molecular weight. Indeed, in our previous study we proposed a hypothesis for the differences between sequence molecular weight of OCX-36 and that detected by SDS-PAGE. For example, possible oligomerization of OCX-36, cross-linking (intramolecular) or even

abnormal mobility on SDS-PAGE due to its highly hydrophobic amino acid sequence [9].

The next step for OCX-36 characterization was purification using ion exchange chromatography. This chromatography method has been extensively used to purify egg proteins because it may not affect the protein structure and the purified proteins remain active [29, 30]. The optimized purification method used for OCX-36 was able to retain and elute OCX-36 from the DEAE-Sepharose column with >98% purity (Figure 2A and 2B) as demonstrated by densitometric analysis (Table 4). Further LC/MS/MS analysis was performed and identified OCX-36 as the most abundant protein. Although, this extraction and purification scheme yielded essentially pure OCX-36 (>98%), other proteins were detected when large amounts of protein were analysed by SDS-PAGE. Most protein contaminants identified in purified OCX-36 samples by MS/MS analysis were egg white proteins (Table 3), of which ovalbumin was the most abundant. These egg white proteins were also found in other compartments such as oviduct fluid and calcified eggshell matrix. However, egg proteins such as Actin cytoplasmic type 5, Tiarin-like and BPI-like 2 were also identified. Certain of these proteins are thought to be nonspecific contaminants that are derived from other oviduct segments or luminal cells during egg formation [31, 32]. Lysozyme, a well-known and potent antimicrobial protein, was not detected in preparations of purified OCX-36.

2. Antimicrobial activity and LPS/LTA binding activity of OCX-36

The natural resistance of the contents of the avian egg to contamination by pathogens depends upon the physical barrier of the eggshell and upon chemical defences due to antimicrobial egg proteins and peptides that are secreted by the luminal cells of the oviduct and become incorporated into egg compartments such as egg white, eggshell membranes and the eggshell [33].

The recognition that avian OCX-36 is a member of the BPI/LBP/PLUNC gene locus, and that its protein sequence has similarity to mammalian BPI/LBP, was the basis for our hypothesis that OCX-36 has an antimicrobial activity [9, 19]. BPI supresses LPS inflammatory activity by binding its N-terminal domain to lipid A moiety in LPS [34]. This interaction of N-terminal domain of BPI to LPS is crucial for its antimicrobial and anti-inflammatory activities of BPI [35, 36]. BPI binds to bacterial LPS and penetrates the inner membrane of bacteria to causes depolarization of its membrane and cell death [37].

We evaluated the antimicrobial activity of purified OCX-36 against a variety of Gram-positive and Gram–negative bacteria, and assessed its capacity to bind LTA and LPS. The results of viability and growth inhibition assays showed that OCX-36 was only effective against *S.aureus* ATCC 6538. OCX-36 possesses both bactericidal and bacteriostatic effects against *S. aureus* ATCC 6538. The bacteriostatic effect of OCX-36 against *S.aureus* ATCC 6538 was dose and time dependent.

As predicted from their structural homology to BPI, PLUNCs have been demonstrated to possess antimicrobial activity and anti-inflammatory properties

[38, 39]. Certain PLUNC proteins showed antimicrobial activity against specific airways pathogens such as *P. aeruginosa* and *Mycoplasma pneumonia* [40]. Moreover, SPLUNC 1 was able to inhibit *P. aeruginosa* growth and binds to LPS [41]. A proposed model for the antimicrobial action of SPLUNC 1 against *P. aeruginosa* in vitro is that SPLUNC 1 protein increases bacterial cell permeability and has a chemoattractant effect upon macrophages and neutrophils at the site of infection [42]. SPLUNC 1 showed a significant antimicrobial activity against *Mycoplasma pneumonia* but only modest inhibition of *E. coli* growth [43].

Recombinant human BPI (rhBPI) is able to directly interact with and neutralize LPS derived from different Gram negative bacteria [44, 45]. The N- terminal of rhBPI at low concentrations in biologic fluids such as serum and whole blood showed antimicrobial activity against Gram-negative bacteria including *E. coli*, *Salmonella typhimurium*, *Shigella* and *Enterobacter* spp. [45]. The rhBPI 21-KDa protein has been used for the treatment of children with meningococcal sepsis [46]. Recombinant BPIs from human and mouse have the ability to neutralize LPS from Gram-negative bacteria. However, mouse recombinant BPI does not inhibit *P. aeruginosa* growth even at higher concentrations than human recombinant BPI [47].

BPI proteins and peptides from BPI at high concentrations showed direct bactericidal activity against L forms of Gram-positive bacteria including *Staphylococcus aureus* and *Streptococcus pyogenes*. This antimicrobial effect suggests that these proteins exert a cytotoxic effect on the cytoplasmatic membrane of these pathogenic bacteria [48]. Murine recombinant BPI 21

promotes the association of *Streptococcus pneumoniae* with murine macrophages through the binding of BPI to this Gram-positive bacterium [49]. Antimicrobial activity against Gram-positive bacteria was also found in GL13K, a modified peptide derived from SPLUNC 2 protein that showed bactericidal activity against *Streptococcus gordonii* [50].

The anti-angiogenic activity of BPI has been demonstrated in chorioallantoic membrane (CAM), which develops in close proximity to the eggshell membranes [16]. The CAM regulates the mobilization of calcium from eggshell to the chick embryo during development to provide the metabolic needs such as skeletal growth and neuromuscular activities [51]. However, we have no evidence whether OCX-36 regulates normal blood vessel development during chick embryonic growth.

We investigated whether OCX-36 binds LPS and LTA, which are bacterial cell wall components of Gram-negative and Gram-positive bacteria, respectively, and are known as pathogen associated molecular patterns (PAMPs). Some examples of PAMPs include lipoproteins, peptidoglycan, lipoteichoic acid (LTA), lipopolysaccharide (LPS) and lipoarabinomannan (LAM) [52]. Interaction with PAMPS such as LPS and/or LTA is a characteristic feature of the family of proteins with BPI/LBP/PLUNC–like domains [53]. OCX-36 showed significant binding of *E. coli* O111:B4 LPS, suggesting that it participates in innate host defense against bacterial challenge similar to BPI or LBP. OCX-36 also binds to LTA from *S. aureus* ATCC 6538, suggesting that its inhibition of *S. aureus* growth is likely dependent on its interaction with bacterial cell wall LTA. The ability to bind to LPS is also found

in human PLUNC isoforms with that are present in nasal lavage fluid. This study identified SPLUNC 1 modified by N-linked glycosylation with LPS binding activity [54]. Parotid secretory protein (PSP), a PLUNC protein found in saliva is an LPS binding protein, as is a corresponding synthetic peptide (GL13NH2). The synthetic peptide showed anti-inflammatory property by inhibiting LPS stimulated secretion of tumor necrosis factor from macrophages [50].

In addition to its LPS binding activity, LBP also interacts with other PAMPs such as peptidoglycan and LTA. LBP binds to peptidoglycan breakdown products and lipopeptides and mediates innate immune responses [52]. In a murine meningitis experimental model, LBP was able to recognize peptidoglycan breakdown products derived from *S. pneumoniae* and modulate the inflammatory response [55]. LBP has been reported to modulate the effect of LTA. LBP might be has a dual role in the innate immune response since this protein is able to increase and decrease the effect of LTA in macrophages and monocytes [56]. All these studies report functional activities detected with recombinant purified LBP/BPI/PLUNC proteins.

3. OCX-36 SNP characterization and functions

Genetic variations in genes such as single nucleotide polymorphisms (SNPs) are associated with host susceptibility and resistance to infectious diseases. SNPs can have an impact on gene expression as well as the biological function of protein, which leads to phenotypic consequences [57]. SNPs in the LPB gene are associated with susceptibility to sepsis and multiple organ dysfunction [58, 59]. For

example, a polymorphism in the human LBP gene affects the risk for Gram-negative bacteremia [60]. A polymorphism in the BPI gene (Lys/Glu - 216) was reported to be associated with increased risk for development of sepsis but is uncorrelated with gender; while a corresponding genetic variation in LBP is related to male gender [61]. A recent study showed that BPI gene polymorphism is associated with susceptibility to bowel disease [62]. An SNP in the SPLUNC1 gene that is associated with enhanced risk of nasopharyngeal carcinoma may be related to altered expression and binding affinity for specificity protein1 (Sp1) transcription factor [63].

OCX-36 SNPs were identified in the OCX-36 gene and one non-synomymous SNP was verified, coding for alternative amino acids at position 71 - Pro/Ser. To address the functional consequences of these alternative versions, OCX-36 was extracted and purified from eggshell membranes harvested from eggs laid by hens that were homozygous for either of the two OCX-36 alleles. The antimicrobial activity of OCX-36 polymorphic forms (Pro-71 and Ser-71) was assessed only against *S. aureus* ATCC 6538 due to limitations in the amount of purified OCX-36 available. Both OCX-36 versions were inhibitory against growth of *S. aureus* ATCC 6538; no significant difference between them was found. Purified OCX-36 (proline-71) and (serine-71) bind to *E. coli* O111:B4 LPS and to LTA from *S. aureus* ATCC 6538. The Pro-71 form binds significantly more strongly to LTA than the Ser-71 form; purified OCX-36 with a mixture of both forms was intermediate. The mechanism by which these alterations in amino acid at position 71 affect the LTA

binding activity of OCX-36 is likely due to differences in tertiary structure due to the significant differences between the properties of serine and proline.

4. Proposed model for OCX-36 function

OCX-36 is specifically expressed in the chicken reproductive and digestive tracts. Our proposal for OCX-36 function is that it is a pattern recognition molecule, which recognizes bacterial endotoxins as a first step to eliminate pathogens. However, a future study to determine if OCX-36 competes for LPS binding or the interaction of OCX-36 with Toll-like receptors (TLR-2 and TLR-4) is necessary for a better understanding of the mechanism of OCX-36 action regarding our hypothetical model for OCX-36 as a pattern recognition receptor.

Acknowledgments

We would like to thank Dr. Syed Sattar (CREM) and his staff, Richard Kibbee in particular, for providing the strains used in our experiments, and Dr. Joel Gautron for providing the OCX-36 antibody. We are grateful to Dr. Yves Nys for valuable discussion and insight during the preparation of this manuscript. We thank Megan Rose-Martel, Jingwen Du, Erica Gifford, Emmanuel Aiyere for their valuable help.

References

1. Hincke MT, Nys Y, Gautron J, Mann K, Rodriguez-Navarro AB, et al. (2012) The eggshell: structure, composition and mineralization. Front Biosci 17:1266-1280.

2. Nys Y, Gautron J, Garcia-Ruiz JM, Hincke MT (2004) Avian eggshell mineralization: biochemical and functional characterization of matrix proteins. Comptes Rendus Palevol 3: 549-562.

3. Fernandez MS, Araya M, Arias JL (1997) Eggshells are shaped by a precise spatio-temporal arrangement of sequentially deposited macromolecules. Matrix Biol 16: 13-20.

4. Hincke MT, Gautron J, Panheleux M, Garcia-Ruiz J, McKee MD, et al. (2000) Identification and localization of lysozyme as a component of eggshell membranes and eggshell matrix. Matrix Biology 19: 443-453.

5. Cordeiro CM, Hincke MT (2011) Recent patents on eggshell: shell and membrane applications. Recent Pat Food Nutr Agric 3: 1-8.

6. Arias JL, Fernandez MS, Dennis JE, Caplan AI (1991) Collagens of the chicken eggshell membranes. Connect Tissue Res 26: 37-45.

7. Kodali VK, Gannon SA, Paramasivam S, Raje S, Polenova T, et al. (2011) A novel disulfide-rich protein motif from avian eggshell membranes. PloS One 6: e18187.

8. Gautron J, Hincke MT, Panheleux M, Garcia-Ruiz JM, Boldicke T, et al.(2001) Ovotransferrin is a matrix protein of the hen eggshell membranes and basal calcified layer. Connect Tissue Res 42: 255-267.

9. Gautron J, Murayama E, Vignal A, Morisson M, McKee MD, et al.(2007) Cloning of ovocalyxin-36, a novel chicken eggshell protein related to lipopolysaccharide-binding proteins, bactericidal permeability-increasing proteins, and plunc family proteins. J Biol Chem 282: 5273-5286.

10. Chiang SC, Veldhuizen EJ, Barnes FA, Craven CJ, Haagsman HP, et al. (2011) Identification and characterisation of the BPI/LBP/PLUNC-like gene repertoire in chickens reveals the absence of a LBP gene. Dev Comp Immunol 35: 285-295.

11. Bingle C, Seal R, Craven, CJ (2011) Systematic nomenclature for the PLUNC/ PSP/BSP30/SMGB proteins as a subfamily of the BPI fold-containing superfamily. Biochem Soc Trans 39: 977-83.

12. Tian X, Gautron J, Monget P, Pascal G (2010) What makes an egg unique? Clues from evolutionary scenarios of egg-specific genes. Biol Reprod 83: 893-900.

13. Krasity BC, Troll JV, Weiss JP, McFallngai MJ (2011) LBP/BPI proteins and their relatives: Conservation over evolution and roles in mutualism. Biochem Soc Trans 39: 1039-1044.

14. Tobias PS, Mathison J, Ulevitch R (1988) A family of lipopolysaccharide binding proteins involved in responses to gram-negative sepsis. J Biol Chem 263: 13479-13481.

15. Yamagata M, Rook SL, Sassa Y, Ma RC, Geraldes P, et al.(2006) Bactericidal/permeability-increasing protein's signaling pathways and its retinal trophic and anti-angiogenic effects. FASEB J 20: 2058–2067.

16. Van der Schaft DW, Toebes EA, Haseman JR, Mayo KH, Griffioen AW (2000) Bactericidal/permeability-increasing protein (BPI) inhibits angiogenesis via induction of apoptosis in vascular endothelial cells. Blood 96:176e81.

17. Schultz H, Weiss JP (2007) The bactericidal/permeability-increasing protein (BPI) in infection and inflammatory disease. Clin Chim Acta 384: 12-23.

18. Wiesner J, Vilcinskas A (2010) Antimicrobial peptides. The ancient arm of the human immune system. Virulence 1: 440–464.

19. Gautron J, Réhault-Godbert S, Pascal G, Nys Y, Hincke M (2011) Ovocalyxin-36 and other LBP/BPI/PLUNC-like proteins as molecular actors of the mechanisms of the avian egg natural defences. Biochem Soc Trans 39: 971-976.

20. Hincke MT, Nairn AC (1992). Phosphorylation of elongation factor 2 during Ca (2+)-mediated secretion from rat parotid acini. Biochem J 282: 877-882.

21. Rose-Martel M, Du J Hincke MT (2012) Proteomic analysis provides new insight into the chicken eggshell cuticle. J Proteomics 75: 2697-2706.

22. Keller A, Nesvizhskii AI, Kolker E, Aebersold R (2002) Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. Anal Chem 74:5383–5392.

23. Nesvizhskii AI, Keller A, Kolker E, Aebersold R (2003) A statistical model for identifying proteins by tandem mass spectrometry. Anal Chem 75:4646–4658.

24. Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, et al. (2005) Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. Mol Cell Proteomics 4:1265–1272.

25. Haigh B, Hood K, Broadhurst M, Medele S, Callaghan M, et al. (2008) The bovine salivary proteins BSP30a and BSP30b are independently expressed BPI-like proteins with anti-Pseudomonas activity. Mol Immunol 45:1944-1951.

26. Nahid AM, Sugii, S (2006) Binding of porcine ficolin-α to lipopolysaccharides from Gram-negative bacteria and lipoteichoic acids from Gram-positive bacteria. Dev Comp Immunol 30:335-343.

27. Kaweewong K, Garnjanagoonchorn W, Jirapakkul W, Roytrakul S (2013) Solubilization and Identification of Hen Eggshell Membrane Proteins During Different Times of Chicken Embryo Development Using the Proteomic Approach. Protein J 4: 297-308.

28. Ahlborn GJ, Clare DA, Sheldon BW, Kelly RW (2006) Identification of eggshell membrane proteins and purification of ovotransferrin and β-NAGase from hen egg white. Protein J 25: 71-81.

29. Levison PR (2003) Large-scale ion-exchange column chromatography of proteins: Comparison of different formats. J Chromatogr B Analyt Technol Biomed Life Sci 790: 17–33.

30. Guérin-Dubiard C, Pasco M, Hietanen A, Quiros del Bosque A, Nau F, et al. (2005) Hen egg white fractionation by ion-exchange chromatography. J Chromatograph A 1090:58-67.

31. Man K (2008) Proteomic analysis of the chicken egg vitelline membrane. Proteomics 8: 2322-2332.

32. Mann K, Maček B, Olsen JV (2006) Proteomic analysis of the acid-soluble organic matrix of the chicken calcified eggshell layer. Proteomics 6: 3801-3810.

33. Rehault-Godbert S, Herve-Grepine V, Gautron J, Cabau C, Nys Y, et al. (2011) Molecules involved in chemical defence of the chicken egg. Improving the safety and quality of eggs and egg products. Volume 1: Egg chemistry, production and consumption. pp. 183-208.

34. Gazzano-Santoro H, Parent JB, Grinna L, Horwitz A, ParsonsT,et al. (1992) High-affinity binding of the bactericidal/permeability-increasing protein and a recombinant amino terminal fragment to the lipid a region of lipopolysaccharide. Infect Immun 60: 4754–4761.

35. Mannion BA, Weiss J, Elsbach P (1990) Separation of sublethal and lethal effects of the bactericidal/permeability increasing protein on Escherichia coli. J Clin Invest 85: 853–860.

36. Ooi CE, Weiss J, Doerfler ME, Elsbach P (1991) Endotoxin- neutralizing properties of the 25 kd n-terminal fragment and a newly isolated 30 kd c-terminal fragment of the 55-60 kd bactericidal/permeability-increasing protein of human neutrophils. J Exp Med 174: 649–655.

37. Shai Y (1999) Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides. Biochim Biophys Acta 1462:55e70.

38. Gorr SU, Abdolhosseini M, Shelar A, Sotsky J (2011) Dual host defence functions of splunc2/psp and synthetic peptides derived from the protein. Biochem Soc Trans 39: 1028–1032.

39. Lukinskiene L, Liu Y, Reynolds SD, Steele C, Stripp BR, et al. (2011) Antimicrobial activity of plunc protects against Pseudomonas aeruginosa infection. J Immunol 187: 382–390.

40. Bartlett J, Gakhar L, Penterman J, Singh P, Mallampalli R, et al. (2011) PLUNC: a multifunctional surfactant of the airways. Biochem Soc Trans 39: 1012-1016.

41. Zhou HD, Li XL, Li GY, Zhou M, Liu HY, Yang YX, et al. (2008) Effect of SPLUNC1 protein on the P. aeruginosa and Epstein-Barr virus. Mol Cell Biochem 309:191–197.

42. Sayeed S, Nistico L, St Croix C, Y. Peter Di, YP (2013) Multifunctional role of human SPLUNC1 in P. aeruginosa infection. Infect Immun 81: 285-291.

43. Chu HW, Thaikoottathil J, Rino JG, Zhang G, Wu Q, et al. (2007) Function and regulation of SPLUNC1 protein in Mycoplasma infection and allergic inflammation. J. Immunol. 179: 3995–4002.

44. Levy O (2000) A neutrophil-derived anti-infective molecule: bactericidal/permeability-increasing protein. Antimicrob Agents Chemother 44: 2925–2931.

45. Levy O (2004) Antimicrobial proteins and peptides: anti-infective molecules of mammalian leukocytes. J Leukoc Biol 76: 909–925.

46. Levin M, Quint PA, Goldstein B, Barton P, Bradley J S, et al.(2000) Recombinant bactericidal/permeability-increasing protein (rBPI21) as adjunctive treatment for children with severe meningococcal sepsis: a randomised trial. rBPI21 Meningococcal Sepsis Study Group. Lancet 356:961–967.

47. Wittmann I, Schönefeld M, Aichele D, Groer G, Gessner A, et al. (2008) Murine bactericidal/permeability-increasing protein inhibits the endotoxic activity of lipopolysaccharide and gram-negative bacteria. J Immunol 180: 7546-7552.

48. Horwitz AH, Williams RE, Li PS, Nadell R (1999) Bactericidal/Permeability-Increasing Protein Inhibits Growth of a Strain of Acholeplasma laidlawii and L Forms of the Gram-Positive Bacteria Staphylococcus aureus and Streptococcus pyogenes. Antimicrob Agents Chemother 43: 2314-2316.

49. Srivastava A, Casey H, Johnson N, Levy O, Malley R (2007) Recombinant bactericidal/permeability-increasing protein rBPI21 protects against pneumococcal disease. Infect Immun 75: 342-349.

50. Abdolhosseini M, Nandula SR, Song J, Hir H, Gorr SU (2012) Lysine substitutions convert a bacterial-agglutinating peptide into a bactericidal peptide that retains anti-lipopolysaccharide activity and low hemolytic activity. Peptides 35:231-238.

51. Tuan RS, Ono T (1986) Regulation of extraembryonic calcium mobilization by the developing chick embryo. J Embryol Exp Morphol 97: 63-74.

52. Akira S, Uematsu S, Takeuchi O (2006) Pathogen recognition and innate immunity. Cell 124: 784-801.

53. Schumann RR (2011) Old and new findings on lipopolysaccharide-binding protein: a soluble pattern-recognition molecule. Biochem Soc Trans 39: 989-993.

54. Ghafouri B, Kihlström E, Tagesson C, Lindahl M (2004) PLUNC in human nasal lavage fluid: multiple isoforms that bind to lipopolysaccharide. Biochim Biophys Acta 1699: 57-63.

55. Schro der NWJ, Heine H, Alexander C, Manukyan M, Eckert J, et al. (2004) Lipopolysaccharide binding protein binds to triacylated and diacylated lipopeptides and mediates innate immune responses. J Immunol 173:2683–2691.

56. Mueller M, Stamme C, Draing C, Hartung T, Seydel U, et al. (2006) Cell activation of human macrophages by lipoteichoic acid is strongly attenuated by lipopolysaccharide-binding protein. J Biol Chem 281:31448–56.

57. Huebner C, Petermann I, Lam WJ, Shelling AN, Ferguson LR (2010) Characterization of single-nucleotide polymorphisms relevant to inflammatory bowel disease in commonly used gastrointestinal cell lines. Inflamm Bowel Dis 2:282-95.

58. Zeng L, Gu W, Zhang AQ, Zhang M, Zhang LY, et al. (2012) A functional variant of lipopolysaccharide binding protein predisposes to sepsis and organ dysfunction in patients with major trauma. Ann Surg 255: 147–157.

59. Barber RC, O'Keefe GE (2003) Characterization of a single nucleotide polymorphism in the lipopolysaccharide binding protein and its association with sepsis. Am J Respir Crit Care Med 167:1316–1320.

60. Chien JW, Boeckh MJ, Hansen JA, Clark JG (2008) Lipopolysaccharide binding protein promoter variants influence the risk for Gram-negative bacteremia

and mortality after allogeneic hematopoietic cell transplantation. Blood 111:2462–2469.

61. Hubacek JA, Stüber F, Fröhlich D, Book M, Wetegrove S, et al. (2001) Gene variants of the bactericidal/permeability increasing protein and lipopolysaccharide binding protein in sepsis patients: gender-specific genetic predisposition to sepsis. Crit Care Med 29: 557-561.

62. Klein W, Tromm A, Folwaczny C, Hagedorn M, Duerig N, et al. (2005) A polymorphism of the bactericidal/permeability increasing protein (BPI) gene is associated with Crohn's disease. J Clin Gastroenterol 39: 282-283.

63. Yew PY, Mushiroda T, Kiyotani K, Govindasamy GK, Yap LF, et al. (2012) Identification of a functional variant in SPLUNC1 associated with nasopharyngeal carcinoma susceptibility among Malaysian Chinese. Mol Carcino 51: E74-E82.

VI. CHAPTER 3. Ovocalyxin-36 is an effector protein modulating the production of proinflammatory mediators.

Kovacs-Nolan J, Cordeiro C, Young D, Mine Y, Hincke MT (2014). Ovocalyxin-36 is an effector protein modulating the production of proinflammatory mediators. *Veterinary immunology and immunopathology*, 160(1): 1-11.

This chapter reports the immune-modulating effect of OCX-36 as characterized with *in vivo* and *in vitro* approaches. The data supports our hypothesis that OCX-36 is an endotoxin-neutralizing protein with potential therapeutic applications in sepsis. This is the first demonstration of therapeutic relevance for a non-recombinant, non-mammalian PLUNC family member. Moreover, these results may ultimately lead to approaches to mitigate the risk of food-borne disease and positively impact poultry food safety.

Author contributions:

Experiments were conducted by Jennifer Kovacs-Nolan (Tables 1, Figures 1A, 2A, 4A, 5, 6 and 7), Cristianne Cordeiro (Tables, Figures 1B, 2B, 3, 4B) and Denise Young. Data interpretation and manuscript preparation was done by Jennifer Kovacs-Nolan and Cristianne Cordeiro. Experiments, data analysis, and manuscript preparation were supervised by Dr. Yoshinori Mine and Dr. Maxwell T. Hincke.

Ovocalyxin-36 is an effector protein modulating the production of proinflammatory mediators.

Jennifer Kovacs-Nolan [b], Cristianne Cordeiro [a], Denise Young [b], Yoshinori Mine [b], Maxwell Hincke [a, c]

[a] Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada, K1H 8M5

[b] Department of Food Science, University of Guelph, Guelph, Ontario, Canada, N1G 2W1

[c] To whom correspondence should be addressed:

Maxwell T. Hincke, Department of Cellular and Molecular Medicine, University of Ottawa, 451 Smyth Road, Ottawa, K1H 8M5, Canada, Tel:(1) 613-562-5800, x8193; Fax: (1) 613- 562- 5687; E-mail: mhincke@uottawa.ca.

Keywords: Lipopolysaccharide, Ovocalyxin-36, Gene expression and Innate immunity

List of abbreviations:

ACE	Angiotensin-I converting enzyme
BCA	Bicinchoninic acid
BPI	Bactericidal permeability-increasing protein
BW	Body weight
CCL2	MCP-1, monocyte chemotactic protein-1
CXCR4	Chemokine receptor 4
Cybβ	Cytochrome b-245, beta polypeptide
DMEM	Dulbecco's modified Eagle's Medium
dOCX-36	Digested Ovocalyxin-36
HRP	Horseradish peroxidase
HTAB	Hexadecyltrimethyl ammonium bromide
iNOS	Inducible nitric oxide
LAL	Limulus Amebocyte Lysate
LBP	Lipopolysaccharide-binding protein
LTA	Lipoteichoic acid
MPO	Myeloperoxidase

MWCO Molecular weight cut-off

MyD88 Myeloid differentiation primary response gene 88

NO Nitric oxide

OCX-36 Ovocalyxin-36

PAMPs Pathogen-associated molecular patterns associated molecular patterns

Pglyrp1 Peptidoglycan recognition protein 1

PLUNC Palate, lung and nasal epithelium clone

PMSF Phenylmethanesulfonyl fluoride

PRR Pattern recognition molecule

Proc Protein C

TMB Tetramethylbenzidine

TLR Toll-like receptor

Abstract

Sepsis is a systemic inflammatory response syndrome during infection. Therapeutic agents are essential to protect the host from sepsis. Ovocalyxin-36 (OCX-36) is a chicken eggshell membrane protein and shares protein sequence and gene organization homology with bactericidal permeability-increasing protein (BPI), lipopolysaccharide-binding protein (LBP) and palate, lung and nasal epithelium clone (PLUNC) proteins that play a major role in innate immune protection. We recently reported that OCX-36 binds to both lipopolysaccharide (LPS) and lipoteichoic acid (LTA) (Cordeiro et al. 2013, PLOS ONE 8, e84112), which is an important activity to neutralize endotoxins and non-endotoxin pyrogens during an inflammatory response. Here we investigated the immune modulating effects of OCX-36 and enzymatically digested OCX-36 (dOCX-36) *in vitro* and in a mouse model of endotoxemia. OCX-36 alone dose-dependently induced both TNF-α and nitric oxide (NO) production by RAW 264.7 macrophage cells, and this immunostimulatory effect was reduced by enzymatic digestion. In the presence of LPS, dOCX-36 was more effective than intact OCX-36 at reducing LPS-induced secretion of TNF-α from RAW 264.7 cells, but did not reduce NO production. In contrast, OCX-36 increased LPS-induced NO production, both in the presence and absence of FBS, PCR array analysis confirmed that OCX-36 and dOCX-36 differentially regulated genes involved in innate immunity, and dOCX-36 down-regulated the expression of genes involved in LPS signaling and inflammatory responses. *In vivo*, dOCX-36 was more effective at reducing LPS-induced inflammatory symptoms and inhibiting the local production of pro-inflammatory

mediators in the small intestine. These results suggest that OCX-36 and OCX-36 derived peptides may differentially modulate innate immune responses, and support our hypothesis that OCX-36 derived peptides have potential therapeutic applications in sepsis.

1. Introduction

Sepsis is a disease characterized by the invasion of bacterial pathogens into the bloodstream that activates an inflammatory response. The uncontrolled immune response leads to septic shock that involves tissue damage and multiple organ dysfunction and failure (Opal, 2007).

LPS is the main component of the Gram-negative bacterial cell wall and the principal activator of the innate immune system which promotes the production of pro-inflammatory mediators during infection (Beutler and Rietschel, 2013). LPS is one of several pathogen-associated molecular patterns (PAMPs) and is recognized by Toll-like receptor (TLR) 4 which is expressed on the surface of macrophages. This stimulates the host cells to secrete a large amount of proinflammatory mediators and cytokines such as nitric oxide (NO), tumor necrosis factor (TNF)-α, and interleukins (ILs) (Kumar et al., 2009). NO and TNF-α are associated with antimicrobial activity, the host innate immune response to pathogens and tumor cell killing (Bogdan, 2001).

The toxic effect of LPS is modulated by a large family of proteins such as the LBP/BPI/PLUNC protein family. These proteins bind LPS and mediate the LPS

signal to innate immune receptors (Wiesner and Vilcinskas 2010). For example, BPI protein suppresses the delivery of LPS to immune receptors and promotes LPS uptake by macrophages via the macrophage phagocytic process (Iovine et al., 2002). On the other hand, low concentrations of LBP deliver LPS to CD14 molecules and then boost the inflammatory response induced by LPS; in contrast, high concentrations of LBP reduce LPS activation of macrophages (Lamping et al., 1998). Some studies have reported that LBP/BPI/PLUNC proteins inhibit proinflammatory activities of LPS in macrophages such as induction of cytokines secretion, stimulation of neutrophil oxidase enzymes and NO formation (Schumann, 2001, Lukinskiene et al., 2011).

The current therapy for severe sepsis and septic shock include treatment of circulatory failure, the administration of antibiotics and the use of activated protein C (Rivers et al., 2001). Newer strategies are the identification and development of improved antimicrobial peptides that also neutralize the LPS functionality that leads to overproduction of proinflammatory mediators (Schuerholz et al., 2012).

OCX-36 is an avian protein enriched in the eggshell membranes of chicken eggs. OCX-36 shares similarity in protein sequence and gene structure with LBP, BPI and PLUNC proteins, which is the origin of our hypothesis that OCX-36 participates in the innate immune protection against pathogens (Gautron et al., 2007). We have recently characterized the biological function of purified OCX-36 extracted from eggshell membranes, demonstrating that it is a pattern recognition molecule (PRR) which has antimicrobial activity against *S. aureus* and the ability to bind to *E. coli* LPS and to *S. aureus* LTA (Cordeiro et al., 2013).

In order to evaluate the potential of OCX-36 for therapeutic and neutraceutical applications, we compared whole and enzymatically digested OCX-36 to determine their immune-stimulating and anti-endotoxin properties *in vivo* and *in vitro*.

2. Material and methods

2.1. Materials

Dulbecco's modified Eagle's Medium (DMEM), sodium pyruvate and penicillin-streptomycin were purchased from Gibco. FBS was purchased from Cansera. 48-well tissue culture plates and 96-well medium binding microplates were purchased from Corning Costar. Recombinant mouse TNF-α, IL-6, and IL-1β, anti-mouse TNF-α, IL-6, and IL-1β antibodies, biotinylated anti-mouse TNF-α, IL-6, and IL-1β antibodies, and avidin-conjugated HRP were purchased from BD Biosciences. Mouse TNF-α and IL-6 ELISA Ready-SET-Go®kits were purchased from eBioscience. WST-1 Cell Proliferation Reagent was purchased from Roche Applied Science. Bicinchoninic acid (BCA) protein assay, Bovine serum albumin (BSA), cell culture grade water (endotoxin-free, < 0.005 EU/mL)) and Limulus Amebocyte Lysate (LAL) Chromogenic Endotoxin Quantification Kit were purchased from Thermo Scientific. Pepsin from porcine gastric mucosa, LPS from *E. coli* O111:B4, PMSF, aprotinin, leupeptin, pepstatin A, (3,3',5,5"-tetramethylbenzidine (TMB) and hexadecyltrimethyl ammonium bromide (HTAB) were purchased from Sigma-Aldrich. The Griess reagent system was purchased

from Promega. Aurum™ Total RNA Mini Kit was purchased from Bio-Rad Laboratories. RT[2] First Strand cDNA Kit and Mouse Innate and Adaptive Immune Response RT[2] Profiler PCR Array were purchased from SA Biosciences.

2.2. Ovocalyxin-36

OCX-36 was extracted from eggshell membranes and purified as previously described (Cordeiro et al., 2013). Purified OCX-36 was dissolved in PBS buffer (10 mM sodium phosphate buffer, 0.154 M NaCl, pH 7.4) prepared with endotoxin-free water (< 0.005 EU/mL) and the concentrations of OCX-36 for all assays were determined by the BCA protein assay using BSA as standard. Endotoxin levels in OCX-36 samples were measured by the LAL assay (Thermo Scientific).

2.3. Enzymatic digestion of OCX-36

To prepare pepsin-digested OCX-36, freeze-dried OCX-36 (2 mg/mL) was dissolved in 0.15 M HCl, and pepsin was added to the OCX-36 solution at an enzyme to substrate ratio of 1:250 (w/w). Samples were incubated at 37°C for 0, 30 s; 1.5, 5 and 30 min; and 1.5h, 5h and 10 h, followed by heating at 90°C for 5 min to inactivate the enzyme. The digested samples were dialyzed against water (MWCO 100Da; Spectrum Laboratories, Inc.) and lyophilized for further use in cell culture and animal studies.

To prepare thermolysin-digested OCX-36, freeze dried OCX-36 was dissolved in endotoxin-free PBS buffer and diluted in thermolysin digestion buffer (50 mM Tris HCl, 0.5mM $CaCl_2$, pH 7.4). Thermolysin was dissolved in the same buffer and

was added at an enzyme to substrate ratio of 1:100 (w/w). Samples were incubated at 65°C for 15 min, 30 min; and 1h, 1.5 h and 4 h. After digestion, the enzyme was inactivated by heating at 95°C for 15 min and then cooled for 10 min at room temperature. Samples were centrifuged at 13,000 rpm (Biofuge Pico Heraeus Instruments) at 4°C for 10 min.

2.4. Electrophoresis for OCX-36 analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described (Hincke and Nairn, 1992), followed by staining with Coomassie Blue and destaining.

2.5. Cell culture

RAW 264.7 cells (ATCC) were cultured in DMEM supplemented with 1mM sodium pyruvate, 50 U/mL of penicillin-streptomycin and 10% FBS, and were grown at 37°C in a 5% CO_2 chamber. Cell passages 2-10 were used. Cells were seeded into 48-well tissue culture plates at a density of 5 x 10^5 cells/ well and allowed to adhere for 16-18 h before treatment. OCX-36 or dOCX-36 (with or without LPS) was either diluted into the culture medium (500 µL) from concentrated stocks (10µL) (for measurements of NO secretion) or prediluted in medium and added to the wells (500 µL) after aspiration of the culture medium (TNF-α secretion), in order to obtain the final, stated, concentrations.

2.6. Evaluation of anti-endotoxin activity *in vitro*

RAW 264.7 cells were treated with OCX-36 or enzymatically-digested OCX-36 (dOCX-36), in the presence or absence of *E. coli* O111:B4 LPS, in DMEM containing 10% FBS. To examine the effect on NO production, cells were incubated for 24 h in the presence of medium alone, 10 ng/mL LPS, OCX-36 or dOCX-36 alone, or LPS pre-incubated for 1 h with OCX-36 or dOCX-36 at the indicated concentrations. For TNF-α secretion and gene expression analysis by PCR array, cells were incubated for 6 h in the presence of medium alone, 100 ng/mL LPS, OCX-36 or dOCX-36 alone, or LPS pre-incubated with OCX-36 or dOCX-36 at the indicated concentrations.

To evaluate the potential role of LBP on the anti-endotoxin activity of OCX-36, cells were washed twice with PBS and treated as described above with OCX-36 in the presence or absence of LPS in serum-free DMEM. After incubation for 6 or 24 h, culture supernatants were collected for measurement of TNF-α and NO, respectively.

2.7. Evaluation of anti-endotoxin activity *in vivo*

Balb/c mice (18-20 g, 6-8 mice/group; Charles River Laboratories, Inc.) were injected intraperitoneally (i.p.) with 2 or 10 mg/kg·body weight (BW) OCX-36 or dOCX-36 combined with 25 µg *E. coli* O111:B4 LPS in sterile saline, in a total volume of 100 µL. Positive control mice received LPS alone, and negative control mice received saline only. Two control groups were given 10 mg/kg BW OCX-36

or dOCX-36 alone in sterile saline. Mice were monitored for clinical symptoms and weighed 24 h after LPS administration.

To further examine the acute local and systemic effects, mice were injected i.p. with 10 mg/kg·BW OCX-36 or dOCX-36 combined with 25 μg *E. coli* O111:B4 LPS in sterile saline. Positive control mice received LPS alone, and negative control mice received saline. After 2 h, mice were humanely euthanized and blood, liver, and small intestine (ileum) samples were collected. Blood was processed for serum and stored at -20°C, and liver and ileum samples were flash frozen and stored at -80°C until further analysis.

All animal procedures were carried out in accordance with the Canadian Council of Animal Care Guide to the Care and Use of Experimental Animals and were approved by the University of Guelph Animal Care Committee (AUP #07R116).

2.8. Cell viability assay

Cell viability was measured using the WST-1 Cell Proliferation Reagent (Roche) according to the manufacturer's instructions.

2.9. Nitric oxide (NO) assay

The NO concentration in culture supernatants was determined using the Griess Reagent System (Promega) according to the manufacturer's protocol.

2.10. Cytokine ELISAs

Measurement of TNF-α concentration in culture supernatants was carried out by ELISA according to the manufacturer's instructions (BD Biosciences), and results are expressed as percent TNF-α secretion relative to positive control (LPS) cells.

To measure TNF-α, IL-6 and IL-1β concentrations in liver and ileum samples, tissues were homogenized in 1 mL ice-cold PBS containing 0.5% Triton X-100, 1 mM PMSF, 10 µg/mL aprotinin, 10 µg/mL leupeptin, and 10 µg/mL pepstatin A using a Polytron® homogenizer (PT 1200; Kinematica, Inc., Switzerland). Homogenates were clarified by centrifugation at 12,000 rpm for 15 min at 4°C (Biofuge Fresco, Heraeus Instruments). TNF-α, IL-6 and IL-1β concentrations were measured by ELISA according to the manufacturer's instructions (BD Biosciences). TNF-α and IL-6 concentrations in serum were determined using mouse TNF-α and IL-6 ELISA Ready-SET-Go® kits (eBioscience) according to the manufacturer's instructions. Cytokine concentrations are expressed as pg or ng cytokine per mL serum, or ng cytokine per g tissue.

2.11. Myeloperoxidase (MPO) activity

Homogenate pellets from liver and ileum samples (section 10) were re-homogenized in 1 mL of 50 mM potassium phosphate, pH 6.0 containing 0.5% (w/v) HTAB, and subjected to two freeze-thaw cycles. Samples were clarified by centrifugation at 12,000 rpm for 10 min at 4°C (Biofuge Fresco, Heraeus Instruments), and supernatants were assayed for MPO

activity. Briefly, samples were diluted in HTAB buffer and mixed with a 1.6 mM TMB solution in 0.3 mM H_2O_2. One unit (U) of enzyme activity was defined as the amount of MPO present that caused a change in absorbance of 1.0/min at 655 nm. MPO activities are expressed as mU MPO per g tissue.

2.12. RNA isolation and PCR array analysis

Total RNA was extracted from RAW 264.7 cells using the Aurum™ Total RNA Mini Kit (Bio-Rad Laboratories) according to the manufacturer's instructions. One microgram of RNA was reverse transcribed using the RT^2 First Strand cDNA Kit (SA Biosciences), and the expression of 84 genes involved in the host response to bacterial infection and sepsis were analyzed simultaneously using a Mouse Innate and Adaptive Immune Response RT^2 Profiler PCR Array (SA Biosciences) according to the manufacturer's instructions. PCR was carried out using a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad) and data was analyzed using the instructions and template provided by the manufacturer (http://www.sabiosciences.com/dataanalysis.php). Results are expressed as -fold change relative to untreated cells. A gene was considered to be differentially expressed when it had a fold change of at least ± 2.0.

2.13. Statistical Analysis

All analyses were performed in triplicate unless specified otherwise. Statistical analyses were carried out using GraphPad Prism version 5.0 (GraphPad). Statistical significance was determined by Student's t-test with $p<0.05$ taken as significant. Results are reported as mean ± SEM.

3. Results

3.1. Anti-endotoxin effects *in vitro*

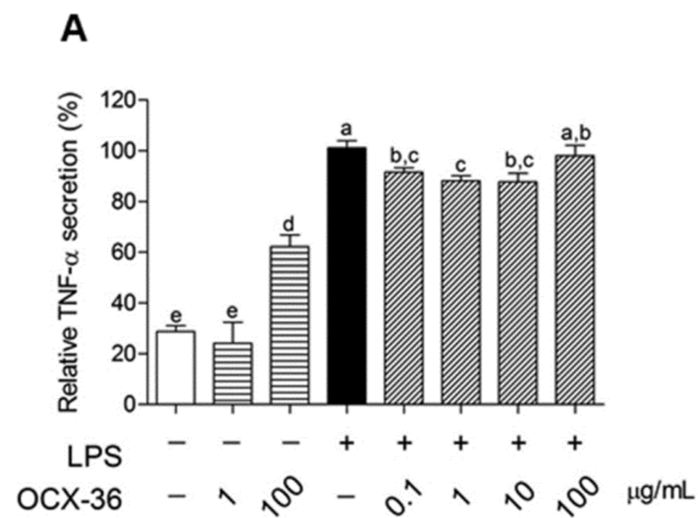
3.1.1. The effect of OCX-36 on LPS-induced secretion of TNF-α and NO in murine macrophages

To investigate the effect of OCX-36 on TNF-α and NO production in murine macrophages, RAW 264.7 cells were incubated with varying concentrations of OCX-36 and in the presence or absence of *E. coli* LPS. Treatment with LPS significantly increased TNF-α production in RAW 264.7 when compared to untreated control cells ($p<0.05$), and this was decreased ($p<0.05$) by OCX-36 at concentrations of 0.1, 1 and 10 μg/mL (Fig. 1A). In contrast, OCX-36 had no pronounced effect on LPS-induced NO production (Fig. 1B).

Surprisingly, treatment of RAW 264.7 cells with OCX-36 alone, without LPS stimulation, significantly increased both TNF-α and NO production ($p<0.05$) compared to untreated control cells, when added at concentrations greater than 1 μg/mL (Fig. 1A and 1B). This was not associated with endotoxin contamination in OCX-36 samples, since the endotoxin levels measured in OCX-36 samples using the LAL assay were lower than 1 ng/mL (0.11 ± 0.04 EU/μg of OCX-36, 0.02 ± 0.01 ng/μg of OCX-36), and this level of endotoxin (LPS) was found not to induce NO production in RAW 264.7 cells.

To further examine the effect of LBP, which is naturally present in FBS, on the anti-endotoxin activity of OCX-36, RAW 264.7 cells were treated with OCX-36 and

LPS in serum-free medium. Similar to the results obtained in medium containing FBS, OCX-36 at concentrations of 0.1, 1 and 10 μg/mL significantly reduced TNF-α secretion ($p<0.05$) (Fig 2A). However, in the absence of serum, OCX-36 treatment at 1 and 10 ug/mL resulted in a >25% decrease in LPS-induced TNF-α secretion, compared to only a 12% reduction in the presence of FBS, at the same OCX-36 concentrations. OCX-36 did not reduce NO production in LPS-activated RAW 264.7 cells in the absence of serum, but rather OCX-36 at 100 μg/mL significantly increased LPS-induced NO secretion when compared to cells treated with LPS alone ($p<0.5$) (Fig. 2B). No effect of OCX-36 or LPS treatment was observed on cell viability (data not shown).


A
Relative TNF-α secretion (%)
120
100
80
60
40
20
0
e
e
d
a
b,c
c
b,c
a,b
LPS
OCX-36
μg/mL

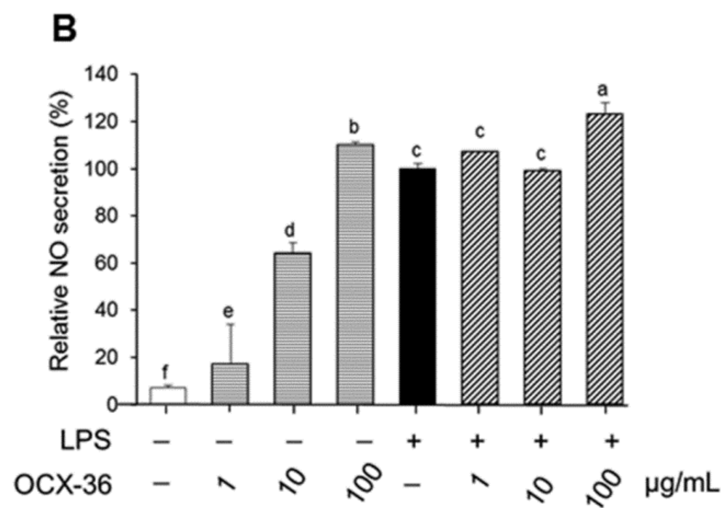
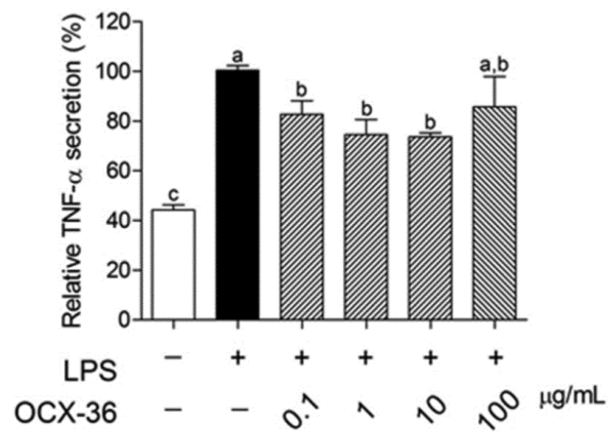

B
Relative NO secretion (%)
140
120
100
80
60
40
20
0
f
e
d
b
c
c
c
a
LPS
OCX-36
μg/mL

Fig. 1. Effect of OCX-36 on TNF-α and NO secretion in LPS-stimulated RAW 264.7 cells. (A) Cells were treated with 0, 0.1, 1, 10 or 100 μg/mL OCX-36 in the presence or absence of 100 ng/mL *E. coli* LPS for 6 h. TNF-α concentration was measured by ELISA, and is presented as percent TNF-α relative to positive control cells treated with LPS alone. (B) Cells were treated with 0, 1, 10 or 100 μg/mL OCX-36 in the presence or absence of 10 ng/mL *E. coli LPS* for 24 h. NO concentration was measured using Griess reagent and is presented as percent NO relative to positive control cells. Data represent means ± SEM, n=3. Values without a common letter are significantly different at $p<0.05$.


A
Relative TNF-α secretion (%)
120
100
80
60
40
20
0
c
a
b
b
b
a,b
LPS
OCX-36
μg/mL

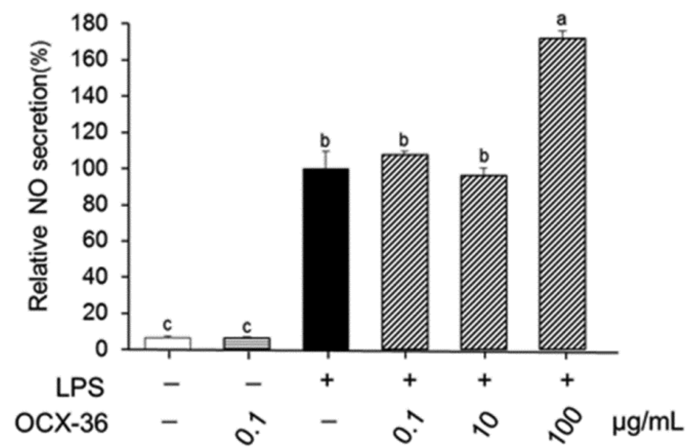

B
Relative NO secretion(%)
180
160
140
120
100
80
60
40
20
0
c
c
b
b
b
a
LPS
OCX-36
μg/mL

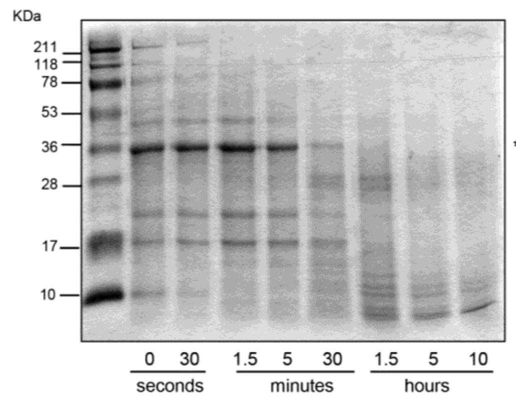
Fig. 2. Effect of OCX-36 on TNF-α and NO production in LPS-stimulated RAW 264.7 cells in the absence of FBS. (A) Cells were treated with 0, 0.1, 1, 10 or 100 μg/mL OCX-36 in the presence of 100 ng/mL *E. coli* LPS for 6 h in serum-free medium. TNF-α concentration was measured by ELISA, and is presented as percent TNF-α relative to positive control cells treated with LPS alone. (B) Cells were treated with 0, 0.1, 10 or 100 μg/mL OCX-36 in the presence of 10 ng/mL *E. coli LPS* for 24 h in serum-free medium, and NO concentration was measured using Griess reagent. Data represent means ± SEM, n=3. Values without a common letter are significantly different at $p<0.05$.

3.1.2. The effect of dOCX-36 on TNF-α and NO production in LPS-activated murine macrophages

The effect of OCX-36-derived peptides (dOCX-36) on TNF-α and NO release by RAW 264.7 macrophages stimulated with *E. coli* LPS was also examined. OCX-36 was digested with pepsin for up to 10 h, and digestion was monitored at intervals by SDS-PAGE. OCX-36 appeared to be completely digested by pepsin after 1.5 h and by 10 h only peptide fragments were visible (Fig. 3A); thus this time point was chosen for OCX-36 digestion. OCX-36 was also digested with thermolysin for up to 4 h, and digestion was similarly monitored by SDS-PAGE (Fig. 3B). The peptides generated from digestion of OCX-36 with thermolysin were only tested for NO analysis in vitro.

dOCX-36 significantly reduced LPS-induced TNF-α secretion from RAW 264.7 cells ($p<0.05$) in a dose-dependent manner, and an almost 50% reduction was observed at the highest dose tested (100 μg/mL) when compared to cells treated with LPS alone (Fig. 4A). Digestion with pepsin also appeared to abrogate the stimulatory effect of OCX-36 on RAW 264.7 cells, as dOCX-36 alone did not induce TNF-α secretion when compared to untreated cells. Peptides derived from OCX-36 digested with pepsin (Fig. 4B) and thermolysin (data not shown), however, did not show any effect on NO secretion in LPS-stimulated cells when compared to cells treated with LPS alone. As with TNF-α, dOCX-36 alone did not induce NO production when compared to untreated cells. Treatment with OCX-36 digested with pepsin or thermolysin did not affect cell viability (data not shown).

A



B

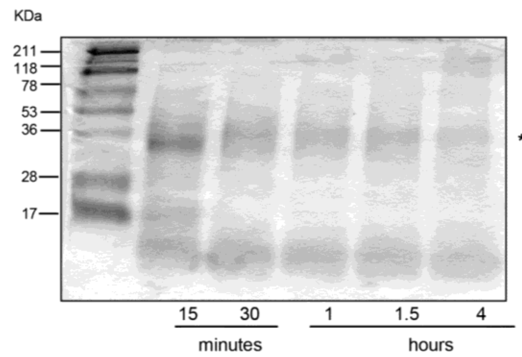
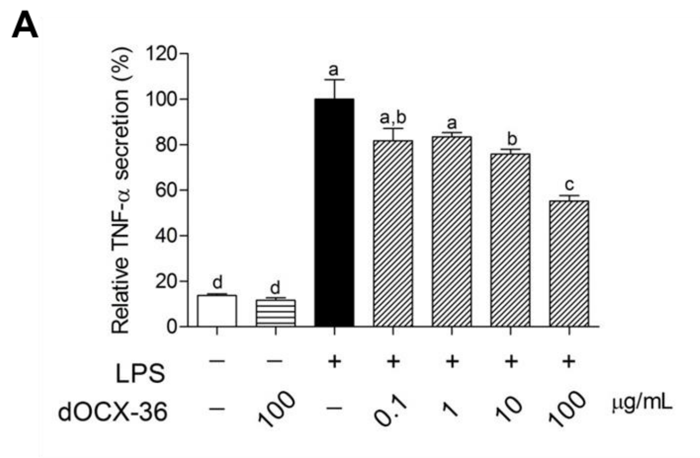


Fig. 3. SDS-PAGE analysis of OCX-36 digested with pepsin and thermolysin. OCX-36 was incubated with (A) pepsin or (B) thermolysin for the times indicated. The position of molecular weight standards (KDa) is indicated and position of OCX-36 is indicated by the asterisk.


A
Relative TNF-α secretion (%)
120
100
80
60
40
20
0
d
d
a
a,b
a
b
c
LPS
–
–
+
+
+
+
+
dOCX-36
–
100
–
0.1
1
10
100
μg/mL

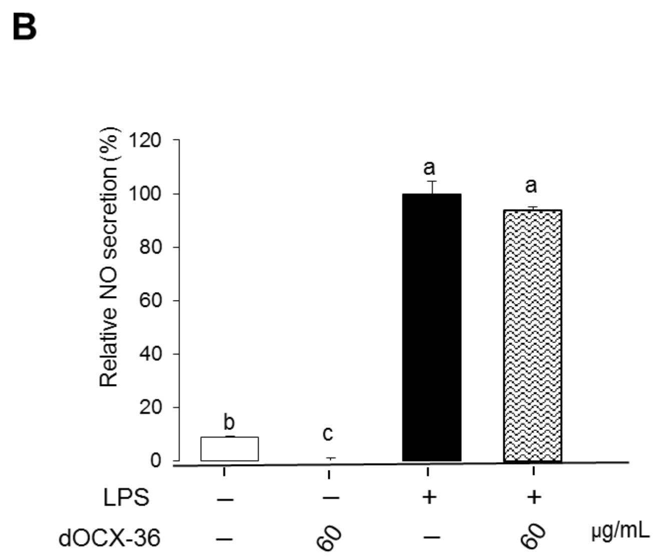

B
Relative NO secretion (%)
120
100
80
60
40
20
0
b
c
a
a
LPS
–
–
+
+
dOCX-36
–
60
–
60
μg/mL

Fig.4. Effect of OCX-36-derived peptides (dOCX-36) on TNF-α and NO secretion in LPS-stimulated RAW 264.7 cells. (A) Cells were treated with 0, 0.1, 1, 10 or 100 μg/mL dOCX-36 in the presence or absence of 100 ng/mL *E. coli* LPS for 6 h. TNF-α concentration was measured by ELISA, and is presented as percent TNF-α relative to positive control cells treated with LPS alone. (B) Cells were treated with 0 or 60 μg/mL of pepsin-digested OCX-36 in the presence or absence of 10 ng/mL *E. coli* LPS for 24 h, and NO concentration was measured using Griess reagent. Data represent means ± SEM, n=3. Values without a common letter are significantly different at $p<0.05$.

3.1.3. PCR array analysis

Since both OCX-36 and dOCX-36 exerted differential effects on TNF-α and NO production in RAW 264.7 cells, we further examined the effects of OCX-36 and dOCX-36 at the transcriptional level. Cells were incubated for 6 h with medium alone, OCX-36 or dOCX-36 alone, LPS alone, or LPS pre-incubated with dOCX-36 (LPS+dOCX-36), and relative gene expression was analyzed by PCR array. Differentially expressed genes are shown in Table 1. Notably, treatment of cells with dOCX-36 alone down-regulated expression of Cybβ (cytochrome b-245, beta polypeptide), IL-1f6 (IL-1 family, member 6), IL-1rn (IL-1 receptor antagonist), Pglyrp1 (peptidoglycan recognition protein 1) and up-regulated Proc (protein C) expression when compared to OCX-36. Treatment with OCX-36 alone down-regulated IL-1β when compared to LPS, and along with dOCX-36 down-regulated

IL-6 expression. Moreover, when combined with LPS (LPS+dOCX-36), dOCX up-regulated expression of CCL2 (MCP-1, monocyte chemotactic protein-1), and down-regulated expression of CXCR4 (chemokine receptor 4), MyD88 (myeloid differentiation primary response gene 88) and Prg2 (Proteoglycan 2).

Table 1. Differentially expressed genes in RAW 264.7 cells treated with OCX-36, dOCX-36, LPS, or LPS+dOCX-36.

Gene Symbol	Fold Change[a]				Functional Gene Grouping
	OCX-36	dOCX-36	LPS	LPS+ dOCX-36	
Camp	–	–	***-2.3***	***-2.1***	Cathelicidin antimicrobial peptide; Defense response to bacteria
CCL2	–	–	–	2.7	Chemokine involved in inflammatory response
CD1d1	2.9	–	3.5	2.4	Innate immune response, detection of bacteria
Colec12	–	***-2.7***	***-2.3***	***-2.0***	Antibacterial humoral response
CXCR4	–	–	–	***-2.07***	Defense response to bacteria, LPS receptor
Cybβ	–	***-2.5***	–	–	Inflammatory response, antibacterial humoral response
IFN-β1	–	–	7.8	5.4	Innate immune response
IL-10	–	***-4.55***	***-4.38***	***-2.2***	Septic shock, apoptosis
IL-1α	–	–	2.3	2.4	Inflammatory response
IL-1β	***-2.4***	–	2.8	–	Inflammatory response, septic shock
IL-1f6	–	***-2.3***	–	–	Member of IL-1 family; Inflammatory response
IL-1rn	_	***-2.4***	–	–	IL-1 receptor antagonist; Inflammatory response, septic shock
IL-6	***-2.0***	***-2.0***	–	–	Inflammatory response, septic shock
MyD88	–	–	–	***-2.5***	NF-κB signalling
Pglyrp1	–	***-2.6***	–	***-2.7***	Detection of bacteria, defense response to bacteria
Prg2	–	–	–	***-3.6***	Defense response to bacteria, inflammatory response
Proc	–	2.6	–	–	Protein C; Septic shock
TLR1	3.0	4.3	2.7	–	Defense response to bacteria, NF-κB signalling
TLR3	2.6	–	2.6	–	Defense response to bacteria, NF-κB signalling
TLR8	2.5	–	–	–	Detection of pathogens, NF-κB signalling

[a] Cells were treated for 6 h with 1 µg/mL OCX-36 or dOCX-36 alone, 100 ng/mL LPS alone, or dOCX-36+LPS, and gene expression was analyzed by PCR array. Results are expressed as fold change relative to untreated cells. A gene was considered differentially expressed when fold change was at least ± 2.0. Numbers in bold and italics indicated down-regulation.

3.1.4. Anti-endotoxin effects *in vivo*

To examine the anti-endotoxin effects *in vivo*, mice were injected with a sublethal dose of *E. coli* O111:B4 LPS and monitored for clinical signs and body weights. Mice given LPS along with native OCX-36 (LPS+OCX-36) or pepsin-digested OCX-36 (LPS+dOCX-36) displayed slightly reduced clinical signs (ruffled fur, lethargy) than mice given LPS alone. While all mice lost weight 24 h after LPS administration, mice given the high dose (10 mg/kg·BW) OCX-36 or dOCX-36 lost less weight than positive control (LPS) mice ($p<0.05$) (Fig. 5). Therefore this dose was chosen for further study. There was no effect of OCX-36 or dOCX-36 administered alone.

To examine the effects of OCX-36 and dOCX-36 on acute LPS-induced endotoxemia, levels of pro-inflammatory mediators in the serum, liver and intestine (ileum) were measured 2 h after i.p. injection of LPS alone, or combined with OCX-36 or dOCX-36. Administration of LPS+dOCX-36 reduced serum IL-6 concentrations ($p<0.05$) when compared to LPS-treated mice (Fig. 6A), but did not significantly affect serum TNF-α (Fig. 7A), or liver IL-6 and TNF-α (Fig. 6B). LPS+OCX-36 had no effect on serum or liver cytokine concentrations when compared to mice treated with LPS alone. Levels of pro-inflammatory cytokines and myeloperoxidase (MPO) activity in the ileum were also measured to examine the effect of OCX-36 and dOCX-36 on local inflammation. IL-6, TNF-α, and IL-1β concentrations were elevated in the ileum of positive control (LPS) mice, but were significantly reduced in mice administered LPS+dOCX-36 ($p<0.05$) (Fig. 7A). LPS+OCX-36 did not significantly affect cytokine levels in the ileum when

compared to mice treated with LPS alone. The activity of MPO, an indicator of neutrophil infiltration into the intestinal mucosa, was significantly decreased by treatment with both LPS+OCX-36 and LPS+dOCX-36 when compared to positive control (LPS) mice ($p<0.05$) (Fig. 7B).

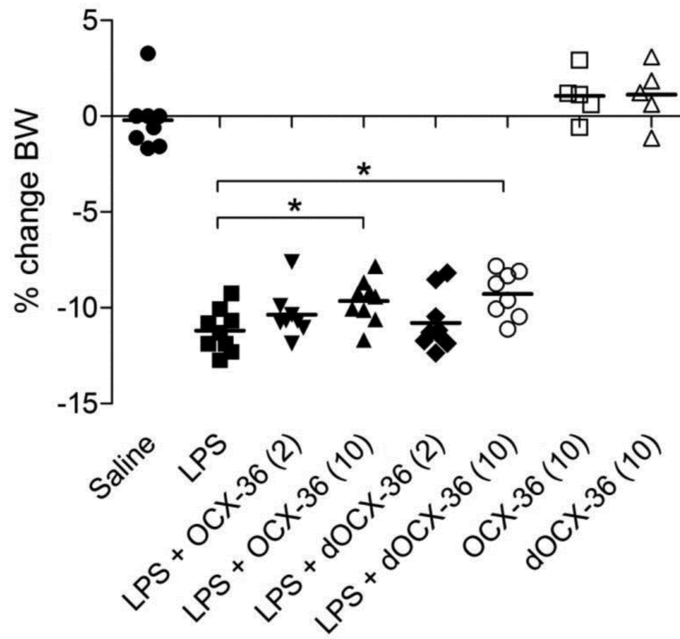
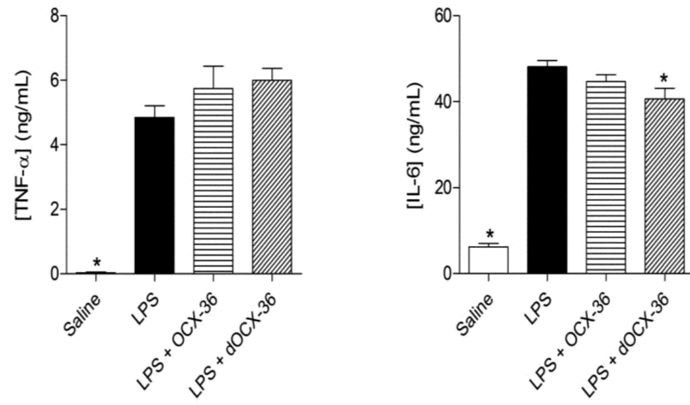


Fig. 5. Effect of OCX-36 and dOCX-36 on LPS-induced weight loss in mice. Mice were injected i.p. with 25 µg LPS combined with 2 or 10mg/kg·BW OCX-36 or dOCX-36 in sterile saline. Positive control (LPS) mice received *E. coli* LPS alone, and negative control (Saline) mice received saline only. Mice were weighed before and 24 h after LPS injection. n = 5-10 mice/group. Each data point represents an individual animal, and horizontal lines indicate mean values.*, $p < 0.05$.


A
[TNF-α] (ng/mL)
[IL-6] (ng/mL)
Saline
LPS
LPS + OCX-36
LPS + dOCX-36

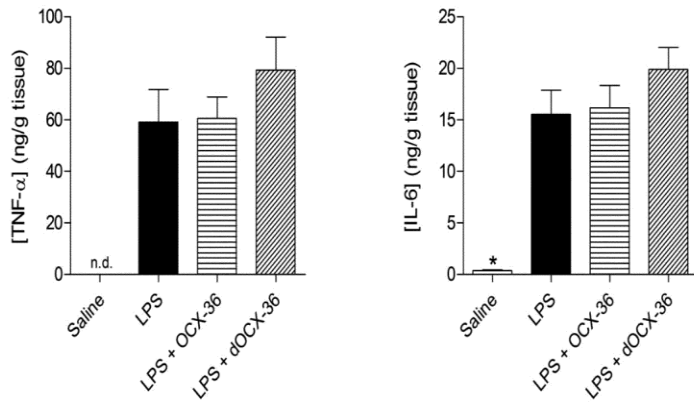
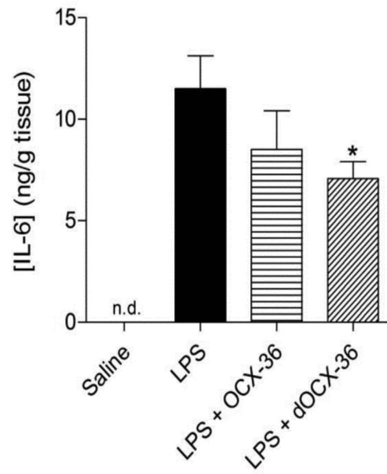
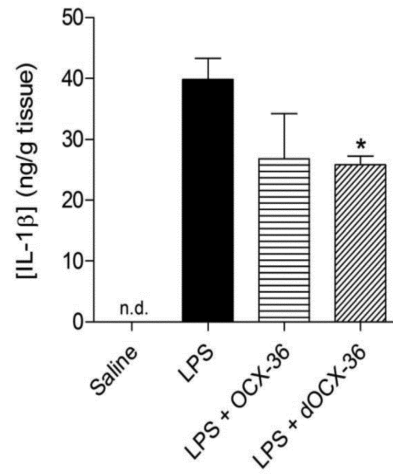
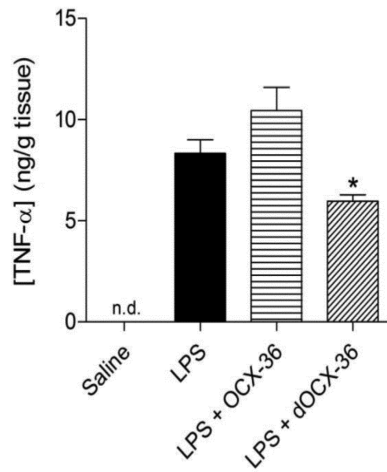

B
[TNF-α] (ng/g tissue)
[IL-6] (ng/g tissue)
n.d.
Saline
LPS
LPS + OCX-36
LPS + dOCX-36

Fig. 6. Effect of OCX-36 and dOCX-36 on TNF-α and IL-6 concentrations in the (A) serum and (B) liver of LPS-treated mice. Mice were injected i.p. with 25 μg *E. coli* LPS combined with 10 mg/kg·BW OCX-36 or dOCX-36 in sterile saline. Positive control (LPS) mice received LPS alone. Blood and liver samples were collected 2 h after injection and cytokine levels in serum and liver homogenates measured by ELISA. Values shown are means ± SEM for n = 6-8 mice/group. *, $p < 0.05$ compared to LPS.

A







B

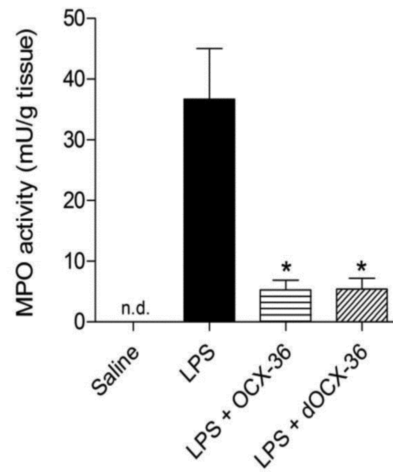


Fig. 7. (A) Cytokine and (B) MPO concentrations in the ileum of LPS-treated mice. Mice were injected i.p. with 25 μg *E. coli* LPS combined with 10 mg/kg·BW OCX-36 or dOCX-36 in sterile saline. Positive control (LPS) mice received LPS alone. Ileum sections were collected 2 h after injection and cytokine levels and MPO activity in ileum homogenates were measured by ELISA and MPO assay, respectively. Values shown are means ± SEM for n = 6-8 mice/group.*, $p < 0.05$ compared to LPS.

4. Discussion

OCX-36 is a chicken eggshell protein specifically expressed in the chicken reproductive and digestive tracts. Based on similarities in the protein sequence and exon / intron gene organization with LBP, BPI and PLUNC family members, it has been proposed that OCX-36 plays a role in host defense (Gautron et al., 2007; Tian et al., 2010). LBP/BPI/PLUNC family proteins are capable of recognizing and neutralizing the effects of LPS (Wurfel et al., 1994; Wiesner and Vilcinskas, 2010). LBP also binds to other bacterial components such as LTA, peptidoglycan breakdown products and lipopeptides, and has been shown to modulate the effects of LTA in macrophages and monocytes (Schumann, 2011). We recently demonstrated that OCX-36 has antimicrobial activity against *S. aureus* and affinity for bacterial endotoxin (*E. coli* LPS) and non-endotoxin pyrogen (*S. aureus* LTA) (Cordeiro et al., 2013). OCX-36 can be readily extracted directly from eggshell membranes. This is a clear difference in comparison to the other innate immune proteins such as LBP, BPI and PLUNC proteins since most studies examining these proteins utilize recombinant proteins (Amura et al.,1998; Lamping et al.,

1998; Chen et al., 2007).In the current study, we generated peptides by OCX-36 digestion and compared the effect of full-length OCX-36 and OCX-36-derived peptides on LPS-induced TNF-α and NO production in RAW 264.7 macrophage cells *in vitro,* and *in vivo* using a mouse model of endotoxemia.

Macrophages are phagocytic cells that attack infectious pathogens through the secretion of immune modulating mediators (Rosenberger and Finlay, 2003). Activation of macrophages *in vitro* by LPS induces production of pro-inflammatory mediators, including TNF-α, IL-6, IL-1β and NO (Zhu et al., 2013). Our *in vitro* experiments demonstrated that OCX-36 displayed a moderate inhibitory effect on LPS-induced TNF-α secretion and a stimulatory activity on NO production in RAW 264.7 cells. Previous studies have shown that human recombinant BPI and LBP inhibited the ability of LPS to stimulate TNF-α, but only BPI was able to suppress the production of TNF-α and NO on mouse macrophages (Amura et al.,1997, 1998). This is in line with our findings that OCX-36 exerted distinct effects on LPS-induced TNF-α and NO secretion from RAW 264.7 cells. The authors were unable to detect differences in TNF-α or inducible nitric oxide synthase (iNOS) gene expression, LPS-induced phosphorylation or activation of the NF-κB transcription factor by either LBP or BPI, and suggested downstream regulation of LPS-mediated signaling events or the presence of two independent membrane binding sites for LPS used by LBP and BPI protein to activate LPS stimulated murine macrophages (Amura et al., 1997, 1998). We also observed that at high doses, OCX-36 alone could significantly enhance the production of TNF-α and NO by

mouse macrophages, indicating that OCX-36 can also exert immunostimulatory effects.

The inhibitory effect of LBP on TNF-α secretion in murine macrophages stimulated with LPS was previously reported to occur in both the presence and absence of murine serum (Lamping et al., 1998). Here, OCX-36 likewise reduced TNF-α secretion by LPS-activated RAW 264.7 cells in both the absence and presence of 10% FBS. FBS is a source of LBP and a percentage of bioactive LBP in FBS is able to promote cell activation with murine and human TLRs (Meszaros et al.,1995). The reduction in TNF-α secretion from LPS-stimulated RAW 26.4 cells treated with OCX-36 in the absence of serum suggested that OCX-36 may compete with LBP for LPS and lead to an inhibition of TNF-α secretion. The observation that OCX-36 did not reduce TNF-α secretion when added to the cells at different time points independent of LPS and without pre-incubation (data not shown) further supports the role of the interaction of OCX-36 with LPS in anti-inflammatory activity.

We next examined the effect of OCX-36-derived peptides on TNF-α and NO secretion in RAW 264.7 cells. Synthetic peptides derived from LBP and BPI have been shown to prevent LPS-induced TNF-α secretion by macrophages, and have been suggested as a potential adjunctive therapy to conventional sepsis treatments (Battafarano et al., 1995; Dankesreiter et al., 2000). More recently, a synthetic peptide (GL13NH$_2$) from parotid secretory protein, a PLUNC member protein, was similarly found to reduce the LPS-stimulated release of TNF-α from RAW 264.7 cells (Abdolhosseini et al., 2012). Here, OCX-36 was digested with

pepsin, to mimic digestion in the gastrointestinal tract. Several studies have shown that peptides derived from chicken egg proteins digested with pepsin exhibit broad antimicrobial activity spectrum, anti-oxidant activity and reduced allergenicity (Kovacs-Nolan et al., 2000; Mine et al., 2004). OCX-36-derived peptides showed a different effect on TNF-α and NO secretion by RAW 264.7 cells when compared to full-length OCX-36. Pepsin-digested OCX-36 had enhanced anti-endotoxin effects and reduced LPS-induced TNF-α secretion by almost 50%. On the other hand, OCX-36 digested with pepsin or thermolysin did not show any inhibitory effect on NO release by LPS-stimulated murine macrophages. Moreover, in contrast to full-length OCX-36, OCX-36-derived peptides alone had no immunostimulatory activity and did not induce NO or TNF-α production.

The differential effects of OCX-36 and OCX-36-derived peptides on NO and TNF-α production in RAW 264.7 macrophages, both alone and in the presence of LPS, was further examined by the expression profiling of several genes involved in innate immunity and inflammation. OCX-36 alone down-regulated the expression of pro-inflammatory cytokines IL-1β and IL-6, but up-regulated molecules involved in bacterial antigen recognition and presentation, such as CD1d and TLR8, which can lead to downstream activation of NF-κB (Joyce, 2001; Cervantes et al., 2012), further supporting its role in activation of innate immunity. Likewise, dOCX-36 alone down-regulated the expression of molecules involved in inflammation, including the oxidative stress-related molecule cytochrome b-245, IL-1F6, a member of the IL-1 cytokine family that activates NF-κB, and IL-1 receptor agonist, which regulates IL-1-mediated inflammation (Towne et al., 2004).

In addition, dOCX-36 up-regulated protein C, which has been shown to exert anti-inflammatory effects in acute inflammation and sepsis (Frommhold et al., 2011). In the presence of LPS, dOCX-36 up-regulated CCL2 (MCP-1), an important mediator of monocyte/macrophage recruitment, which has been shown to induce NO production in mouse macrophages *in vitro* (Biswas et al., 2001). dOCX-36 also down-regulated the expression of MyD88 and CXCR4 in LPS-activated RAW 264.7 cells. MyD88 is a TLR adapter protein important for TLR4 cell signaling pathways and is involved in systemic inflammation and mortality during sepsis. The CXCR4 receptor plays important functions, which, along with CD14, TLR4 and MD-2, may also play a role in LPS binding and signaling (Triantafilou and Triantafilou, 2002; Feng et al., 2011). Surprisingly, expression of the anti-inflammatory cytokine IL-10 was down-regulated in cells treated with dOCX-36, LPS, and to a lesser extent, LPS+dOCX-36. Decreased IL-10 production in LPS-stimulated macrophages has been described in response to anti-inflammatory treatments (Babcock et al., 2002), and Amura et al. (1998) found that BPI and LBP did not alter IL-10 expression in LPS-stimulated mouse macrophages despite a significant reduction in LPS-induced TNF-α secretion. While these results suggest that IL-10 may not be involved in the anti-endotoxin effects of dOCX-36 observed here, further gene expression analysis at different time points may be required to elucidate the role of IL-10 in response to OCX-36/dOCX-36 treatment.

The anti-endotoxin activity of pepsin-digested OCX-36 (dOCX-36) *in vitro* motivated us to use a mouse model of endotoxemia to examine the effects of dOCX-36 on the levels of pro-inflammatory cytokines in various organs. Our data

showed that dOCX-36 significantly reduced IL-6, but not TNF-α levels in the serum of mice following i.p. treatment with LPS. While TNF-α is important in inflammation, IL-6 plays a key role in the acute phase response during sepsis and endotoxemia, and in fact circulating IL-6 levels have been found to be more closely correlated with disease severity and mortality than other inflammatory cytokines in patients with septic shock (Damas et al., 1992; Liaw et al., 1994). In addition, dOCX-36 was able to decrease the levels of IL-6, TNF-α and IL-1β, as well as MPO activity, in the ileum of mice administered LPS+dOCX-36. Both OCX-36 and dOCX-36 were able to decrease local LPS-induced MPO activity, suggesting the potential to reduce the recruitment of neutrophils in the ileum of septic mice. TNF-α, IL-6 and IL-1β are pro-inflammatory cytokines that play important roles in the intestinal mucosa during endotoxemia. Increased levels of these cytokines might affect the intestine as well as the function and integrity of remote organs and tissues (Meyer et al., 1995; Pritts et al., 2002). The intestinal anti-inflammatory activity of OCX-36 peptides suggested that dOCX-36 can modulate the intestinal mucosal immune response during endotoxemia. Both LBP and BPI are present in the intestinal epithelium, and overexpression of BPI was shown to attenuate bacteria-induced inflammation in intestinal epithelial cells, suggesting the potential for oral administration of OCX-36-derived peptides (Vreugdenhil et al., 2000; Canny et al., 2006). There are a number of reports describing the anti-endotoxin activity of BPI and recombinant BPI peptides *in vivo* (Levy, 2002). Recombinant chimeric protein BPI_{23}-Fcγ1 displayed anti-endotoxin and bactericidal activity and increased the survival rate of mice with sepsis caused by Gram-negative infection (Chen et al.,

2007), and Jiang et al. (2004) found that a synthetic BPI peptide at a dose of 10 mg/kg could protect animals from lethal endotoxemia and reduce production of TNF-α and IL-6. While recombinant LPB at high doses has also been shown to protect mice against septic shock *in vivo*, it is highly dependent on LBP concentration, as lower doses have been found to pontentiate cell responses to LPS (Lamping et al., 1998), and as such LBP-derived peptides that can prevent LPS-induced TNF-α secretion *in vitro* and *in vivo*, independent of LBP concentration, have also been reported (Araña et al., 2003). Likewise, the OCX-36-derived peptides tested here lacked the immunostimulatory activity of native OCX-36, and may therefore be beneficial for the treatment of sepsis and inflammation.

Overall, *in vitro* studies revealed that purified OCX-36 reduces LPS-induced secretion of TNF-α from macrophages and that OCX-36-derived peptides possess potent anti-endotoxin properties. The neutralizing activity of digested OCX-36 was confirmed by its capability to down-regulate the expression of genes involved in LPS signaling and inflammatory responses. OCX-36 might promote LPS activation in RAW 264.7 cells to augment some macrophage functions such as NO and TNF-α production. This suggests that OCX-36 is also a potential candidate as an immunostimulator of NO and TNF-α, which are important cytotoxic mediators contributing to the bactericidal activity of macrophages. Similar to our *in vitro* data, OCX-36-derived peptides were found to have an inhibitory effect on the production of LPS-induced pro-inflammatory mediators associated with endotoxemia *in vivo*. A future study to isolate and identify the effective anti-inflammatory OCX-36

peptides may lead to development of a novel endotoxin-neutralizing therapeutic agent or to delivery of OCX-36 as a nutraceutical by oral ingestion is conceivable.

Acknowledgements

These studies were supported by the Canadian NSERC Strategic grant program (STPGP 365046 - 08). CMMC is grateful to Dr. Chantal Matar and her post-doctoral fellow, Dr. Tri Vuong for providing cell culture training and lab facilities. We would like to thank Dr. Yves Nys for valuable discussion and insight during the preparation of this manuscript, and are grateful to Hamed Esmaili, Megan Rose-Martel, Dr. Prithy Rupa and Hua Zhang for their valuable help.

References

Abdolhosseini, M., Sotsky, J.B., Shelar, A.P., Joyce, P.B., Gorr, S.U., 2012. Human parotid secretory protein is a lipopolysaccharide-binding protein: identification of an anti-inflammatory peptide domain. Mol. Cel. Biochem. 359, 1-8.

Amura, C.R., Kamei, T., Ito, N., Soares, M.J., Morrison, D.C., 1998. Differential regulation of lipopolysaccharide (LPS) activation pathways in mouse macrophages by LPS-binding proteins. J. Immunol. 161, 2552-2560.

Amura, C.R., Chen, L.C., Hirohashi, N., Le, M.G., Morrison, D.C., 1997. Two functionally independent pathways for lipopolysaccharide-dependent activation of mouse peritoneal macrophages. J. Immunol.159, 5079-5083.

Araña, M.J., Vallespi, M.G., Chinea, G., Vallespi, G.V., Rodriguez-Alonso, I., Garay, H.E., Buurman, W.A., Reyes, O., 2003. Inhibition of LPS-responses by synthetic peptides derived from LBP associates with the ability of the peptides to block LBP-LPS interaction. J. Endotoxin Res. 9, 281-291.

Babcock, T.A., Novak, T., Ong, E., Jho, D.H., Helton, W.S., Espat, N.J., 2002. Modulation of lipopolysaccharide-stimulated macrophage tumor necrosis factor-α production by ω-3 fatty acid is associated with differential cyclooxygenase-2 protein expression and is independent of interleukin-10. J. Surg. Res. 107, 135-139.

Battafarano, R.J., Dahlberg, P.S., Ratz, C.A., Johnston, J.W., Gray, B.H., Haseman, J.R., Mayo, K.H., Dunn, D.L., 1995. Peptide derivatives of three distinct lipopolysaccharide binding proteins inhibit lipopolysaccharide-induced tumor necrosis factor-α secretion in vitro. Surgery 118, 318-324.

Beutler, B., Rietschel, E.T., 2013. Innate immune sensing and its roots: the story of endotoxin. Nat. Rev. Immunol. 3, 169–176.

Biswas, S.K., Sodhi, A., Paul, S., 2001. Regulation of nitric oxide production by murine peritoneal macrophages treated in vitro with chemokine monocyte chemoattractant protein 1. Nitric Oxide 5, 566-579.

Bogdan, C., 2001. Nitric oxide and the immune response. Nat. Immunol. 2, 907-916.

Canny, G., Cario, E., Lennartsson, A., Gullberg, U., Brennan, C., Levy, O., Colgan, S.P.,2006. Functional and biochemical characterization of epithelial bactericidal/permeability-increasing protein. Am. J. Physiol. Gastrointest. Liver. Physiol. 290, G557-G567.

Cervantes, J.L., Weinerman, B., Basole, C., Salazar, J.C., 2012. TLR8: the forgotten relative revindicated. Cell. Mol. Immunol. 9, 434-438.

Chen, J., Li, C., Guan, Y., Kong, Q., Li, C., Guo, X., Chen, Q., Jing, X., An, Y., 2007. Protection of mice from lethal Escherichia coli infection by chimeric human bactericidal/permeability-increasing protein and immunoglobulin G1 Fc gene delivery. Antimicrob. Agents. Chemother. 51, 724-731.

Cordeiro, C.M.M., Esmaili, H., Ansah, G., Hincke, M.T., 2013. Ovocalyxin-36 is a pattern recognition protein in chicken eggshell membranes. PLOS ONE 8, e84112.

Damas, P., Ledoux, D., Nys, M., Vrindts, Y., De Groote, D., Franchimont, P., Lamy, M., 1992. Cytokine serum level during severe sepsis in human IL-6 as a marker of severity. Ann. Surgery 215, 356-362.

Dankesreiter, S., Hoess, A., Schneider-Mergener, J., Wagner, H., Miethke, T., 2000. Synthetic endotoxin-binding peptides block endotoxin-triggered TNF-α

production by macrophages in vitro and in vivo prevent endotoxin-mediated toxic shock. J. Immunol. 164, 4804-4811.

Feng, Y., Zou, L., Zhang, M., Li, Y., Chen, C., Chao, W., 2011. MyD88 and Trif signaling play distinct roles in cardiac dysfunction and mortality during endotoxin shock and polymicrobial sepsis. Anesthesiology 115, 555-567.

Frommhold, D., Tschada, J., Braach, N., Buschmann, K., Doerner, A., Pflaum, J., Stahl, M.S., Wang, H., Koch, L., Sperandio, M., Bierhaus, A., Isermann, B., Poeschl, J., 2011. Protein C concentrate controls leukocyte recruitment during inflammation and improves survival during endotoxemia after efficient in vivo activation. Am. J. Pathol. 179, 2637-2650.

Gautron, J., Murayama, E., Vignal, A., Morisson, M., McKee, M.D., Réhault, S., Labas, V., Belghazi, M., Vidal, M.L., Nys, Y., Hincke, M.T., 2007. Cloning of ovocalyxin-36, a novel chicken eggshell protein related to lipopolysaccharide-binding proteins, bactericidal permeability-increasing proteins, and plunc family proteins. J. Biol. Chem. 282, 5273-5286.

Hincke, M.T., Nairn, A.C., 1992. Phosphorylation of elongation factor 2 during Ca (2+)-mediated secretion from rat parotid acini. Biochem. J. 282, 877-882.

Iovine, N., Eastvold, J., Elsbach, P., Weiss, J.P., Gioannini, T.L., 2002. The carboxyl-terminal domain of closely related endotoxin-binding proteins determines the target of protein-lipopolysaccharide complexes. J. Biol. Chem. 277, 7970-7978.

Jiang, Z., Hong, Z., Guo, W., Xiaoyun, G., Genfa, L., Yongning, L., Guangxia, X., 2004. A synthetic peptide derived from bactericidal/permeability-increasing protein neutralizes endotoxin in vitro and in vivo. Int. Immunopharmacol. 4, 527-537.

Joyce, S., 2001. CD1d and natural T cells: how their properties jump-start the immune system. Cell. Mol. Life. Sci. 58, 442-469.

Kovacs-Nolan, J., Zhang, J.W., Hayakawa, S., Mine, Y., 2000. Immunochemical and structural analysis of pepsin-digested egg white ovomucoid. J. Agric. Food. Chem. 48, 6261-6266.

Kumar, H., Kawai., T, Akira, S., 2009. Pathogen recognition in the innate immune response. Biochem. J. 420, 1-16.

Lamping, N., Dettmer, R., Schroder, N.W.J., Pfeil, D., Hallatschek, W., Burger, R., Schumann, R.R., 1998. LPS-binding protein protects mice from septic shock caused by LPS and gram negative bacteria. J. Clin. Invest. 101, 2065–71.

Levy, O., 2002. Therapeutic potential of the bactericidal/permeability-increasing protein. Expert Opin. Investig. Drugs 11, 159-167.

Liaw, Y.S., Yu, C.J., Wu, H.D., Yang, P.C., 1997. Comparison of inflammatory cytokine concentration and physiologic parameters in septic shock. J. Formos. Med. Assoc. 96, 685-690.

Lukinskiene, L., Liu, Y., Reynolds, S.D., Steele, C., Stripp, B.R., Leikauf, G.D., Kolls, J.K., Di, P., 2011. Antimicrobial activity of plunc protects against Pseudomonas aeruginosa infection. J. Immunol. 187, 382–390.

Meszaros, K., Aberle, S., White, M., Parent, J.B., 1995. Immunoreactivity and bioactivity of lipopolysaccharide-binding protein in normal and heat-inactivated sera. Infect. Immun. 63, 363-365.

Meyer, T.A., Wang, J., Tiao, G.M., Ogle, C.K., Fischer, J.E., Hasselgren, P.O.,1995 Sepsis and endotoxemia stimulate intestinal interleukin-6 production. Surgery 118, 336-342.

Mine, Y., Lauriau, S., Ma, F., 2004. Antimicrobial peptides released by enzymatic hydrolysis of hen egg white lysozyme. J. Agric. Food. Chem. 10, 1088-1094.

Opal, S.M., 2007. The host response to endotoxin, antilipopolysaccharide strategies, and the management of severe sepsis. Int. J. Med. Microbiol. 297, 365-377.

Pritts, T., Hungness, E., Wang, Q., Robb, B., Hershko, D., Hasselgren, P.O., 2002. Mucosal and enterocyte IL-6 production during sepsis and endotoxemia – role of transcription factors and regulation by the stress response. Am. J. Surg. 183, 372-383.

Rivers, E., Nguye, B., Havstad, S., Ressler, J., Muzzin, A., Knoblich, B., Peterson, E., Tomlanovich, M., 2001. Early goal-directed therapy in the treatment of severe sepsis and septic shock. N. Engl. J. Med. 19, 1368–1377.

Rosenberger, C.M., Finlay, B.B., 2003. Phagocyte sabotage: disruption of macrophage signalling by bacterial pathogens. Nat. Rev. Mol. Cell. Biol. 4, 385–396.

Schuerholz, T., Brandenburg, K., Marx, G., 2012. Antimicrobial peptides and their potential application in inflammation and sepsis. Crit. Care. 16, 1-8.

Schumann, R.R,, 2001. High concentrations of lipopolysaccharide-binding protein in serum of patients with severe sepsis or septic shock inhibit the lipopolysaccharide response in human monocytes. Blood 98, 3800–3808.

Schumann, R.R., 2011. Old and new findings on lipopolysaccharide-binding protein: a soluble pattern-recognition molecule. Biochem. Soc. Trans. 39, 989-993.

Tian, X., Gautron, J., Monget, P., Pascal, G., 2010. What makes an egg unique? Clues from evolutionary scenarios of egg-specific genes. Biol. Reprod. 83, 893-900.

Towne, J.E., Garka, K.E., Renshaw, B.R., Virca, G.D., Sims, J.E., 2004. Interleukin (IL)-1F6, IL-1F8, and IL-1F9 signal through IL-1Rrp2 and IL-1RAcP to activate the pathway leading to NF-kappaB and MAPKs. J. Biol. Chem. 279, 13677-13688.

Triantafilou, M., Triantafilou, K., 2002. Lipopolysaccharide recognition: CD14, TLRs and the LPS-activation cluster. Trends. Immunol. 23, 301-304.

Vreugdenhil, A.C., Snoek, A.M., Greve, J.W., Buurman, W.A., 2000. Lipopolysaccharide-binding protein is vectorially secreted and transported by

cultured intestinal epithelial cells and is present in the intestinal mucus of mice. J. Immunol. 165, 4561–4566.

Wiesner, J.,Vilcinskas, A., 2010. Antimicrobial peptides: The ancient arm of the human immune system. Virulence 1, 440–464.

Wurfel, M.M., Kunitake, S.T., Lichenstein, H., Kane, J.P., Wright, S.D.,1994. Lipopolysaccharide (LPS)-binding protein is carried on lipoproteins and acts as a cofactor in the neutralization of LPS. J. Exp. Med. 180,1025-1035.

Zhu, J., Luo, C., Wang, P., He, Q., Zhou, J., 2013. Saikosaponin A mediates the inflammatory response by inhibiting the MAPK and NF-κB pathways in LPS-stimulated RAW 264.7 cells. Exp. Ther. Med. 5, 1345-1350.

VII. CHAPTER 4. Quantitative proteomics analysis of eggshell membrane proteins during chick embryonic development

Cordeiro CMM, Hincke MT (2015). Quantitative proteomics analysis of eggshell membrane proteins during chick embryonic development. *Journal of Proteomics,* 130 (1):11-25.

This chapter reports the first comprehensive analysis of the ESM proteome with bioinformatics assessment to understand the functional roles of ESM proteins during chick embryonic development. Cross-analysis of proteomics data from two models, eggshell membranes from fertilized and unfertilized eggs, identified proteins associated with eggshell membranes that have important functions in the three phases of chick embryonic development, and in the protection of unfertilized eggs against pathogen invasion. We report that a subset of these proteins is likely to be involved in innate immune protection of the chick embryo against bacterial invasion.

Author contributions:

Experiments were conducted by Cristianne Cordeiro. Data interpretation and manuscript preparation was done by Cristianne Cordeiro. Experimental design, data analysis, and manuscript preparation were supervised by Dr. Maxwell T. Hincke.

Abstract

The avian eggshell membrane (ESM) is a meshwork made up of highly cross-linked protein fibers and it is a scaffold upon which biomineralization of the eggshell is initiated. The ESM and associated shell participates in embryonic development by providing physical and chemical protection against pathogen invasion. We performed quantitative proteomic analysis of ESM proteins on multiple days during the three phases of embryonic development. The ESMs were stripped from both fertilized and unfertilized eggs at different days of incubation, and solubilized in a novel manner using TCEP-HCl (Tris (2-carboxyethyl) phosphine hydrochloride). The changes in ESM proteins between occurred during incubation were analysed. Bioinformatics analysis revealed that of the 12 functional protein clusters identified, protease inhibitors were present at all phases of chick development. A group of proteins involved in calcium binding and oxygen transport were only present during the second phase. Extracellular matrix, cell adhesion proteins related to the vascularization of chorioallantoic membrane (CAM), antimicrobial proteins and proteins involved in the binding and transport of lipids were found in the second and third phases of development. These findings provide insight into the functionality and evolving nature of ESM associated proteins involved in chick embryonic development.

Biological significance

The eggshell membranes (ESMs) are a fibrous scaffold that consists of highly crosslinked collagens (types I, V but mainly X), glycoproteins and CREMPs (cysteine-rich eggshell membrane proteins). The ESMs aid in the development of the chick embryo and protect it against pathogen invasion. This biopolymeric fibrous net functions as a platform for nucleation of the calcitic eggshell which provides a primary physical barrier against bacterial ingress. Comparative proteomic analyses of proteins in the ESMs from fertilized eggs and unfertilized eggs showed changes in their levels which varied between the specific phases of chick embryogenesis across 19 days of incubation. Bioinformatics characterization of these ESM proteins provides understanding of their evolving nature during chick embryonic development.

1. Introduction

The eggshell membranes (ESMs) are composed of a meshwork of fibers that are organized into inner and outer layers of different calibres ranging from 0.1 to 3 μm (inner ESM) to 1 and 7 μm (outer ESM). The fibers of the inner ESM are connected to the outer ESM; the fibers of the outer ESM penetrate the mammillary cones of the shell [1, 2]. The ESMs are a fibrous scaffold for calcium carbonate deposition where nucleation occurs to initiate mineralization of the mammillary cones and the beginning of eggshell formation [3, 4]. The mineralized structure of the eggshell physically protects the developing embryo from physical insults, pathogen invasion and dehydration. The eggshell also plays an important role in gas exchange and provides calcium to the developing embryo via shell dissolution from the calcium reserve body located at the base of each mammillary cone [5, 6].

The ESM is a bioactive material which consists of glycoproteins, cysteine-rich eggshell membrane proteins (CREMPs) and collagens (types I, V and X) [7, 8]. The ESM fibers are extensively cross-linked by lysine-derived desmosine and isodesmosine covalent linkages which are believed to be responsible for the insolubility of ESM fibers. Therefore, the effective characterization of protein constituents of ESMs is a challenge. Soluble proteins with functional activities extracted from ESM have been reported in several studies. For instance, lysozyme, ovotransferrin and ovocalyxin-36 (OCX-36) are ESM proteins with antimicrobial activity [9-11]. Although a number of studies have utilized different extraction buffers to solubilize protein constituents from the ESMs of both fertilized and unfertilized eggs, a complete inventory of ESM proteins has not yet been

established, since the structural units and associated cross-linked proteins have remained insoluble during extraction procedures [8, 12-16].

Chick embryonic development is divided into three phases. The first phase takes place from days 0-7 and it is characterized by the establishment of germ layers. The blastula cells migrate and reorganize into embryonic tissues such as the ectoderm, the mesoderm, and the endoderm that will give rise to different tissues and organs during gastrulation [17, 18]. The functional organs of the chick embryo such as limb buds for the wings and legs, heart and reproductive organs, develop between days 3 and 7 [19, 20]. The formation of the extra-embryonic membranes (amnion, chorion, allantois and yolk sac) also occurs during this phase to support the growing embryo. The second phase, embryo completion (from days 8-18) involves the development of the highly vascularized chorioallantoic membrane (CAM) to provide gas exchange for the embryo [21]. At day 10, the chick embryo is structurally complete with its beak hardened and toes fully formed. The chick moves into the hatching position by turning its head toward the broad end of the egg by day 14. At the end of this phase, the embryo has all the necessary physical conditions to support its transition to an independent ambulatory chick [19, 20]. The third phase, emergence (days 19-21), is characterized by oral consumption of amnion, as well as some metabolic and physiologic changes, such as the accumulation of glycogen reserves in the liver and the development of pulmonary respiration [22, 23].

The aim of this present study was to determine the changes in protein levels in the ESMs of fertilized eggs, compared with the ESMs of control, unfertilized eggs

and, to correlate these changes in protein functionality with specific stages of chick embryogenesis. Two novel elements to this study are the use of unfertilized eggs as a control for nonspecific alterations due to prolonged incubation at 37 ^{o}C, and to discern the impact of embryonic development on the ESMs. Moreover, chemical degradation of the insoluble membranes fibers using the reducing agent TCEP-HCl was performed to obtain a novel inventory of additional protein constituents of the ESM fibers. Bioinformatic analysis was subsequently performed to assess biological functionality at the levels of the ESM constituents related to chick embryonic development.

2. Materials and Methods

2.1. Materials

Sodium chloride (NaCl) and dialysis tubing (MWCO 3.5 kDa) were purchased from Fisher Scientific, USA. Prestained protein molecular weight marker (Blue Standards 161-0373) and precast SDS-PAGE gels (mini protean TGX) were from BioRad Laboratories, Inc. The BCA protein assay reagent (bicinchoninic acid) was purchased from Thermo Fisher Scientific, Illinois, USA. The 96-well untreated microplates were from Corning Costar, USA. Filter paper (Whatman grade No. 2) was purchased from VWR International. 3-Mercaptopropionic acid (MPA) and Tris (2-carboxyethyl) phosphine hydrochloride (TCEP-HCl) were from Sigma-Aldrich. Dithiothreitol (DTT) and glacial acetic acid were purchased from Bioshop Canada Inc., and NuPAGE 4-12% Bis-Tris gels from Life Technologies, California, USA.

2.2. ESM sampling from fertilized and unfertilized eggs

Fertilized chicken eggs from White Leghorn hens, laid within a 24 h period, were obtained from the Animal Diseases Research Institute (ADRI, Ottawa, Canada). Unfertilized eggs (BurnBrae Farms, size large), were purchased from a local grocery store. Both fertilized and unfertilized eggs were incubated obtuse end up, at 37°C in a humidified, rocking Petersime Model 1 incubator and removed at days 0, 3, 7, 11, 15 and 19 for immediate processing. The ESM from unfertilized eggs collected at the same sampling time were used as controls, in order to identify changes in protein levels that were solely due to incubation conditions. The average weights of fertilized and size large unfertilized eggs were 59.9 ± 3.1g and 61.9 ± 2.0g, respectively. This difference was not significant ($p > 0.05$). Fertilized eggs (viable embryos) and unfertilized eggs (4 eggs from each model) were collected and opened to remove their contents. Embryos were removed and euthanized by decapitation. Eggshell interiors were rinsed under running demineralized water (DM). ESMs were mechanically stripped from the interior of washed eggs and collected on Whatman No. 2 filter paper. The experimental protocol was approved by the University of Ottawa Animal Care Ethics Committee Guidelines (CMM-129).

2.3. Protein extraction from ESMs

ESM proteins were extracted as described in Cordeiro *et al.*, [11] with some modifications. The moist ESMs were sliced into small pieces and proteins were sequentially extracted which increasingly harsher conditions. Initially, ESMs were

stirred in 1M NaCl (80 mL/ g of ESM) using an overhead mixer (IKA RW 20 digital, Cole-Parmer Canada) for 1h at 4 °C. The mixture was filtered on a Whatman No. 2 filter paper under vacuum, followed by the collection of pieces of ESMs for further processing. The clear NaCl filtrate was dialyzed and then concentrated by freeze-drying for future analysis. The moist membranes were weighed and transferred to a second extraction buffer (50 mM Tris-HCl, pH 8.5 containing 10 mM DTT at room temperature) (30 mL of extraction buffer per gram of membrane) and stirred for 19h at room temperature. The suspension was filtered (Whatman No. 2) to collect the membranes and the filtrate was centrifuged (3,500 x g, 20 minutes, 4°C) to remove the finer membrane particles. The resulting supernatant was dialyzed and freeze-dried. Moist membranes were weighed and incubated with the third extraction buffer containing 100 mM TCEP-HCl, 0.17M acetic acid at 80°C, for 72h. After cooling, the solution was vacuum-filtered through Whatman No. 2 filter paper to remove traces of insoluble materials. The pH of the resulting filtrate was adjusted to pH 8.5 with 5 M NaOH and centrifuged to remove fine particles. The clear supernatant obtained from filtrate sample extracted from fertilized and unfertilized ESMs using the TCEP-HCl extraction buffer was dialyzed (MWCO 3.5 kDa) versus 6 changes of water, and freeze-dried. Different chemical conditions (3-MPA, TCEP, etc) were explored in order to optimize this extraction (Table S1).

2.4. Sampling of chick embryo blood and chorioallantoic membrane (CAM)

Fertilized eggs were incubated obtuse end up, at 37°C in a humidified, rocking Petersime Model 1 incubator for 19 days. Viable fertilized eggs (5 eggs) were collected, broken, and CAM samples dissected free of visible adherent egg

compartments. Combined CAM samples were gently washed with 30 mL PBS buffer (10 mM sodium phosphate buffer, 0.15 M NaCl, pH 7.4) and centrifuged at 5000 rpm for 20 min at 4ºC three times, with resuspension. The samples were sonicated in 5 mL of PBS on ice with five bursts of 30 sec (Sonic Dismembrator, Fisher Scientific 60). Blood samples from the beating chick embryo heart were obtained using a syringe (28G 1/2 (0.36 x13 mm)) and cleared by centrifugation at 13,000 rpm for 10 min at 4ºC in order to collect the serum. CAM and chick blood serum samples were stored at −20ºC.

2.5. Sample preparation for Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS) analysis

The amount of protein extracted from fertilized and unfertilized ESMs, CAM and chick heart blood samples was measured with the bicinchoninic acid (BCA) protein assay using bovine serum albumin as a standard. The CAM and chick blood serum samples were fractionated on a 4–12% Bis–Tris gel, and gel lanes stained with Coomassie Blue before sectioning into 10 equal gel slices. For each of the 36 ESM extract samples collected at days 0, 3, 7, 11, 15 and 19 from both fertilized and unfertilized ESMs with the three extraction conditions, a brief migration (5 min) on a 4−12% Bis−Tris gel was performed in order to concentrate into a narrow band. Gels were stained with Coomassie Blue. Bands were excised from the gels and sent to the Proteomics Platform of the Eastern Quebec Genomics Centre (Laval, QC) for LC/MS/MS analysis (in-gel digestion, mass spectrometry analysis and Mascot data-base searching). The procedures for these analyses were performed as previously described [16, 24]. Briefly, protein in-gel were digested with trypsin

and peptides were separated by reversed-phase (RP) nanoscale capillary liquid chromatography (nanoLC) performed using an Agilent 1200 nanopump connected to a 5600 mass spectrometer (AB Sciex, Framingham, Massachusetts, USA) with a nanoelectrospray ion source and mass spectra were detected using Analyst software (Version 1.6, AB Sciex, Framingham, Massachusetts, USA) (ES-MS/MS). MS/MS peak lists were generated using ProteinPilot (Version 4.5, AB Sciex, Framingham, Massachusetts, USA) and analyzed using Mascot (Version 2.4.0, Matrix Science, London, UK) and X!Tandem (CYCLONE version, 2010.12.01.1), both programmed to search the TAX_GallusGallus_9031_20141114 database (unknown version, 222250 entries) with carbamidomethyl(C) as a fixed modification and deamidation (NQ), Gln pyro-Glu (N-term Q), and oxidation (MP) as variable modifications.

2.6. Criteria for protein identification

Validation of MS/MS based peptide and protein identification was performed using Scaffold (version Scaffold_ 4.3.4, Proteome Software Inc, Portland, OR, USA). MS/MS spectra were searched against the Uniprot (http://www.uniprot.org) and NCBI (http://www.ncbi.nlm.nih.gov/protein) chicken databases. Protein identification was accepted at a false discovery rate (FDR) of 1% at protein and peptide level, with at least two unique peptides. The quantification of proteins in ESMs extracted from fertilized and unfertilized eggs with greater than two unique peptides identified was determined using adjusted normalized spectral counts to combine the data from the different extraction conditions. The adjusted normalized spectral counts (ANSC) were calculated to combine data for 3 extractions

conditions for each ESM sample. The normalized spectral counts (NSC) obtained from Scaffold software (version 4.3.4.) was multiplied by the protein yield (%) obtained for each buffer extraction condition (Table 1).

$$ANSC= \sum ((NSC_{NaCl} * 0.019)+(NSC_{Tris/DTT} * 0.028)+ (NSC_{TCEP\text{-}HCl} * 53))$$

The -fold change of protein abundance in the fertilized eggs was calculated by comparison with the unfertilized eggs at the same time intervals. A heat map was generated in this study to represent the different profiles of abundance of significant proteins identified in ESMs from fertilized and unfertilized eggs during chick embryonic development (Fig. 2).

Gene ontology (GO) terms for proteins found in the ESMs from fertilized and unfertilized eggs were obtained from the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Functional Annotation Tool (DAVID Bioinformatics Resources 6.7, NIAID/NIH).

Table 1 - Yield of soluble proteins extracted from eggshell membranes.

Conditions	T(°C)	NaCl (M)[a]	Tris/DTT (mM)[b]	TCEP-HCl (mM)[c]	A.A(M)[d]	Yield (%)[e]
1	4	1	0	0	0	0.019
2	37	0	50/10	0	0	0.028
3	80	0	0	100	0.17	53

[a] Concentration of NaCl.

[b] Concentration of Tris-HCl, pH 8.5 and dithiothreitol.

[c] Concentration of tris(2-carboxyethyl)phosphine hydrochloride.

[d] Concentration of acetic acid.

[e] Yield of product which remains soluble at pH 8.5.

3. Results

3.1. Extraction of ESM proteins from fertilized and unfertilized eggs

Previous studies [13] established that 3-MPA under certain conditions can solubilize the ESMs; however, we are not aware that proteomics analysis of this material has been reported. The ability of different concentrations of reducing agents such as 3-MPA and TCEP-HCl was investigated to solubilize ESMs, in the presence of acetic acid at different concentrations and temperatures (Table S1). The solubilization approach using 100 mM TCEP-HCl containing 0.17 M acetic acid at 80°C was selected to solubilize the ESMs because we found this condition more effective than 3-MPA (Table S1). The ESM proteins were extracted from the fertilized and unfertilized eggs using a three-step sequential extraction procedure: 1) 1M NaCl at 4°C , 50 mM Tris-HCl at room temperature and pH 8.5, 2) 10 mM DTT and 3) 100 mM TCEP-HCl containing 0.17 M acetic acid at 80°C (Table 1). The NaCl and Tris-HCl/DTT extraction conditions were selected to provide continuity with our previous work [11].

3.2. Quantitative proteomics analysis

3.2.1. Fertilized and unfertilized ESM proteomes during incubation

Overall, 228 different proteins were identified in ESM after combining all the data from both unfertilized and fertilized samples at all days of incubation. When the data from days 0, 3, 11, 15, and 19 of chick embryonic development are combined, a total of 201 proteins were identified in ESMs from fertilized eggs (Tables S2-S7), whereas 114 proteins were in the inventory of the ESM proteome from unfertilized

eggs (Tables S8-S13) (Fig.1). Proteins from both independent conditions were compared and 87 proteins were found to be in common between the fertilized and unfertilized models (Fig.1). Therefore, during embryonic development, a large number of additional proteins (114) become associated with the ESMs. On the other hand, only a small number (27) of proteins diffuse from some egg compartments to ESM during the incubation of unfertilized eggs. In addition, 63 ESM proteins from a total of 228 proteins (fertilized and unfertilized ESM proteins) are novel to this study, compared to previous works [11,15, 16] (Table 2). Most of these proteins (40) were only detected in the ESM from fertilized eggs, including alpha-fetoprotein (**AFP**), caldesmon (**CALD1**) and pleiotrophin (**PTN**) whereas a minority (11) were only identified in the ESM from unfertilized eggs, including apolipoprotein L domain-containing protein 1 (**APOLD1**), dedicator of cytokinesis protein 4 isoform X4 (**DOCK4**) and semaphorin-3G isoform X4 (**SEMA3G**).

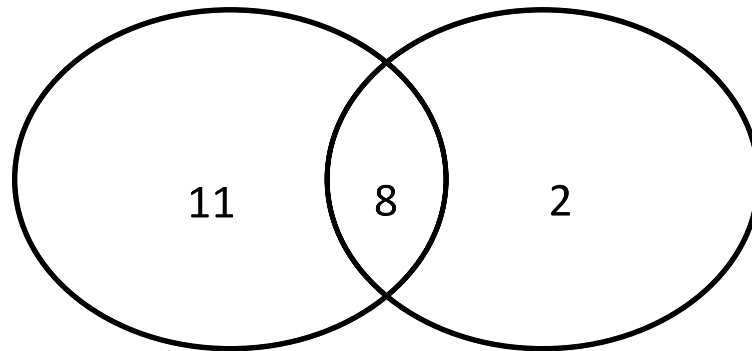


Figure 1. Venn diagrams representing the specific and overlapping proteins identified in the fertilized and the unfertilized eggshell membrane conditions at combined days 0, 3, 7, 11, 15 and 19 of chick embryonic development.

Table 2- List of eggshell membrane proteins identified for the first time in our study.

Identified Proteins	Official Gene Symbols	Also present present in [a]
Acidic mammalian chitinase	CHIA	
Agrin	AGRN	vm
Alpha-amylase	AMY1A	
Alpha-fetoprotein	AFP	y
Apolipoprotein L	APOLD1	
Caldesmon	CALD1	
Centrosome associated protein CEP250 isoform X14	CEP250	
Dedicator of cytokinesis protein 4 isoform X4	DOCK4	
Diacylglycerol kinase zeta	DGKZ	
DNA-dependent protein kinase catalytic subunit	PRKDC	
Dystonin isoform X3	DST	
Dystrophin	DMD	
ELKS/Rab6-interacting/ CAST family member 1 isoform X8	ERC1	
Fatty acid synthase	FASN	
Folate receptor alpha precursor	FOLR1	
Gastric intrinsic factor isoform X2	GIF	
GON-4-like protein isoform X2	GON4L	
HEMCAM	MCAM	
Hemoglobin subunit epsilon	HBE	
Homeobox protein cut-like 1	CUX1	
Hyaluronan and proteoglycan link protein 1 precursor	HAPLN1	
Hypothetical protein RCJMB04_3p21	YWHAQ	
la-related protein 4	LARP4	
IgGFc-binding protein-like	LOC429249	
Inactive heparanase-2 isoform X10	HPSE2	
Inner centromere protein-like isoform X6	LOC423138	
Insulin-like growth factor binding protein 7	IGFBP7	
Intraflagellar transport protein 172 isoform X6	IFT172	
Laminin subunit alpha-5	LAMA5	
Laminin subunit gamma-1 chain precursor	LAMC1	y

Lipocalin 8	LNC8	
Meiosis-specific nuclear structural protein 1	LOC426295	
Melanotransferrin	MFIA	w
Microtubule-associated tumor suppressor candidate 2 isoform X2	MTUS2	
Myb-related protein B	MYBL2	
Na(+)/H(+) exchange regulatory cofactor NHE-RF2	SLC9A3R2	
Nesprin-1 isoform X6	SYNE1	y
Nidogen-1	NID1	y
Nidogen-2	NID2	
Obscurin	OBSCN	y
Phosphofurin acidic cluster sorting protein 1 isoform X5	PACS1	
Phosvitin	CSNK2B	
Pleiotrophin	PTN	s,w
Pol-like protein ENS3	ENS-3	
Profilin	PFN2	
Prostaglandin-H2 D isomerase precursor	PTGDS	w
Protein piccolo	PCLO	
6-pyruvoyl tetrahydrobiopterin synthase isoform X7	PTS	
Radial spoke head protein 4 homolog A	RSPH4A	
Receptor-type tyrosine-protein phosphatase F isoform X5	PTPRF	
Rho guanine nucleotide exchange factor isoform X1	ARHGEF10	
Secretoglobin family 1C member 1-like, partial	LOC101749303	
Semaphorin-3G isoform X4	SEMA3G	
Serine/arginine repetitive matrix protein 2-like	LOC101751537	
Serpin B6	SERPINB6	
SHC-transforming protein 2 isoform X1	SHC2	
Syntaphilin	SNPH	
Trinucleotide repeat-containing gene 6A protein isoform X3	TNRC6A	
Vacuolar protein sorting 13 homolog D	VPS13D	
Vesicular integral-membrane protein (Lectin, manose binding 2)	LMAN2	y,s
Vitronectin	VTN	
WAP four-disulfide core domain protein 3 isoform X7	WFDC2	
Zona pellucida C	ZP3	

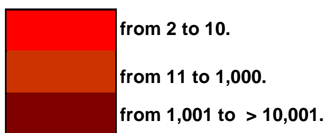
[a]Egg compartment where previously detected: y, yolk; vm, vitelline membrane; w, egg white; s, eggshell [30, 37, 97-101]

To perform quantitative analysis of our proteomic data, the normalized spectral counts were adjusted to take into account the differing yields obtained with the different extraction conditions, so as to combine extraction data from each condition (incubation, time and fertilization status) into a relative abundance (see Material and Methods 2.6). This permitted a comparative analysis of the proteins present in the ESMs between the fertilized and unfertilized conditions, in order to detect the -fold change in ESM protein abundance due to the presence of the embryo during incubation. Only increases or decreases of at least 2-fold in protein abundance were considered to be remarkable and the resulting data set was represented as a heat map (Fig.2). This heat map representation revealed 228 proteins that showed different levels of abundance in the ESM from fertilized and unfertilized eggs during different days of embryonic development. Among these 228 ESM proteins, proteins with increased (red colors) or decreased (green colors) levels in ESM were observed (Fig.2). For example, beta-microseminoprotein-like (**B2M**) and antimicrobial proteins such as ovocalyxin-32 (OCX-32) (**RARRES1**) were (10,200-fold) and (3,700-fold) enriched, respectively, in the ESMs from fertilized eggs at day 3, compared with the unfertilized sample. However, some proteins such as ovocalyxin-36 (OCX-36) (**BPIFB8**) (2,400-fold) and ovocleidin-116 (OC-116) (**MEPE**) (200-fold) were higher in abundance in the ESMs from unfertilized eggs compared with the ESMs from fertilized eggs at day 0 (Fig.2). Proteins with equivalent levels or unchanged protein (gray colors) were also detected in the fertilized and unfertilized conditions (Fig.2). These unchanging proteins were also represented in Figure 2 according to the average of their

adjusted normalized spectral values. The levels of some ESM proteins did not change between fertilized and unfertilized models, such as collagen alpha-1 (X) (**COL10A1**) which is a major ESM structural constituent, at all days of incubation (Fig. 2). This observation supports the validity of our analysis since collagen alpha-1 (X) (**COL10A1**) abundance in ESM is not expected to change during incubation.

Identified Proteins	Official Gene Symbols	day 0	day 3	day 7	day 11	day 15	day 19
Agrin	AGRN						
Acidic mammalian chitinase	CHIA						
Actin cytoplasmic type 5	ACTG1						
Adenomatous polyposis coli protein 2	APC2						
A-kinase anchor protein 9 isoform X22	PCNT						
Alpha-1-acid glycoprotein	ORM1						
Alpha-2-antiplasmin isoform X1	SERPINF2						
Alpha-2-macroglobulin-like 1 isoform X3	A2ML1						
Alpha-amylase	AMY1A						
Alpha-fetoprotein	AFP						
Alternatively spliced tenascin 190, 200 and 230 kd variants	TNC						
Anchorin CII	ANXA5						
Annexin A2	ANXA2						
Annexin A8	ANXA8						
Apolipoprotein A-I	APOA1						
Apolipoprotein AIV	APOA4						
Apolipoprotein B	APOB						
Apolipoprotein L domain-containing protein 1	APOLD1						
Astacin-like metalloendopeptidase	ASTL						
Avidin	AVD						
Basement membrane-specific heparan sulfate proteoglycan core protein	HSPG2						
Beta-actin	ACTB						
Beta-2-microglobulin	B2M						
Beta-H globin	HBE1						
Beta-microseminoprotein A1-like	LOC101750594						
Beta-microseminoprotein-like	LOC101750704						
BPI fold containing family B, member 3 precursor	BPIFB3						
BPI fold-containing family C protein isoform X2	BPIFCB						
Bromodomain adjacent to zinc finger domain protein 2B	BAZ2B						
Cadherin-1	CDH1						
Caldesmon	CALD1						
Cathepsin B	CTSB						
Centrosome-associated protein CEP250 isoform X14	CEP250						
Chondrogenesis associated lipocalin	PTGDS						
Clusterin	CLU						
Cochlin	COCH						
Collagen alpha-1(X)	COL10A1						
Collagen alpha-2(I)	COL1A2						
Collagen alpha-2(V) chain isoform X2	COL5A2						
Collagen alpha-3(IV)	COL4A3						
CREMP	CREMP						
CREMP-like 1	N/A						
CREMP-like 2	N/A						
CREMP-like 3	N/A						
Cystatin	CST3						
Cytochrome C	CYCS						
Dedicator of cytokinesis protein 4 isoform X4	DOCK4						
Diacylglycerol kinase zeta	DGKZ						
Dickkopf-related protein 3	DKK3						
DNA-dependent protein kinase catalytic subunit	PRKDC						
Dystonin isoform X3	DST						
Dystrophin	DMD						

Fold increase in the fertilized model.

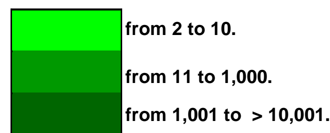


from 2 to 10.

from 11 to 1,000.

from 1,001 to > 10,001.

Fold decrease in the fertilized model.



from 2 to 10.

from 11 to 1,000.

from 1,001 to > 10,001.

Levels of proteins whose levels are equivalent in both fertilized and unfertilized models (average of adjusted normalized spectral values).

from 0.10 to 10.

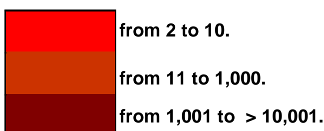
from 11 to 1,000.

from 1,001 to > 10,001.

Not detected either fertilized or unfertilized models at this time (< 2 unique peptides).

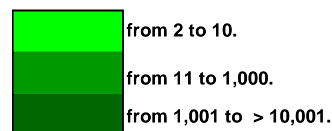
EGF-like repeats and discoidin I-like domains 3 isoform X2	EDIL3						
EGF-like repeats and discoidin I-like domains 3 isoform X3	EDIL3						
ELKS/Rab6-interacting/CAST family member 1 isoform X8	ERC1						
Enhancer of mRNA-decapping protein 3	EDC3						
Erythroid-specific folate receptor	FOLR1						
EW135	N/A						
Extracellular fatty acid-binding protein	LCN8						
Fatty acid synthase	FASN						
Fibronectin	FN1						
Folate receptor alpha precursor	FOLR1						
Galactocerebrosidase isoform X3	GALC						
Galactosylceramidase	GALC						
Gallin	GALN2						
Gallinacin-11	GAL11						
Gallinacin-9	GAL9						
Gastric intrinsic factor isoform X2	GIF						
Gastrokine 2 (OCX-21)	GKN2						
Glutathione peroxidase	GPX3						
Glutathione peroxidase 3 precursor	GPX3						
GON-4-like protein isoform X2	GON4L						
HEMCAM	MCAM						
Hemoglobin subunit alpha-A	HBAA						
Hemoglobin subunit alpha-D	HBAD						
Hemoglobin subunit beta	HBG2						
Hemoglobin subunit epsilon	HBE						
Hemopexin	HPX						
Hep21 protein	HEP21						
Heterochromatin-associated protein MENT	SERPINB10						
High mobility group protein B1	HMGB1						
Histone H1.01	HIST1H101						
Histone H1.11L	HIST1H111L						
Histone H3.2 like	LOC769852						
Histone-lysine N-methyltransferase, H3 lysine-36 and H4 lysine-20 specific	NSD1						
Homeobox protein cut-like 1-like, partial	CUX1						
Hyaluronan and proteoglycan link protein 1	HAPLN1						
Hyaluronan and proteoglycan link protein 3	HAPLN3						
Hypothetical protein RCJMB04_3p21	YWHAQ						
la-related protein 1 isoform X4	LARP1						
la-related protein 4	LARP4						
Ig alpha heavy chain	N/A						
Ig gamma chain (clone 36)	N/A						
Ig gamma H	N/A						
Ig heavy chain	N/A						
Ig heavy chain variable region	N/A						
Ig J polypeptide	N/A						
Ig lambda chain	N/A						
Ig lambda chain V-1 region precursor	N/A						
Ig lambda light chain VLJ region	N/A						
Ig light chain	N/A						
Ig light chain precursor V-J region	N/A						
Ig mu chain C region	LOC101748478						
IgGFc-binding protein-like	LOC429249						
Inactive heparanase-2 isoform X10	HPSE2						

Fold increase in the fertilized model.



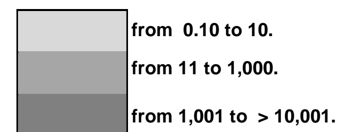
from 2 to 10.
from 11 to 1,000.
from 1,001 to > 10,001.

Fold decrease in the fertilized model.



from 2 to 10.
from 11 to 1,000.
from 1,001 to > 10,001.

Levels of proteins whose levels are equivalent in both fertilized and unfertilized models (average of adjusted normalized spectral values).

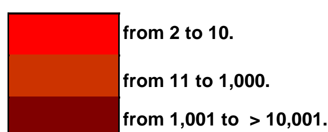


from 0.10 to 10.
from 11 to 1,000.
from 1,001 to > 10,001.

Not detected either fertilized or unfertilized models at this time (< 2 unique peptides).

Inner centromere protein-like isoform X6	LOC423138						
Insulin-like growth factor binding protein 7	IGFBP7						
Intraflagellar transport protein 172 homolog isoform X6	IFT172						
Lactadherin	MFGE8						
Lactadherin isoform 2	MFGE8						
Lamin-A	LOC396224						
Lamin-B2	LMNB2						
Laminin alpha-5 chain	LAMA5						
Laminin gamma-1 chain precursor	LAMC1						
Laminin subunit alpha-5 isoform X7	LAMA5						
Laminin subunit beta-1	LAMB1						
Lipocalin 8	LCN8						
Lysosomal-trafficking regulator	LYST						
Lysozyme C	LYZ						
Lysyl oxidase homolog 2	LOXL2						
Lysyl oxidase homolog 3	LOXL3						
Meiosis-specific nuclear structural protein 1	LOC426295						
Melanotransferrin	MFI2						
Mesothelin-like protein-like	MSLNL						
Metalloproteinase inhibitor 3	TIMP3						
Microtubule-associated tumor suppressor candidate 2 isoform X2	MTUS2						
Mucin-5AC	LOC100859916						
Mucin-5B	LOC395381						
Mucin-6	MUC6						
Myb-related protein B	MYBL2						
Myosin regulatory light chain 2	MYL2						
Na(+)/H(+) exchange regulatory cofactor NHE-RF2	SLC9A3R2						
Nesprin-1 isoform X6	SYNE1						
Neuroserpin	SERPINI1						
Nidogen 1	NID1						
Nidogen 2	NID2						
Obscurin	OBSCN						
Olfactomedin-4 precursor	OLFM4						
Ovalbumin	OVAL						
Ovalbumin-related protein X	OVALX						
Ovalbumin-related protein Y	OVALY						
Ovocalyxin-32	RARRES1						
Ovocalyxin-36	BPIFB8						
Ovocleidin-17	OC-17						
Ovocleidin-116	MEPE						
OvoglobulinG2 type AA (TENP)	BPIFB7						
Ovoinhibitor	SPINK5						
Ovomucoid	SPINK7						
Ovostatin	OVST						
Ovotransferrin	TF						
Pantetheinase precursor	VNN1						
Partial anti-prion protein Ig light chain variable region	N/A						
Partial Ig heavy chain variable region	N/A						
Partial Ig lambda chain	N/A						
Partial Ig lambda light chain	N/A						
Partial Ig light chain variable region	N/A						
Partial Ig heavy	N/A						
Partial Ovomucin beta-subunit	MUC6						
Peptidyl-prolyl cis-trans isomerase	PPIC						
Peroxiredoxin-1	PRDX1						

Fold increase in the fertilized model.

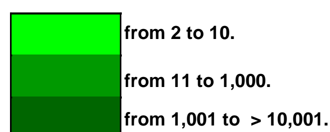


from 2 to 10.

from 11 to 1,000.

from 1,001 to > 10,001.

Fold decrease in the fertilized model.



from 2 to 10.

from 11 to 1,000.

from 1,001 to > 10,001.

Levels of proteins whose levels are equivalent in both fertilized and unfertilized models (average of adjusted normalized spectral values).

from 0.10 to 10.

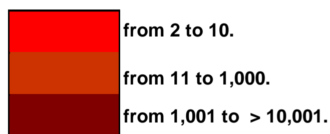
from 11 to 1,000.

from 1,001 to > 10,001.

Not detected either fertilized or unfertilized models at this time (< 2 unique peptides).

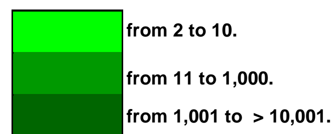
Phosphofurin acidic cluster sorting protein 1 isoform X5	PACS1						
Phosphodiesterase 4D interacting protein /Myomegalin-like isoform X13	LOC424382						
Phosvitin	CSNK2B						
PIT54 protein	PIT54						
Plasma protease C1 inhibitor isoform X1	SERPING1						
Pleckstrin homology domain-containing family G member 3	PLEKHG3						
Pleiotrophin	PTN						
Pol-like	N/A						
Pol-like protein ENS-3	ENS-3						
Polymeric Ig receptor	PIGR						
Polyubiquitin-C-like isoform X2	LOC101747587						
Profilin	PFN2						
Prostaglandin-H2 D-isomerase precursor	PTGDS						
Prostate stem cell antigen	PSCA						
Protein Jade-3 isoform X2	PHF16						
Protein piccolo	PCLO						
Protein S100-A9-like	LOC101747463						
Protein S100-A11	S100A11						
14-3-3 Protein theta	YWHAQ						
Protein Smaug homolog 2 isoform X2	N/A						
Protocadherin beta-15-like	LOC100858025						
Protocadherin Fat 1 isoform X11	FAT1						
Protocadherin Fat 2	FAT2						
Prothrombin	F2						
6-pyruvoyl tetrahydrobiopterin synthase	PTS						
Quiescence-specific protein	LCN8						
Radial spoke head protein 4 homolog A	RSPH4A						
Receptor-type tyrosine-protein phosphatase F isoform X5	PTPRF						
Rho guanine nucleotide exchange factor 10 isoform X1	ARHGEF10						
Riboflavin-binding protein	RTBDN						
Secretory trypsin inhibitor	N/A						
Semaphorin-3G isoform X4	SEMA3G						
Serine/arginine repetitive matrix protein 2-like	LOC101751537						

Fold increase in the fertilized model.



from 2 to 10.
from 11 to 1,000.
from 1,001 to > 10,001.

Fold decrease in the fertilized model.



from 2 to 10.
from 11 to 1,000.
from 1,001 to > 10,001.

Levels of proteins whose levels are equivalent in both fertilized and unfertilized models (average of adjusted normalized spectral values).

from 0.10 to 10.
from 11 to 1,000.
from 1,001 to > 10,001.

Not detected either fertilized or unfertilized models at this time (< 2 unique peptides).

Serpin B6	SERPINB6						
Serum albumin precursor	ALB						
SHC-transforming protein 2 isoform X1	SHC2						
SNF-related serine/threonine-protein kinase	SNRK						
Spectrin alpha chain, non-erythrocytic 1	SPTAN1						
Spectrin beta chain, non-erythrocytic 5	N/A						
Sulfhydryl oxidase 1	QSOX1						
Syntaphilin	SNPH						
Tenascin	TNC						
Thioredoxin	TXN						
Tiarin-like	OLFM4						
Titin isoform X2	TTN						
Titin isoform X3	TTN						
Transmembrane protease serine 9 isoform X3	TMPRSS9						
Transmembrane protease serine 9 isoform X4	TMPRSS9						
Transthyretin	TTR						
Trinucleotide repeat-containing gene 6A protein isoform X3	TNRC6A						
Tropomyosin alpha-3 chain	TPM3						
Trypsin inhibitor CITI-1	SPINK2						
Ubiquitin	UBB						
Vacuolar protein sorting 13 homolog D	VPS13D						
Vesicular integral-membrane protein VIP36	LMAN2						
Vitelline membrane outer layer protein 1	VMO1						
Vitellogenin	VTG2						
Vitellogenin-1	VTG2						
Vitellogenin-2	VTG2						
Vitronectin	VTN						
WAP four-disulfide core domain protein 3 isoform X6	WFDC2						
WAP four-disulfide core domain protein 3 isoform X7	WFDC2						
WAP four-disulfide core domain protein 3 isoform X8	WFDC2						
Zinc finger MYM-type protein 2 isoform X2	ZMYM2						
Zona pellucida glycoprotein 1	ZP1						
Zona pellucida C	ZP3						
Zona pellucida sperm-binding protein 3	ZP3						

Fold increase in the fertilized model.

from 2 to 10.
from 11 to 1,000.
from 1,001 to > 10,001.

Fold decrease in the fertilized model.

from 2 to 10.
from 11 to 1,000.
from 1,001 to > 10,001.

Not detected either fertilized or unfertilized models at this time (< 2 unique peptides).

Levels of proteins whose levels are equivalent in both fertilized and unfertilized models (average of adjusted normalized spectral values).

from 0.10 to 10.
from 11 to 1,000.
from 1,001 to > 10,001.

Figure 2. Heat-map showing an overview of comparative analysis of the eggshell membrane proteins with different profiles of abundance found in the fertilized and unfertilized eggs at days 0, 3, 7, 11, 15 and 19 of incubation. Intensity of red in cells indicates the fold increase in abundance of eggshell membrane proteins from fertilized eggs in comparison to eggshell membrane proteins from unfertilized condition. Intensity of green in cells indicates the fold increase in abundance of eggshell membrane proteins for unfertilized eggs, in comparison to the embryonated eggs. Intensity of gray in cells indicates the levels of eggshell membrane proteins which are present in both fertilized and unfertilized conditions at similar levels. White cells indicate that specific proteins were not detected at that day of incubation.

Functional annotation of proteins whose levels changed in the ESMs from fertilized eggs during chick embryonic development was carried out using Gene ontology (GO) term enrichment analysis. The GO term analysis generated 12 unique functional groups that were found to be significantly enriched ($p < 0.05$): enzyme inhibitor activity, skeletal muscle tissue development, extracellular structure organization, cell adhesion, tetrapyrrole binding, glycosaminoglycan binding, calcium ion binding, cellular homeostasis, lipid binding, lipid transport, copper ion binding and defense response (Table 3). There was a progressive change in number and specificity of functional clusters of proteins in the ESMs from fertilized eggs during development of the chick embryo (Table 3). Some functional clusters were only identified in the ESMs from fertilized eggs at day 15, such as calcium ion binding. However, a group of proteins with enzyme inhibitor activity was found to be enriched in the ESMs from fertilized eggs at all days, which reinforces the importance of proteins with protease inhibitor activity from the earliest stages of development until hatching. In contrast, proteins with increased abundance in the ESMs of unfertilized eggs were found in significant GO term clusters at days 0 and 3 (enzyme inhibitor activity, response to bacterium and cell adhesion).

Table 3 - Functional annotation analysis for abundant eggshell membrane proteins from fertilized eggs during embryonic development.

Days [a]	GO terms		Official Gene Symbols [b]
0	GO:0004857	Enzyme inhibitor activity	OVAL, OVALY, SPINK7, WFDC2
3	GO:0004857	Enzyme inhibitor activity	CST3, SPINK5, SPINK7, OVALX, OVALY, SERPINB10, A2ML1, WFDC2
7	GO:0004857	Enzyme inhibitor activity	CST3, SPINK7 ,OVALX, OVALY, A2ML1, WFDC2, OVST
	GO:0007519	Skeletal muscle tissue development	DMD, AGRN, TNC
	GO:0043062	Extracellular structure organization	AGRN, TNC, VMO1
	GO:0007155	Cell adhesion	EDIL3, HAPLN3, MFGE8,TNC
11	GO:0004857	Enzyme inhibitor activity	TIMP3, A2ML1, OVST,ANXA2,CST3,OVAL,OVALX
	GO:0043062	Extracellular structure organization	ANXA2, COL1A2, VMO1, TNC
	GO:0046906	Tetrapyrrole binding	GIF, HBAA, HBE
15	GO:0004857	Enzyme inhibitor activity	CST3, SPINK5, SPINK7, OVALX, OVALY, A2ML1, WFDC2, OVST, SERPINI1
	GO:0043062	Extracellular structure organization	ANXA2, NID1, VMO1
	GO:0007155	Cell adhesion	EDIL3, CDH1, FN1, HAPLN1, LAMB1, MFGE8, NID1, NID2
	GO:0005539	Glycosaminoglycan binding	FN1, HAPLN1, PTN
	GO:0005509	Calcium ion binding	EDIL3, ANXA2, CDH1, MFGE8, NID1, NID2, MYL12A
	GO:0019725	Cellular Homeostasis	MFI2, TF, PRDX1, QSOX1
19	GO:0004857	Enzyme inhibitor activity	CST3, OVAL, OVALX, ANXA2, WFDC2, A2ML1
	GO:0008289	Lipid binding	BPIFB3, BPIFB7, ALB, ANXA2, APOA4
	GO:0006869	Lipid transport	APOA4, VTG2, APOB
	GO:0005507	Copper ion binding	ALB, APOA4, AFP
	GO:0006952	Defense response	APOA4, FN1, GAL11

[a] Stage 1 (days: 0, 3 and 7); Stage 2 (days 11 and 15) and Stage 3 (day 19).

[b] Correspond to abundant proteins in the eggshell membranes from fertilized eggs at a specific day of chick embryonic development.

3.2.2. Chick embryonic blood and chorioallantoic membrane (CAM) proteome at day 19 of chick embryonic development

Proteins identified in the ESMs of fertilized eggs at day 19 were compared with the proteomes of the isolated CAM and chick embryonic blood, in order to investigate their tissue origins (Table 4). Proteomics data from the ESMs, blood and CAM at day 19 showed that 16 blood-related proteins were present in all three sources, including for instance, alpha-fetoprotein (**AFP**), apolipoprotein AIV (**APOA4**), apolipoprotein B (**APOB**) and serum albumin precursor (**ALB**). These are likely present in the vascularized CAM that is well developed at this phase (Fig.3) (Table 4). Annexin 2 (**ANXA2**), cystatin (**CST3**), lysozyme C (**LYZ**) and transiently expressed in neural precursor (TENP) (**BPIFB7**) are examples of 22 proteins that were present in both the ESMs and the CAM at day 19, but not in the blood suggesting their origin could be from the CAM. In contrast, 32 proteins were exclusively present in the ESM and not in blood or CAM; these included eggshell matrix proteins (OC-17 (**OC-17**), OCX-21 (**GKN2**), OCX-32 (**RARRES1**), OCX-36 (**BPIFB8**)), proteins associated with ESM fibers (CREMP (**CREMP**) and lysyl oxidase homolog 2 (**LOXL2**), egg white proteins (avidin (**AVD**), clusterin (**CLU**), dickkopf-related protein 3 (**DKK3**), mucin 6B (**MUC6**), ovostatin (**OVST**) and sulfhydryl oxidase 1 (**QSOX1**)) (Table 4).

Table 4 - Presence and absence of proteins identified in the blood, chorioallantoic membrane (CAM) and eggshell membrane (ESM) from fertilized eggs at day 19.

Identified Proteins	Official Gene [a] Symbols	Accession number	Presence (+) / Absence (-)		
			Blood [b]	CAM [b]	ESM [b]
Actin (cytoplasmic type 5)	ACTG1	NP_001007825.1	+	+	+
Alpha-1-acid glycoprotein	ORM1	AAT39530.1	+	+	+
Alpha-2-antiplasmin isoform X1	SERPINF2	XP_003642447.1	-	-	+
Alpha-2-macroglobulin-like 1	A2ML1	XP_416480.4	-	+	+
Alpha-fetoprotein	AFP	XP_003641248.2	+	+	+
Anchorin CII	ANXA5	AAB39917.1	-	-	+
Annexin A2	ANXA2	NP_990682.1	-	+	+
Annexin A8	ANXA8	E1C8K3	-	+	+
Apolipoprotein A-I	APOA1	AAA48597.1	+	+	+
Apolipoprotein AIV	APOA4	CAA76273.1	+	+	+
Apolipoprotein B	APOB	ABF70173.1	+	+	+
Astacin-like metalloendopeptidase	ASTL	P0DJJ2	-	-	+
Avidin	AVD	P02701	-	-	+
Beta-H globin	HBE1	Q90864	+	+	+
Clusterin	CLU	AAD17257.1	-	-	+
CREMP	CREMP	UPI0000E8213B	-	-	+
Cystatin	CST3	P01038	-	+	+
Dickkopf-related protein 3	DKK3	NP_990456.1	-	-	+
EGF-like repeats and discoidin I-like domains 3 isoform X2	EDIL3	XP_424906.3	-	-	+
Fibronectin	FN1	P11722.3	+	+	+
Gallinacin-11	GAL11	Q6IV20	-	-	+
Gastrokine 2 (OCX-21)	GKN2	XP_417666.1	-	-	+
Hemoglobin subunit alpha-A	HBAA	P01994	+	+	+
Hemoglobin subunit alpha-D	HBAD	P02001	+	+	+
Hemoglobin subunit beta	HBG2	P02112	+	+	+
Hemopexin	HPX	XP_417267.3	-	+	+
Histone H1.01	HIST1H111R	NP_001035732.1	-	+	+
Ig alpha heavy chain	N/A	AAB22614.2	-	+	+
Ig mu chain C region	LOC101748478	P01875	-	+	+
Lipocalin 8	LCN8	P21760.2	-	-	+
Lactadherin isoform 2 precursor	MFGE8	NP_001264040.1	-	+	+
Lysozyme C	LYZ	P00698	-	+	+
Lysyl oxidase homolog 2	LOXL2	E1C3U7	-	-	+
Mesothelin-like protein-like	MSLNL	XP_001234087.3	-	-	+
Mucin-5AC	LOC100859916	XP_003641370.2	-	+	+
Mucin-5B	LOC395381	Q98UI9.1	-	+	+
Mucin 6	MUC6	XP_426405.4	-	-	+
Ovalbumin	OVAL	P01012.2	+	+	+
Ovalbumin-related protein X	OVALX	P01013.1	-	+	+
Ovalbumin-related protein Y	OVALY	P01014.1	-	+	+
Ovocalyxin-32	RARRES1	CAC44378.2	-	-	+

Ovocalyxin-36	BPIFB8	CAI91279.1	-	-	+
Ovocleidin-17	OC-17	Q9PRS8	-	-	+
OvoglobulinG2 type AA (TENP)	BPIFB7	NP_990357.1	-	+	+
Ovoinhibitor	SPINK5	P10184	-	+	+
Ovomucoid	SPINK7	P01005	-	+	+
Ovostatin	OVST	XP_423478.4	-	-	+
Ovotransferrin	TF	P02789.2	+	+	+
Peroxiredoxin-1	PRDX1	P0CB50	+	+	+
Phosvitin	CSNK2B	P67869.1	-	-	+
Plasma protease C1 inhibitor isoform X1	SERPING1	XP_003641424.1	-	-	+
PIT54 protein precursor	PIT54	NP_997063.1	-	-	+
Polymeric Ig receptor	PIGR	AAP69598.1	-	+	+
Polyubiquitin-C-like isoform X2	LOC101747587	XP_004946720	-	-	+
Protein S100-A9-like	LOC101747463	XP_004948279.1	-	-	+
Prothrombin	F2	NP_989936.1	-	+	+
Riboflavin-binding protein	RBP	CAD44564.1	+	+	+
Secretoglobin family 1C member 1-like, partial	LOC101749303	XP_004941584.1	-	-	+
Serum albumin precursor	ALB	NP_990592.2	+	+	+
Spectrin alpha chain non-erythrocytic 1	SPTAN1	NP_001036003.1	-	-	+
Sulfhydryl oxidase 1	QSOX1	Q8JGM4	-	-	+
Tiarin-like	OLFM4	Q25C35	-	-	+
Transmembrane protease serine 9 isoform X3	TMPRSS9	XP_425880.4	+	-	+
Vacuolar protein sorting 13 homolog D	VPS13D	ENSGALP00000006816	-	-	+
Vitelline membrane outer layer protein 1	VMO1	P41366	-	-	+
Vitellogenin	VTG2	AAA49139.1	-	-	-
Vitellogenin-1	VTG2	NP_001004408.1	+	-	+
Vitellogenin-2	VTG2	NP_001026447.1	-	+	+
Vitronectin	VTN	O12945	-	-	+
Zona pellucida glycoprotein 1	ZP1	Q9DER4	-	+	+
Zona pellucida sperm-binding protein 3	ZP3	P79762	-	+	+
WAP four-disulfide core domain protein 3 isoform X7	WFDC2	XP_004947252.1	-	-	+

[a] Official gene symbols corresponds to eggshell membranes proteins from fertilized eggs (Table S7).
[b] Values obtained from Scaffold software, version 4.3.4 and at day 19 of embryonic development.

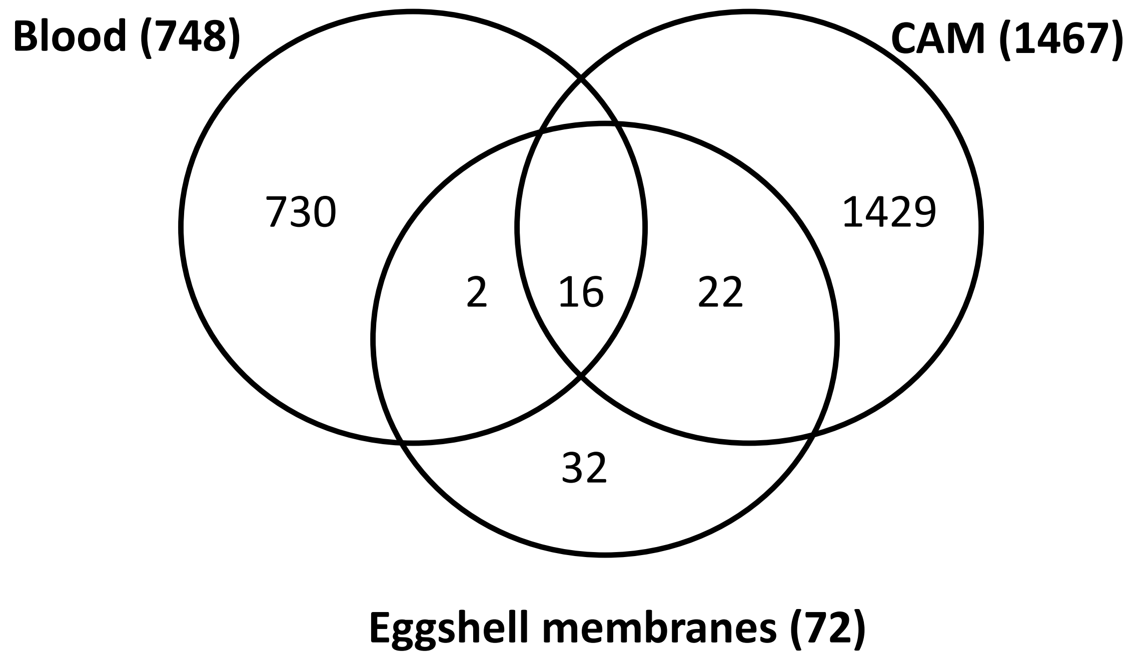


Figure 3. Venn diagrams representing the specific and overlapping proteins identified in the fertilized eggshell membranes, blood and chorioallantoic membrane at day 19 of embryonic development.

4. Discussion

4.1. Eggshell Membranes overview: structure and function

The eggshell membranes, associated eggshell and the overlaying cuticle are physical barriers that protect the chick embryo against pathogen invasion during incubation. The ESM is a tight meshwork of interlaced fibers consisting of highly cross-linked proteins such as collagens (types I, V and mainly X) and the cysteine-rich eggshell membrane protein (CREMP) that resist microbial invasion [8, 14]. The ESMs are essential for eggshell mineralization and provide a fibrous support for calcification. Organic aggregates are deposited on the surface of the outer ESM fibers in a quasi-periodic pattern where nucleation of calcium carbonate mineral begins with progressive deposition of amorphous calcium carbonate; giving rise to the mammillary cones and subsequently the palisade layer [4, 25]. The spacing and dimensions of the mammillary cones determines the biomechanical properties of the completed eggshell and therefore its physical strength [26, 27]. In conjunction with the ESMs, the eggshell plays a role in the exchange of gases and water, as well as providing calcium to the developing embryo [4]. In addition to their function as a physical barrier, the eggshell and its membranes act as a chemical barrier, due to the antimicrobial proteins that are present. For instance, lysozyme (**LYZ**), ovotransferrin (**TF**), OCX-32 (**RARRES1**) and OCX-36 (**BPIFB8**) are proteins with antimicrobial activity present in the shell and membranes [9, 10, 11, 24]. Endogenous antimicrobial protection from the ESMs is essential because bacteria can contaminate the surface and penetrate to the interior of avian eggs leading to an increase of embryonic death during incubation or pose a risk to the

consumer of unfertilized table eggs. Gram-positive bacteria are predominant in the microflora of the shell exterior, possibly because of their tolerance of dry conditions and might originate from dust, soil or faeces. However, Gram-negative bacteria are abundantly present in the interior of decaying eggs [28]. The shell and contents of eggs can be contaminated by a variety of pathogens such as *Campylobacter jejuni*, *Listeria monocytogenes, Escherichia coli, Yersinia enterocolitica* and especially *Salmonella enterica* serovar enteritidis [28]. *Staphylococcus spp.* are among the Gram-positive bacteria that are mainly found in the eggshells of contaminated eggs [29].

Although cuticle and eggshell proteomes have been reported, the ESM proteome is not yet fully characterized [24, 30]. Our previous work [11] showed proteomic LC/MS/MS analysis of proteins extracted with Tris-HCl/DTT from the ESM of unincubated unfertilized eggs, which identified OCX-36 (**BPIFB8**), ovalbumin (**OVAL**), tiarin-like (**OLFM4**) and actin (cytoplasmatic type 5) (**ACTG1**) as abundant proteins. This study revealed that OCX-36 is a protein with specific antimicrobial activity against *S. aureus*. OCX-36 is also a pattern recognition molecule that recognizes bacterial lipopolysaccharides [11]. Moreover, OCX-36 (**BPIFB8**) and OCX-36-derived peptides were able to modulate the innate immune response by neutralizing endotoxins *in vitro* and in a mouse model of endotoxemia [31]. The identification of functional proteins, such as OCX-36, in the ESMs from unfertilized eggs provides impetus to fully characterize the ESM proteome, and to understand how it changes during embryonic development.

One of the challenges encountered in previous studies of the ESM proteome is the extreme stability and insolubility of the highly cross-linked ESM fibers. Previous proteomics studies of the chicken ESM [8, 11, 15, 16] were incomplete because the fibers were not fully solubilized, resulting in an incomplete assessment of the ESM proteome. In the current study, we developed methods to dissolve the ESMs based on chemical degradation of peptides bonds and concomitant cross-linkages. A variety of conditions were explored, based on the prior demonstration that 3-MPA treatment under acid conditions can dissolve the ESMs [13]. We extended these observations by testing another powerful reducing agent, TCEP, which is also stable and effective at an acidic condition [32]. TCEP-HCl in the presence of acetic acid at 80°C was a novel approach that successfully solubilized the membranes and yielded a material suitable for proteomics analysis. A progressive extraction and solubilization approach was taken: 1) 1 M NaCl at 4°C; 2) 50 mM Tris-HCl, pH 8.5, 10 mM DTT at room temperature; and 3) 100 mM TCEP-HCl, 0.17 M acetic acid at 80°C. This approach allowed us to characterize the protein inventory of the ESM fibers during embryonic development, and compare these results to the proteome of ESM from unfertilized eggs. One limitation of this study is that the fertilized and unfertilized eggs were not from the same chicken flock with the same breed, age and raising environment. We did address this aspect by ensuring that hens were of white leghorn background, with similar weight of eggs,.

4.2. Changes of protein levels in the ESM proteome during incubation (unfertilized condition)

One objective was the identification and quantification of the ESM proteins in an unfertilized model, in order to discern changes in the ESM proteins in the fertilized model that are associated with chick embryonic development. In common with other ESM proteomics studies using unfertilized eggs [8], a number of consistently detected proteins were identified: i.e., CREMP proteins (**CREMP**), collagen alpha-1 (X) (**COL10A1**), lysyl oxidase-like 3 (**LOXL3**), lysozyme C (**LYZ**) and OCX-36 (**BPIFB8**) (Fig.2). In this current study, high levels of ESM proteins such as OCX-36 (**BPIFB8**), lysozyme C (**LYZ**), CREMP (**CREMP**), OC-116 (**MEPE**) and avian beta-defensin-11 (AvBD-11) (**GAL11**) were extracted from unfertilized eggs and detected at day 0 (Fig.2) (Table S8). Lysozyme (**LYZ**) is an antimicrobial ESM protein that shows antimicrobial activity against Gram-positive bacteria, and might be active during deposition of calcium carbonate for shell calcification [9, 33]. CREMP (**CREMP**) is a cysteine-rich eggshell membrane protein similar to spore coat protein SP75 from cellular slime molds such as *Dictyostelium discoideum* and *Polysphondilium pallidum*, and this protein is proposed to be the major structural component of the avian ESM [8]. OC-116 (**MEPE**) is the most abundant eggshell specific matrix protein which regulates shell calcification [34, 35].

AvBD-11 (**GAL11**) and beta-2 microglobulin (beta 2M) (**B2M**) are proteins involved in innate immune protection and were included in the functional cluster: response to bacterium. AvBD-11 (**GAL11**) is a member of the β–defensin family of antimicrobial proteins, which has been previously detected in ESM and other egg

compartments [16, 36, 37, 38]. Beta 2M (**B2M**) was also found to be enriched in the ESM from unfertilized eggs at day 0. **B2M** is an antimicrobial protein secreted in the hen uterus that possesses immunoglobulins-like domains which are involved in cell-cell recognition, cell-surface receptors and immune responses [39].

Increased levels of egg white proteins such as avidin (**AVD**) at day 3 and ovomucoid (**SPINK7**) at day 19 in the ESM of unfertilized eggs in comparison with the ESMs from fertilized eggs were observed in our study (Fig.2). This increase might be explained by the diffusion of these proteins towards the ESM from the egg white. Since this egg compartment that is in closest contact with the eggshell membranes. During incubation, complex changes in the egg compartments occur that include thinning of egg white, weakening and stretching of the vitelline membrane and an increase in the pH of egg white. These changes might lead to diffusion of proteins from one egg compartment to another one: i.e. an egg white to eggshell membranes.

4.3. Changes in the ESM proteome associated with embryonic development (fertilized model)

Chick embryonic development can be divided into three main phases. Pooling the proteomic data from fertilized egg ESM at specific days of incubation into the three different phases of chick embryonic development was performed in order to provide a window into changes in the ESM proteome as the extra-embryonic membranes develop. The first phase is the germ establishment during the first week of incubation and is characterized by the development of four extra-

embryonic membranes—the yolk sac, chorion, allantois and amnion, which develop from the ventral portion of the embryo at day 5 to support the growth of the embryo [22]. The second phase of development is embryo completion (from days 7 to 18), including maturation of the chorioallantoic membrane (CAM). The third phase (emergence) occurs during the last three days of development and involves physiological changes necessary for hatching at day 21. During embryonic development, components and constituents of these extra-embryonic membranes become associated with the ESMs, and there are likely induced changes in the ESMs themselves.

The Adjusted Normalized Spectral Values (ANSC) from each ESM protein found in the fertilized model were corrected relative to the unfertilized model at each day of incubation in order to evaluate the changes in protein levels associated with the developing embryo (Fig.2).

4.3.1. Functional analysis of ESM proteins associated with the first phase of chick embryonic development

Our functional analysis of proteins enriched in the ESMs from fertilized eggs during the first phase of embryonic development identified an enrichment of proteins with protease inhibitory activity. Serpin 10 or Myeloid and erythroid nuclear termination stage-specific protein (MENT) (**SERPIN 10**), a member of the serine protease inhibitor (serpin) family which is involved in the regulation of proteolytic activity of various biological processes such as angiogenesis, immune system development and embryogenesis. Protease inhibitors inactivate proteases secreted by

microorganisms which are important for their metabolism and virulence; furthermore, protease inhibitors frequently possess antimicrobial activity [24, 40]. This is important since the immune system of the chick embryo is not fully developed during the first stage of chick embryonic development. The chicken embryo is protected by antibodies from the chicken hen, but these antibodies are finite and decrease during embryonic development [41, 42]. Ovalbumin (**OVAL**), a storage protein from egg white, is a member of serpin family that lacks protease inhibitory activity, but antimicrobial peptides derived from its digestion by chymotrypsin are antimicrobial against *Bacillus subtilis* [43]. Ovalbumin related protein X (**OVALX**) possesses antimicrobial activity against Gram-positive and Gram-negative bacteria [44, 45]. Ovalbumin related protein Y (**OVALY**) has been shown to be associated with fertilization [46, 47]. In addition, ovoinhibitor (**SPINK5**) is a protein that belongs to the category of Kazal-type protease inhibitors and has anti-protease activity against *Bacillus* spp. growth [48]. Furthermore, a cysteine protease inhibitor, cystatin, possesses bactericidal activity against a variety of pathogenic bacteria such as *E.coli*, *P. aeruginosa, Oligella sp.*, and *Actinobacter Iwoffi* [49]. Among these protease inhibitors, OCX-32 (**RARRES1**) was identified as an overabundant protein in the ESMs of fertilized eggs and shows a large increase (4,000-fold) at day 3 in comparison with the ESMs of unfertilized eggs (Fig. 2). OCX-32 is an eggshell matrix-specific protein that inhibits *B.* Subtilis growth and also inhibits carboxypeptidase A activity [50,51]. Ovocalyxin-36 (BPI fold-containing family B, member 3 precursor, **BPIFB3**) was another enriched protein in the ESMs at day 3. Ovocalyxin-36 belongs to the

bactericidal/permeability-increasing protein (BPI)/ (lipopolysaccharide)-binding protein) LBP protein family involved in innate immunity [52]. BPI proteins play an important role in the host-defense against Gram-negative bacteria [53, 54].

Functional groups corresponding to proteins associated with extracellular structure organization and cell adhesion were enriched in the first stage at day 7 (Table 3). We observed that tenascin (**TNC**), an extracellular matrix protein, is enriched in the ESMs at day 7 during chick embryo development. **TNC** is upregulated during vertebrate embryo development [55]. The expression of tenascin (**TNC**) in the chick limb bud has been reported in tendons and in denervated limbs at day 7 [56, 57].

In summary it seems that the first stage of the development is associated with antibacterial defense, protease inhibitory activity to prevent protein degradation and to ensure protein, function integrity and structural maintenance.

4.3.2. Functional analysis of ESM proteins associated with the second phase of chick embryonic development

The maturation of CAM involves its vascularization which takes place in the second phase of embryonic development. CAM vascularization starts from day 4 and occurs until day 14 of embryonic development [58, 59]. The highly vascularized CAM becomes the functional respiratory organ for the embryo from day 8 until day 19, when pulmonary respiration of the embryo is initiated [21, 60]. By day 8 of incubation, the CAM contains the developed capillary plexus, which becomes

progressively associated with the outer surfaces of chorionic epithelial cells. The capillary plexus is localized at the surface of the ectoderm adjacent to the shell membranes at day 14 [61,62].

Our proteomics analyses of the ESMs identified enriched proteins involved in CAM vascularization, which were functionally categorized as extracellular matrix proteins and cell adhesion proteins (Table 3). Fibronectin (**FN1**) is one of the most abundant extracellular matrix protein identified at day 15 of the developing embryo. This protein is a homodimeric, multidomain glycoprotein involved in cell adhesion that binds to nidogen and glycosaminoglycans [63]. **FN1** was found in the extracellular matrix beneath the chorion at early stages of development when the subepithelial capillary plexus is not yet developed [64]. **FN1** induces the migration of endothelial cells merging by sprouting from the mesodermal blood vessels [65]. It was reported that fibronectin provides a matrix along which capillaries migrate [66]. Moreover, fibronectin induces elongation of sprouting microvessels, endothelial cell assembly and the development of multicellular capillaries [67]. The elevated content of **FN1** in the extracellular matrix is in accordance with the vasoproliferative processes stimulated by angiogenic stimuli in the CAM [68].

Different types of laminins, such as laminin subunit alpha 5 isoform X7 (**LAMA5**), laminin subunit beta-1 (**LAMB1**), laminin gamma-1 chain precursor (**LAMC1**), nidogen 1 (**NID1**) and nidogen 2 (**NID2**) were identified as significant proteins associated with cellular adhesion at day 15 of incubation (Fig.2). The laminins are large heterotrimeric glycoproteins present in the extracellular matrix

of developing chick embryos [69-71]. The immunoreactive laminin has been detected at all stages of CAM blood vessel development [64].

Nidogen is an extracellular matrix protein found during chick embryogenesis and might be associated with the directional migration of cells [72]. This protein binds collagen IV and fibronectin (**FN1**), and forms a particularly stable complex with laminin [73-75].

Pleiotrophin (**PTN**) is a heparin binding protein, and enhanced levels of chicken pleiotrophin were identified at day 15. The angiogenic effect of human recombinant pleiotrophin in the chick embryo CAM model has been confirmed *in vivo* and *in vitro* by the activation of growth factors and metalloproteinases that are important players in angiogenesis [76].

Many physical changes in the avian ESMs take place during embryonic development, i.e., an increase in oxygen permeability between days 2 and 5 of incubation, with a progressive increase to its highest level at day 16 [77, 78]. As chicken embryogenesis progresses toward hatch, there is an increase in the capacity for oxygen to diffuse across the CAM as well as increases in circulating hemoglobins [79, 80].

In our study, chicken hemoglobin proteins, especially alpha A (**HBAA**) and alpha D (**HBAD**), subunit beta (**HBG2**) and subunit epsilon (**HBE**), were only found in the ESM after day 11, representing the proximity of the developing vasculature CAM and its erythrocytes from day 11 onwards [81, 82] (Fig.2).

The CAM is also engaged in calcium transport from the eggshell to the embryo. Calcium is mobilized by the CAM from day 10-12 until hatching, and a high level of calcium accumulation by the embryo occurs around day 17 [83]. The chorionic epithelial cells present in the CAM secrete protons that dissolve the calcium reserve bodies at the bases of the mammillary cones from day 11 onwards and liberate Ca ions which are taken up by the chorionic cells and transferred to the developing embryo via the blood stream [82, 84]. Proteins involved in binding calcium ions were abundant in the ESMs at day 15, including EGF-like repeats and discodin I-like domains 3 (**EDIL3**); lactadherin (**MFGE8**) also possesses calcium binding EGF-like domains. These were previously found at the initiation stage of the shell mineralization and are associated with the calcification process [35]. **EDIL3** protein was previously identified in the ESMs at days 15 and 21 [15]. The enrichment of **EDIL3** in the ESMs during the later stages of embryonic development is expected, due to the association of this protein with calcium transport from day 11 until day 21 [15].

Annexin 2 (**ANXA2**) is a calcium binding protein and binds to membrane phospholipids in calcium dependent manner, representing a link between calcium signaling and plasma membrane functions. The **ANXA2** was previously shown to be expressed in the chicken CAM between days 8-12 of incubation [85, 86]. We found that **ANXA2** was enriched in the ESMs of fertilized eggs at day 11 and 15 in comparison with the unfertilized condition.

In summary, the proteins identified in the second phase are mainly structural matrix proteins, antimicrobial and protease inhibitors.

4.3.3. Functional analysis of ESM proteins associated with the third phase of chick embryonic development

Emergence occurs in the last three days of chick development and is initiated by internal pipping, when the beak of the developing chick embryo pierces the CAM and the inner ESM at the edges of the air cell during days 19 to 20. The oxygen supply for the embryo via blood circulation through the vascularized CAM declines after day 19 and internal pipping facilitates pulmonary respiration [87]. After a further 24 h, the chick embryo initiates external pipping by beginning to rupture the eggshell [20]. The main source of energy during mid- to late-embryonic development comes from the oxidation of yolk lipids that are transported to the embryo through the yolk sac membrane and vascular system [21, 88].

The origin of the circulating plasma and liver proteins that we detected in the ESMs is likely from the remaining vascularized CAM that adheres tenaciously to the extracted ESM samples from fertilized eggs (Table 4). Abundant plasma proteins associated with lipid binding and transport were observed at day 19, including alpha fetoprotein (**AFP**), serum albumen precursor (**ALB**), and apolipoproteins (AI (**APOA1**), AIV (**APOA4**) and B (**APOB**)) (Fig.2). **AFP,** which binds fatty acids reversely, is the main plasma protein synthesized by the yolk sac during chick embryogenesis [88, 90]. It is also synthesized by other extra-embryonic membranes such as the CAM and amniotic membrane at low levels [90]. The synthesis of serum albumin (**ALB**) in the yolk sac occurs at the late stage of chick embryogenesis (between days 16 and 18); and is thought to bind and transport of fatty acids and estrogens [91].

Vitellogenin 1 (**VTG2**) and Vitellogenin 2 (**VTG2**) were also enriched in the ESMs at day 19. The proteolytic cleavage of vitellogenin generates yolk proteins, such as lipovitellin and phosvitin, which are sources of nutrients for the chick embryo. Apolipoproteins are a component of very low density lipoprotein (VLDL) and were found in the fertilized eggshell membrane at the late stage of embryo development when the metabolism of lipids is very intense in the chick embryo liver. Apolipoproteins are involved in the recruitment of lipids for the growing embryo [22, 23].

Proteins in the ESMs associated with innate immunity and antimicrobial protection at day 19 were OCX-36 (**BPIFB8**), TENP (**BPIFB7**) and AvBD-11 (**GAL11**), which would contribute to innate protection against pathogens as the shell is weakened before hatching. Previous immunolocalization studies of OCX-36 showed that it is also a matrix protein of the calcified shell, particularly in the mammillary cone layer [92]. Antimicrobial shell proteins such as OCX-36 are likely solubilized and released during the course of mammillary cone dissolution. We have previously reported OCX-36 protein localization within the ESMs [11, 92]; in this study high levels of the OCX-36 in the ESMs of fertilized eggs were detected compared to the ESMs from unfertilized eggs (Fig.2). However, these ESMs were mechanically removed from the shell to extract the proteins at day 19, and some fragments of mammillary cones are expected to be attached to membranes at this time point [16]. The chemical degradation of ESM samples using TCEP-HCl in acid would dissolve the calcitic cones and therefore release OCX-36 and other proteins associated with the cones for detection by proteomic analysis.

TENP (**BPIFB7**) is another member of the LBP/BPI/PLUNC family of antimicrobial proteins. The LPS binding domains of TENP implies an innate immune protection of eggs against pathogens [93]. Purified TENP possesses antibacterial activity specificity against Gram-positive bacteria including *Micrococcus luteus* and *Bacillus subtilis* [94].

One of the most abundant defense response proteins in fertilized eggs at day 19 is AvBD-11 (**GAL11**), which was previously detected in the eggshell, egg white, ESM and is abundantly found in the vitelline membrane [37,38, 95]. **GAL11** has a defensin-like motif which however consists of six disulfide bridges instead of three, which might be the result of gene duplication [38]. **GAL11** possesses antimicrobial activity against Gram-negative and Gram-positive bacteria such as *S. enteritidis* and *L. monocytogenes,* and plays an important role in the innate immune system [38, 96].

It is conceivable that there is local synthesis in the cells of the CAM in order to upregulate antimicrobial protection. However, no GAL 11 was detected in the CAM extraction at day 19; while TENP was detected in both in the extraction of CAM at day 19 and the ESM proteins extracted from fertilized eggs.

5. Conclusion

This current study quantifies the changes in abundance of ESM proteins during chick embryonic development. A comparative analysis of ESM proteins is essential to understand the changes in protein abundance during embryogenesis as well as to confirm whether the proteins are specifically related to embryonic events or, if

the presence of some protein might be due to thermally induced changes in protein localization during incubation. Therefore, the incorporation of proteins with different biological functions from egg compartments and extra-embryonic membranes such as CAM to the ESMs creates a protective and nutritional environment for a sustainable development of the chicken embryo from the earliest stages of development until hatching. .

Acknowledgments

Funding for this study was provided by the Canadian Natural Sciences and Engineering Research Council (NSERC) Discovery (155449) and Strategic (STPGP 365046) grant programs. We would like to thank Dr. Sylvie Bourassa and Daniel Defoy from the Proteomics Platform of the Quebec Genomics Center (Laval, QC) for their proteomics services and their expertise. We are grateful to Dr. Ahmed and Dr. Rose-Martel for providing extremely useful insight and discussions during the preparation of this manuscript. The suggestions of several anonymous reviewers were helpful to significantly improve the manuscript.

References

[1] Bellairs R, Boyde A. Scanning electron microscopy of the shell membranes of the hen's egg. Z Zellforsch Mikrosk Anat 1969;96: 237–49.

[2] Liong JWW, Frank JF, Bailey S. Visualization of eggshell membranes and their interaction with Salmonella enteritidis using confocal scanning laser microscopy. J Food Prot 1997; 60:1022–8.

[3] Hincke MT, Nys Y, Gautron J, Mann K, Rodriguez-Navarro AB, McKee, MD. The eggshell: structure, composition and mineralization. Front Biosci 2012;17:1266- 1280.

[4] Nys Y, Gautron J, Garcia-Ruiz JM, Hincke MT. Avian eggshell mineralization: biochemical and functional characterization of matrix proteins. Comptes Rendus Palevol 2004; 3: 549-562.

[5] Burley RW, Vadhera DV. The Avian Egg: Chemistry and Biology. Wiley and Sons, New York;1989.

[6] Arias JL, Fink DJ, Xiao SQ, Heuer AH, Caplan AI. Biomineralization and eggshells: cell-mediated acellular compartments of mineralized extracellular matrix. Int Rev Cytol 1993;145: 217–250.

[7] Arias JL, Fernandez MS, Dennis JE, Caplan AI. Collagens of the chicken eggshell membranes. Connect Tissue Res 1991; 26:37-45.

[8] Kodali VK, Gannon SA, Paramasivam S, Raje S, Polenova T, Thorpe C. A novel disulfide-rich protein motif from avian eggshell membranes. PloS One 2011; 6: e18187.

[9] Hincke MT, Gautron J, Panheleux M, Garcia-Ruiz J, McKee MD, Nys Y. Identification and localization of lysozyme as a component of eggshell membranes and eggshell matrix. Matrix Biol 2000; 19: 443-453.

[10] Gautron, J, Hincke, MT, Panheleux, M, Garcia-Ruiz, JM, Boldicke, T, Nys, Y. Ovotransferrin is a matrix protein of the hen eggshell membranes and basal calcified layer. Connect Tissue Res 2001; 42: 255-267.

[11] Cordeiro CM, Esmaili H, Ansah G, Hincke MT. Ovocalyxin-36 is a pattern recognition protein in chicken eggshell membranes. PloS One 2013; 8: e84112.

[12] Wong M, Hendrix MJ, von der Mark K, Little C, Stern R. Collagen in the egg shell membranes of the hen. Deve Biol 1984; 104: 28-36.

[13] Yi F, Yu J, Guo ZX, Zhang LX, Li Q. Natural bioactive material: a preparation of soluble eggshell membrane protein. Macromol Biosci 2003; 3: 234-237.

[14] Ahlborn GJ, Clare DA, Sheldon BW, Kelly RW. Identification of eggshell membrane proteins and purification of ovotransferrin and β-NAGase from hen egg white. The Protein J 2006; 25: 71-81.

[15] Kaweewong K, Garnjanagoonchorn W, Jirapakkul W, Roytrakul S. Solubilization and identification of hen eggshell membrane proteins during different times of chicken embryo development using the proteomic approach. The Protein J 2013; 32: 297-308.

[16] Rose-Martel M, Smiley S, Hincke MT. Novel identification of matrix proteins involved in calcitic biomineralization. J Proteomics 2015; 116: 81-96.

[17] Lemaire L, Kessel M. Gastrulation and homeobox genes in chick embryos. Mech Develop 1997; 67: 3-16.

[18] Sheng G. Day-1 chick development. Dev Dynam 2014; 243: 357-367.

[19] Mellor DJ, Diesch TJ. Birth and hatching: Key events in the onset of awareness in the lamb and chick. N Z Vet J 2007; 55:51–60.

[20] Tong Q, Romanini CE, Exadaktylos V, Bahr C, Berckmans D, Bergoug H, etal. Embryonic development and the physiological factors that coordinate hatching in domestic chickens. Poultry Sci 2013; 92:620-628.

[21] Moran ET. Nutrition of the developing embryo and hatchling. Poultry Sci 2007; 86: 1043-1049.

[22] Romanoff AL. Biochemistry of the Avian embryo; 1967.

[23] De Oliveira JE, Uni Z, Ferket PR. Important metabolic pathways in poultry embryos prior to hatch. World Poultry Sci J 2008; 64:488-499.

[24] Rose-Martel M, Du J, Hincke MT. Proteomic analysis provides new insight into the chicken eggshell cuticle. J Proteomics 2012; 75: 2697-2706.

[25] Rodríguez-Navarro AB, Marie P, Nys Y, Hincke MT, Gautron J..Amorphous calcium carbonate controls avian eggshell mineralization: a new paradigm for understanding rapid eggshell calcification. J Struct Biol 2015; 190: 291-303.

[26] Bain MM. Eggshell strength: a relationship between the mechanism of failure and the ultrastructural organization of the mammillary layer. Brit Poultry Sci 1992; 33: 303–319.

[27] Rodriguez-Navarro A, Kalin O, Nys Y, Garcia-Ruiz JM. Influence of the microstructure on the shell strength of eggs laid by hens of different ages. Brit Poultry Sci 2002; 43: 395-403.

[28] Board RG, Tranter, HS. The microbiology of eggs. In: W. J. Stadelman and Coterill O. J. (eds). Egg Science and technology. 4th ed. Haworth Press Inc. New York; 1995.

[29] Abdullah IN. Isolation and identification of some bacterial isolates from table egg. J Vet Sci 2010; 3: 59–67.

[30] Mann K, Maček B, OlsenJV. Proteomic analysis of the acid-soluble organic matrix of the chicken calcified eggshell layer. Proteomics 2006: 6; 3801-3810.

[31] Kovacs-Nolan J, Cordeiro C, Young D, Mine Y, Hincke M. Ovocalyxin-36 is an effector protein modulating the production of proinflammatory mediators. Vet Immunol Immunop 2014; 160:1-11.

[32] Winther JR, Thorpe C. Quantification of thiols and disulfides. (BBA) Gen Subjects 2014; 1840: 838-846.

[33] Pellegrini A, Thomas U, Bramaz N, Klauser S, Hunziker P, Von Fellenberg R. Identification and isolation of a bactericidal domain in chicken egg white lysozyme. J Appl Microbiol 1997; 82: 372-378.

[34] Rose ML, Hincke MT. Protein constituents of the eggshell: eggshell-specific matrix proteins. Cell Mol Life Sci 2009; 66: 2707-2719.

[35] Marie P, Labas V, Brionne A, Harichaux G, Hennequet-Antier C, Nys Y, Gautron J.Quantitative proteomics and bioinformatic analysis provide new insight into protein function during avian eggshell biomineralization. J Proteomics 2015; 113: 178-193.

[36] Mann K. The chicken egg white proteome. Proteomics 2007; 7: 3558-3568.

[37] Mann K. Proteomic analysis of the chicken egg vitelline membrane. Proteomics 2008; 8: 2322-2332.

[38] Hervé-Grépinet V, Réhault-Godbert S, Labas V, Magallon T, Derache C, Lavergne M, et al. Purification and characterization of avian β-defensin 11, an antimicrobial peptide of the hen egg. Antimicrob Agents Ch 2010; 54: 4401-4409.

[39] Smith DK, Xue H. Sequence profiles of immunoglobulin and immunoglobulin-like domains. J Mol Biol 1997; 274:530-545.

[40] Armstrong PB. Proteases and protease inhibitors: a balance of activities in host–pathogen interaction. Immunobiology 2006; 211: 263-281.

[41] Hamal KR, Burgess SC, Pevzner IY, Erf GF. Maternal antibody transfer from dams to their egg yolks, egg whites, and chicks in meat lines of chickens. Poultry Sci 2006; 85: 1364-1372.

[42] Meade KG, Higgs R, Lloyd AT, Giles S, O'Farrelly C. Differential antimicrobial peptide gene expression patterns during early chicken embryological development. Dev Comp Immunol 2009; 33: 516-524. [43] Pellegrini A, Hülsmeier AJ, Hunziker P, Thomas U. Proteolytic fragments of ovalbumin display antimicrobial activity. (BBA) Gen Subjects 2004; 1672: 76-85.

[43] Pellegrini A, Hülsmeier AJ, Hunziker P, Thomas U. Proteolytic fragments of ovalbumin display antimicrobial activity. (BBA) Gen Subjects 2004; 1672: 76-85.

[44] Benarafa C, Remold-O'Donnell E. The ovalbumin serpins revisited: perspective from the chicken genome of clade B serpin evolution in vertebrates. P Natl Acad Sci USA 2005; 102: 11367-11372.

[45] Réhault-Godbert S, Labas V, Helloin E, Hervé-Grépinet V, Slugocki C, Berges M, et al.Ovalbumin-related protein X is a heparin-binding ov-serpin exhibiting antimicrobial activities. J Biol Chem 2013; 288: 17285-17295.

[46] Liu Y, Qiu N, Ma M. Comparative proteomic analysis of hen egg white proteins during early phase of embryonic development by combinatorial peptide ligand library and matrix-assisted laser desorption ionization-time of flight. Poult Sci 2013; 92: 1897-1904.

[47] Qiu N, Liu W, Ma M, Zhao L, Li Y. Differences between fertilized and unfertilized chicken egg white proteins revealed by 2-dimensional gel electrophoresis-based proteomic analysis. Poult Sci 2013; 92: 782-786.

[48] Bourin M, Gautron J, Berges M, Attucci S, Le Blay G, Labas V, et al. Antimicrobial potential of egg yolk ovoinhibitor, a multidomain Kazal-like inhibitor of chicken egg. J Agr Food Chem 2011; 59: 12368-12374.

[49] Wesierska E, Saleh Y, Trziszka T, Kopec W, Siewinski M, Korzekwa K. Antimicrobial activity of chicken egg white cystatin. World J Microb Biot 2005; 21: 59-64.

[50] Gautron J, Hincke MT, Mann K, Panheleux M, Bain M, McKee, et al. Ovocalyxin-32, a novel chicken eggshell matrix protein — isolation, amino acid sequencing, cloning, and immunocytochemical localization. J Biol Chem 2001;276:39243–52.

[51] Xing J, Wellman-Labadie O, Gautron J, Hincke MT. Recombinant eggshell ovocalyxin-32: expression, purification and biological activity of the glutathione S-transferase fusion protein. Comp Biochem Physiol B Biochem Mol Biol 2007;147:172–7.

[52] Bingle CD, Craven CJ. Meet the relatives: a family of BPI-and LBP-related proteins. Trends Immunol 2004; 25: 53-55.

[53] Elsbach P, Weiss J. The bactericidal/permeability-increasing protein (BPI), a potent element in host-defense against gram-negative bacteria and lipopolysaccharide. Immunobiology 1993; 187: 417-429.

[54] Elsbach P. The bactericidal/permeability-increasing protein (BPI) in antibacterial host defense. Journal Leukocyte Biol 1998; 64: 14-18.

[55] Riou JF, Umbhauer M, Shi DL, Boucaut JC. Tenascin: a potential modulator of cell-extracellular matrix interactions during vertebrate embryogenesis. Biol Cell 1992; 75: 1-9.

[56] Wehrle-Haller B, Koch M, Baumgartner S, Spring J, Chiquet M. Nerve-dependent and-independent tenascin expression in the developing chick limb bud. Development 1991; 112: 627-37.

[57] Edom-Vovard F, Schuler B, Bonnin MA,Teillet MA, Duprez D. Fgf4 positively regulates scleraxis and tenascin expression in chick limb tendons. Dev Biol 2002; 247:351–366

[58] Vico PG, Kyriacos S, Heymans O Louryan S, Cartilier L. Dynamic study of the extraembryonic vascular network of the chick embryo by fractal analysis. J. Theor Biol 1998; 195:525–532.

[59] Ribatti D, Nico B, Vacca A, Roncali L, Burri PH, Djonov V. Chorioallantoic membrane capillary bed: A useful target for studying angiogenesis and anti-angiogenesis in vivo. Anat Rec 2001; 264:317–324.

[60] Ribatti D. Chick embryo chorioallantoic membrane as a useful tool to study angiogenesis. Int Rev Cell Mol Bio 2008; 270: 181-224.

[61] Ausprunk DH, Knighton DR, Folkman J. Differentiation of the vascular endothelium in the chick chorioallantois: a structural and autoradiographic study. Dev Biol 1974; 38:237-247.

[62] Baumann R, Meuer HJ. Blood oxygen transport in the early avian embryo. Physiol Rev 1992; 72: 941-965.

[63] Ruoslahti E, Öbrink B. Common principles in cell adhesion. Exp Cell Res1996; 227: 1-11.

[64] Ribatti D, Bertossi M, Nico B, Vacca A, Ria R, Riva A, et al. Role of basic fibroblast growth factor in the formation of the capillary plexus in the chick embryo chorioallantoic membrane. An *in situ* hybridization, immunohistochemical and ultrastructural study. J Submicrosc Cytol Pathol 1998; 30:127-136.

[65] Ribatti D, Nico B, Vacca A, Iurlaro M, Roncali L. Temporal expression of the matrix metalloproteinase MMP-2 correlates with fibronectin immunoreactivity during the development of the vascular system in the chick embryo chorioallantoic membrane. J Anat 1999; 195: 39-44.

[66] Risau W, Lemmon V. Changes in the vascular extracellular matrix during embryonic vasculogenesis and angiogenesis. Devel Biol 1988; 125: 441-450.

[67] Nicosia RF, Bonanno E, Smith M. Fibronectin promotes the elongation of microvessels during angiogenesis in vitro. J Cell Physiol 1993; 154: 654-661.

[68] Ribatti D, Vacca A, Costantino F, Minischetti M, Locci P, Becchetti E, et al. Exogenous heparin induces fibronectin overexpression parallel to angiogenesis in the extracellular matrix of the chick embryo chorioallantoic membrane. Tissue Cell 1997; 29: 131-136.

[69] Engel J. Laminins and other strange proteins. Biochemistry 1992; 31:10643-10651.

[70] Timpl R, Brown JC. The laminins. Matrix Biol 1994; 14: 275-281.

[71] Zagris N, Stavridis V. The expression of the genes for laminin in the early embryo. In: Zagris, N., Duprat, A.M., Durston, A. (Eds.). Organization of the Early Vertebrate Embryo, vol. 279. Plenum Press, New York, pp. 169-182;1995.

[72] Zagris N, Stavridis V, Chung AE. Appearance and distribution of entactin in the early chick embryo. Differentiation 1993; 54, 67-71.

[73] Martin GR, Timpl R. Laminin and other basement membrane components. Annu Rev Cell Biol 1987; 3: 57-85.

[74] Yurchenco PD, O'Rear J. Basal lamina assembly. Curr Opin Cell Biol 1994; 6: 674-681.

[75] Chung AE. The extracellular matrix in development. In: Zagris, N, Duprat, A.M., Durston, A. (Eds.). Organization of the Early Vertebrate Embryo, vol. 279. Plenum Press, New York, pp. 149±167, 1995.

[76] Papadimitriou E, Polykratis A, Courty J, Koolwijk P, Heroult M, Katsoris P. HARP induces angiogenesisin vivo and in vitro: implication of N or C terminal peptides. Biochem Biophys Res Commun 2001; 282:306-313.

[77] Kutchai H, Steen JB. Permeabilty of the shell and shell membraes of hens' eggs during development. Resp Physiol 1971; 11: 265-278.

[78] Lomholt JP. The development of the oxygen permeability of the avian egg shell and its membranes during incubation. J Exp Zool 1976; 198: 177-184.

[79] Tazawa H. Gas transfer in the chorioallantois. In. Piiper (ed.), Respiratory function in birds, adult and embryonic, pp. 274-291. Springer-Verlag,Berlin; 1978.

[80] Tazawa H. Oxygen and CO2 exchange and acid-base regulation in the avian embryo. Am Zool 1980; 20: 395-404.

[81] Romanoff AL. The Avian Embryo: Structural and Functional Development, Macmillan, New York, NY, USA; 1960.

[82] Melkonian G, Munoz N, Chung J, Tong C, Marr R, Talbot P. "Capillary plexus development in the day five to day six chick chorioallantoic membrane is inhibited by cytochalasin D and suramin," J Exp Zool 2002; 292: 241–254.

[83] Gabrielli MG, Accili D. The chick chorioallantoic membrane: a model of molecular, structural, and functional adaptation to transepithelial ion transport and barrier function during embryonic development. J Biomed Res 2010; 1-12.

[84] Chien YC, Hincke MT, McKee MD. Ultrastructure of avian eggshell during resorption following egg fertilization. J Struct Biol 2009; 168: 527-538

[85] Gerke V, Creutz CE, Moss SE. Annexins: linking Ca2+ signalling to membrane dynamics. Nat Rev Mol Cell Bio 2005; 6: 449-461.

[86] Matschke K, Da Silva-Azevedo L, Hlushchuk R, Djonov V, Baum O. Annexins as cell-type-specific markers in the developing chicken chorionallantoic membrane. Cell Tissue Res 2006; 323: 395-404.

[87] Menna TM, Mortola JP. Metabolic control of pulmonary ventilation in the developing chick embryo. Respir Physiolol Neurobiol 2002; 130:43–55.

[88] Speake BK, Murray AM, Noble RC. Transport and transformations of yolk lipids during development of the avian embryo. Prog Lipid Res 1998; 37:1–32.

[89] Slade B, Milne J. Localization and synthesis of alpha fetoprotein in the chicken. Cell Tissue Res 1977; 180: 411-9.

[90] Palazón LS, Rodríguez-Burgos A. Protein synthesis by chick (Gallus domesticus) extraembryonic membranes.Comp Biochem Physiol Part B: Comp Biochem 1993; 104: 689-693.

[91] Gitlin D, Kitzes J. Synthesis of serum albumin, embryo-specific α-globulin and conalbumin by the chick yolk sac. (BBA)-Protein Struct 1967; 147: 334-340.

[92] Gautron J, Murayama E, Vignal A, Morisson M, McKee MD, Réhault S, et al. Cloning of ovocalyxin-36, a novel chicken eggshell protein related to lipopolysaccharide-binding proteins, bactericidal permeability-increasing proteins, and plunc family proteins. J Biol Chem 2007; 282: 5273-5286.

[93] Whenham N, Wilson PW, Bain MM, Stevenson L, Dunn IC. Comparative biology and expression of TENP, an egg protein related to the bacterial permeability-increasing family of proteins. Gene 2014; 538: 99-108.

[94] Maehashi K, Ueda M, Matano M, Takeuchi J, Uchino M, Kashiwagi Y, et al. Biochemical and Functional Characterization of Transiently Expressed in Neural Precursor (TENP) Protein in Emu Egg White. J Agr Food Chem 2014; 62: 5156-5162.

[95] Mageed AMA, Isobe N, Yoshimura Y. Immunolocalization of avian beta-defensins in the hen oviduct and their changes in the uterus during eggshell formation. Reproduction 2009; 138: 971–978.

[96] Cuperus T, Coorens M, van Dijk A, Haagsman HP. Avian host defense peptides. Dev Comp Immunol 2013; 41: 352-369.

[97] Rehault-Godbert S, Mann K, Bourin M, Brionne A, Nys Y. Effect of Embryonic Development on the Chicken Egg Yolk Plasma Proteome after 12 Days of Incubation. J Agr Food Chem 2014; 62: 2531-2540.

[98] Mann K, Mann M. The chicken egg yolk plasma and granule proteomes. Proteomics 2008; 8: 178-191.

[99] Farinazzo A, Restuccia U, Bachi A, Guerrier L, Fortis F, Boschetti E, Fasoli E, Citterio A, Righetti PG. Chicken egg yolk cytoplasmic proteome, mined via combinatorial peptide ligand libraries. J Chromatogr A 2009; 1216:1241–52.

[100] D'Ambrosio C, Arena S, Scaloni A, Guerrier L, Boschetti E, Mendieta ME, Citterio A, Righetti PG. Exploring the chicken egg white proteome with combinatorial peptide ligand libraries. J Proteome Res 2008; 7: 3461-3474.

[101] Guérin-Dubiard C, Pasco M, Mollé D, Désert C, Croguennec T, Nau F. Proteomic analysis of hen egg white. J Agri Food Chem 2006; 54: 3901-3910.

Supplementary Data

Table S1. Solubilization of eggshell membranes.

Conditions	T(°C)	MPA(M)[a]	TCEP-HCl (mM)[b]	A.A(M)[c]	Yield (%)[d]
1	80	1.25	0	0.17	45
2	80	1.25	0	1.7	40
3	80	2.5	0	0	25
4	70	2.5	0	0	40
5	80	0	100	0	32
6	80	0	100	0.17	53
7	80	0	100	1.7	49

[a] Concentration of 3-mercaptopropionic acid.

[b] Concentration of tris(2-carboxyethyl)phosphine hydrochloride.

[c] Concentration of acetic acid.

[d] Yield of product which remains soluble at pH 8.5.

Table S2. List of proteins identified in the eggshell membranes from fertilized eggs at day 0.

Identified Proteins (Day 0 Fertilized)	Acession Number NCBI/UNIPROT	MW (kDa)	Gene ID [d]	Official Gene Symbol [d]	Unique peptides [a]			Normalized spectral counts (NSC) [a,b]			ANSC [a,c]	Percentage Coverage [a] (%)		
					NaCl	Tris	TCEP	NaCl	Tris	TCEP		NaCl	Tris	TCEP
Acidic mammalian chitinase	XP_418051.2	87	419931	CHIA	0	2	0	0	5	0	0.13	0.00	4.20	0.00
Alpha-1-acid glycoprotein	AAT39530.1	22	395220	ORM1	5	4	0	22	23	0	1.07	23.00	20.00	0.00
Alpha-amylase	NP_001001528.1	58	414139	AMY1A	0	8	0	0	22	0	0.61	0.00	21.00	0.00
Avidin	CAC34569.1	17	396260	AVD	6	0	0	12	0	0	0.23	36.00	0.00	0.00
Beta-microseminoprotein A1-like	XP_004942175.1	13	101750594	LOC101750594	0	3	0	0	11	0	0.30	0.00	18.00	0.00
Bromodomain adjacent to zinc finger domain protein 2B	NP_990008.1	236	395400	BAZ2B	2	0	0	2	0	0	0.04	0.99	0.00	0.00
Clusterin	AAD17257.1	51	395722	CLU	0	0	2	0	0	27	1420.93	0.00	0.00	3.80
Collagen alpha-1(X) chain	F1NRH2	66	100858979	LOC100858979	0	4	9	0	22	647	34302.22	0.00	14.00	5.50
CREMP-like 3	UPI000044A8C0	34	N/A	N/A	0	2	2	0	14	195	10351.82	0.00	11.00	9.30
Cystatin	P01038	15	396497	CST3	0	2	0	0	12	0	0.35	0.00	20.00	0.00
EGF-like repeats and discoidin I-like domains 3 isoform X3	XP_004949505.1	54	427326	EDIL3	0	2	3	0	6	42	2233.06	0.00	4.20	4.80
EW135	BAO03774.1	104	N/A	N/A	8	0	0	30	0	0	0.57	10.00	0.00	0.00
Galactocerebrosidase isoform X3	XP_421304.3	77	423394	GALC	0	7	0	0	37	0	1.04	0.00	12.00	0.00
Gastrokine 2 (OCX-21)	XP_417666.1	21	419515	GKN2	0	0	3	0	0	119	6292.16	0.00	0.00	13.00
Glutathione peroxidase	F1NPJ8	25	427638	GPX3	0	0	0	0	0	15	795.00	0.00	0.00	12.00
Hyaluronan and proteoglycan link protein 3	XP_413868.3	41	415495	HAPLN3	0	0	5	0	0	50	2638.61	0.00	0.00	13.00
Ig alpha heavy chain	AAB22614.2	62	N/A	N/A	4	17	0	9	224	0	6.44	7.70	25.00	0.00
Ig gamma H	1405234A	54	N/A	N/A	0	3	0	0	17	0	0.48	0.00	3.60	0.00
Ig lambda chain V-1 region Precursor	NP_001265474.1	24	416928	IGLL1	0	7	0	0	106	0	2.96	0.00	33.00	0.00
Ig mu chain C region	P01875	48	101748478	LOC101748478	0	6	0	0	17	0	0.48	0.00	17.00	0.00
Insulin-like growth factor binding protein 7	XP_420577.3	32	422620	IGFBP7	0	2	0	0	5	0	0.13	0.00	9.80	0.00
Lipocalin 8	P21760.2	20	396393	LCN8	0	11	0	0	71	0	2.00	0.00	48.00	0.00
Lysozyme C	P00698	16	396218	LYZ	32	4	5	508	48	111	5894.00	86.00	27.00	35.00
Lysyl oxidase homolog 2	E1C3U7	87	419533	LOXL2	0	8	19	0	36	850	45060.01	0.00	7.90	21.00
Mucin-5AC	XP_003641370.2	383	100859916	LOC100859916	0	5	0	0	17	0	0.48	0.00	1.50	0.00
Mucin-5B	Q98UI9	234	395381	LOC395381	0	3	0	0	9	0	0.26	0.00	1.70	0.00
Mucin 6	XP_426405.4	294	414878	MUC6	2	0	0	7	0	0	0.13	0.92	0.00	0.00
Ovalbumin	P01012.2	43	396058	OVAL	10	15	5	30	137	142	7513.97	36.00	41.00	20.00
Ovalbumin-related protein X	P01013.1	45	420898	OVALX	2	0	0	6	0	0	0.11	6.50	0.00	0.00
Ovalbumin-related protein Y	P01014.1	44	420897	OVALY	0	3	0	0	11	0	0.30	0.00	7.70	0.00
Ovocalyxin-36	Q53HW8	58	419289	BPIFB8	0	3	0	0	19	0	0.52	0.00	8.00	0.00
Ovocleidin-17	Q9PRS8	15	100313508	OC-17	2	0	3	4	0	57	3044.40	22.00	0.00	15.00
Ovocleidin-116	NP_989900.1	77	395256	MEPE	5	22	0	27	207	0	6.30	12.00	30.00	0.00
Ovomucoid	P01005	23	416236	SPINK7	6	3	0	21	11	0	0.70	33.00	25.00	0.00
Ovotransferrin	P02789.2	78	396241	TF	19	12	0	44	39	0	1.93	30.00	24.00	0.00
Partial Ig heavy chain variable region	CAO79236.1	13	N/A	N/A	0	2	0	0	9	0	0.26	0.00	14.00	0.00
Peptidyl-prolyl cis-trans isomerase	F1NZW7	25	768427	PPIC	0	2	0	0	5	0	0.13	0.00	15.00	0.00
Polymeric Ig receptor	AAP69598.1	71	419848	PIGR	0	4	0	0	17	0	0.48	0.00	10.00	0.00
Prostaglandin-H2 D-isomerase Precursor	NP_989590.1	21	374110	PTGDS	0	6	0	0	26	0	0.74	0.00	27.00	0.00
Riboflavin-binding protein	P02752.2	25	396449	RTBDN	5	0	0	9	3	0	0.26	17.00	0.00	0.00
Secretory trypsin inhibitor	227660	8	N/A	N/A	0	2	0	0	5	0	0.14	0.00	29.00	0.00
Sulfhydryl oxidase 1	Q8JGM4	83	373914	QSOX1	0	5	0	0	23	0	0.65	0.00	10.00	0.00

Tenascin	F1N8F4	199	396440	TNC	0	11	0	0	28	0	0.78	0.00	8.20	0.00
Tiarin-like	Q25C35	56	418826	OLFM4	0	2	0	0	6	0	0.17	0.00	14.00	0.00
Thioredoxin	P08629	12	396437	TXN	0	13	0	0	85	0	2.38	0.00	29.00	0.00
Trypsin inhibitor ClTI-1	P85000.1	6	770729	SPINK2	0	3	0	0	50	0	1.39	0.00	51.00	0.00
Ubiquitin	NP_001280103.1	41	396190	UBB	0	3	0	0	9	0	0.26	0.00	8.60	0.00
Vacuolar protein sorting 13 homolog D	E1BYT6	492	419481	VPS13D	0	2	0	0	6	0	0.17	0.00	0.36	0.00
Vesicular integral-membrane protein VIP36	XP_003642101.2	38	100859676	LMAN2	0	4	0	0	16	0	0.43	0.00	14.00	0.00
Vitelline membrane outer layer protein 1	NP_001161233.1	20	418974	VMO1	3	0	0	7	0	0	0.13	27.00	0.00	0.00
WAP four-disulfide core domain protein 3 isoform X7	XP_004947252.1	38	419300	WFDC2	0	2	0	0	5	0	0.14	0.00	6.80	0.00
WAP four-disulfide core domain protein 3 isoform X8	XP_004947253.1	31	419300	WFDC2	0	2	0	0	30	0	0.84	0.00	27.00	0.00

[a] Information obtained from Scaffold softwarer, version 4.3.4.

[b] Normalized spectra counts.

[c] Adjusted normalized spectra counts.

[d] Information obtained from PubMed database.

Table S3. List of proteins identified in the eggshell membranes from fertilized eggs at day 3.

Identified Proteins (Day 3 Fertilized)	Acession Number NCBI/UNIPROT	MW (kDa)	Gene ID [d]	Official Gene Symbol [d]	Unique peptides [a]			Normalized spectral counts (NSC) [a,b]			ANSC [a,c]	Percentage Coverage [a] (%)		
					NaCl	Tris	TCEP	NaCl	Tris	TCEP		NaCl	Tris	TCEP
Acidic mammalian chitinase	XP_418051.2	87	419931	CHIA	0	3	0	0	6	0	0.17	0.00	3.60	0.00
A-kinase anchor protein 9 isoform X22	XP_004942619.1	500	424039	PCNT	0	2	0	0	2	0	0.06	0.00	1.00	0.00
Alpha-1-acid glycoprotein	AAT39530.1	22	395220	ORM1	8	4	0	48	9	0	1.16	0.00	0.00	19.00
Alpha-2-macroglobulin-like 1 isoform X3	XP_416480.4	178	418254	A2ML1	2	0	0	2	0	0	0.04	2.00	0.00	0.00
Alpha-amylase	NP_001001528.1	58	414139	AMY1A	0	11	0	0	19	0	0.53	0.00	27.00	0.00
Beta-microseminoprotein-like	XP_004942176.1	12	101750704	LOC101750704	0	0	3	0	0	11	583.00	0.00	25.00	0.00
BPI fold containing family B member 3 precursor	NP_001026032.1	146	419289	BPIFB3	0	0	2	0	0	23	1219.00	0.00	0.00	10.00
Centrosome-associated protein CEP250 isoform X14	XP_004947006.1	330	419138	CEP250	0	0	3	0	0	19	1007.00	0.00	0.00	1.30
Chondrogenesis associated Lipocalin	AAL99254.1	21	374110	PTGDS	2	4	0	2	6	0	0.21	17.00	27.00	0.00
Clusterin	AAD17257.1	51	395722	CLU	0	0	3	0	0	6	318.00	0.00	0.00	7.60
Collagen alpha-1(X) chain isoform X1	F1NRH2	66	100858979	LOC100858979	0	0	19	0	0	90	4770.00	0.00	0.00	20.00
Cochlin	O42163.1	59	395779	COCH	0	2	0	0	8	0	0.22	0.00	6.60	0.00
Collagen alpha-2(V) chain isoform X2	XP_421846.3	34	100006366	COL5A2	0	0	5	0	0	9	477.00	0.00	0.00	6.70
CREMP-like 1	UPI0000ECBE15	33	N/A	N/A	0	0	7	0	0	24	1272.00	0.00	0.00	19.00
CREMP-like 2	UPI0000ECBEAB	35	N/A	N/A	0	4	4	0	11	15	795.31	0.00	17.00	18.00
CREMP-like 3	UPI000044A8C0	35	N/A	N/A	0	0	15	0	0	92	4876.00	0.00	0.00	30.00
Cystatin	P01038	15	396497	CST3	2	2	0	4	4	0	0.19	0.00	20.00	20.00
DNA-dependent protein kinase catalytic subunit	NP_989989.2	473	395376	PRKDC	0	0	3	0	0	3	159.00	0.00	0.00	1.10
Dystrophin	NP_990630.1	1397	396236	DMD	0	0	4	0	0	4	212.00	0.00	0.00	0.42
EGF-like repeats and discoidin I-like domains 3 isoform X3	XP_004949505.1	51	427326	EDIL3	0	2	7	0	2	15	795.06	0.00	7.40	14.00
Erythroid-specific folate Receptor	Q9PW81	31	395638	FOLR1	0	2	0	0	3	0	0.08	0.00	6.60	0.00
Extracellular fatty acid-binding protein precursor	P21760.2	20	396393	LCN8	4	9	0	10	27	0	0.95	23.00	43.00	0.00
Fatty acid synthase	NP_990486.2	274	396061	FASN	0	0	3	0	0	3	159.00	0.00	0.00	1.30
Fibronectin	P11722.3	259	396133	FN1	0	2	0	0	3	0	0.08	0.00	1.40	0.00
Galactocerebrosidase isoform X3	XP_421304.3	73	423394	GALC	0	4	3	0	6	2	106.17	0.00	7.50	4.20
Gallin	CBE70278.1	7	422031	GALN2	0	0	4	0	0	9	477.00	0.00	0.00	46.00
Gastrokine-2	XP_417666.1	17	419515	GKN2	0	2	8	0	3	22	1166.08	0.00	23.00	49.00
GON-4-like protein isoform X2	XP_422861.4	194	425067	GON4L	0	0	2	0	0	3	159.00	0.00	0.00	1.10
Hemopexin	XP_417267.3	29	419076	HPX	4	0	0	7	0	0	0.13	22.00	0.00	0.00
Hep21 protein	BAF62984.1	12	395192	HEP21	0	2	0	0	2	0	0.06	0.00	16.00	0.00
Heterochromatin-associated protein MENT	NP_990228.1	42	395715	SERPINB10	2	0	0	14	0	0	0.27	16.00	0.00	0.00
Histone-lysine N-methyltransferase H3 lysine-36 H4 lysine-20 specific isoform X7	XP_004944950.1	393	416214	NSD1	0	2	0	0	2	0	0.06	0.00	0.47	0.00

Hyaluronan and proteoglycan link protein 1	NP_990813.1	20	396475	HAPLN1	0	0	4	0	0	6	318.00	0.00	0.00	27.00
Ig alpha heavy chain	AAB22614.2	62	N/A	N/A	0	18	0	0	91	0	2.55	0.00	32.00	0.00
IgGFc-binding protein-like	XP_426805.4	22	429249	LOC429249	0	0	5	0	0	10	530.00	0.00	0.00	14.00
Ig heavy chain	AAA48833.1	26	N/A	N/A	0	3	0	0	11	0	0.31	0.00	21.00	0.00
Ig lambda chain	BAB47316.1	11	N/A	N/A	0	2	0	0	3	0	0.08	0.00	17.00	0.00
Ig light chain	AAA48859.1	22	N/A	N/A	0	8	0	0	35	0	0.98	0.00	55.00	0.00
Inner centromere protein-like isoform X6	XP_421068.4	102	423138	LOC423138	0	0	2	0	0	2	106.00	0.00	0.00	2.70
Insulin-like growth factor binding protein 7 precursor	XP_420577.3	29	422620	IGFBP7	0	2	0	0	3	0	0.08	0.00	7.20	0.00
Intraflagellar transport protein 172 homolog isoform X6	XP_004935865.1	220	421983	IFT172	0	0	2	0	0	2	106.00	0.00	0.00	1.30
Lactadherin isoform 2	NP_001264040.1	50	415494	MFGE8	0	2	0	0	3	0	0.08	0.00	5.00	0.00
Laminin alpha-5 chain	XP_004947142.1	359	428148	LAMA5	0	0	3	0	0	3	159.00	0.00	0.00	1.60
Lipocalin 8	P21760.2	20	396393	LCN8	4	9	0	10	27	0	0.95	23.00	43.00	0.00
Lysozyme C	P00698	14	396218	LYZ	17	13	2	473	237	50	2665.62	88.00	81.00	0.00
Lysyl oxidase homolog 2	E1C3U7	82	419533	LOXL2	0	2	50	0	4	230	12190.11	0.00	4.20	47.00
Lysyl oxidase homolog 3	XP_423667.4	101	425986	LOXL3	0	0	2	0	0	2	106.00	0.00	0.00	2.10
Microtubule-associated tumor suppressor candidate 2 isoform X2	XP_417117.2	249	418923	MTUS2	0	0	2	0	0	2	106.00	0.00	0.00	1.00
Mucin-5AC	XP_003641370.2	274	100859916	LOC100859916	0	10	2	0	27	2	106.76	0.00	5.80	0.65
Mucin-5B	Q98UI9	234	395381	LOC395381	6	3	0	10	5	0	0.33	4.30	1.50	0.00
Myb-related protein B	NP_990649.1	91	396258	MYBL2	0	0	2	0	0	2	106.00	0.00	0.00	4.50
Nesprin-1 isoform X6	XP_419679.4	999	421640	SYNE1	0	0	2	0	0	2	106.00	0.00	0.00	0.29
Obscurin	XP_418501.4	1312	420395	OBSCN	0	2	0	0	2	0	0.06	0.00	0.33	0.00
Ovalbumin	P01012	43	396058	OVAL	5	2	0	1714	500	0	46.57	87.00	86.00	0.00
Ovalbumin-related protein X	P01013	56	420898	OVALX	16	7	0	53	12	0	1.34	43.00	25.00	0.00
Ovalbumin-related protein Y	P01014	44	420897	OVALY	22	0	2	172	0	3	162.27	56.00	0.00	6.40
Ovocalyxin-32	Q90YI1	31	395209	RARRES1	0	0	2	0	0	4	212.00	0.00	0.00	16.00
Ovocalyxin-36	Q53HW8	49	419289	BPIFB8	3	7	9	5	14	26	1378.49	3.90	24.00	26.00
Ovocleidin-17	Q9PRS8	15	100313508	OC-17	0	2	5	0	3	12	636.08	0.00	15.00	34.00
Ovocleidin-116	NP_989900.1	77	395256	MEPE	0	14	7	0	28	12	636.78	0.00	27.00	12.00
OvoglobulinG2 type AA (TENP)	NP_990357.1	47	395882	BPIFB7	12	0	0	38	0	0	0.72	53.00	0.00	0.00
Ovoinhibitor	XP_004944964.1	57	416235	SPINK5	19	0	0	51	0	0	0.97	57.00	0.00	0.00
Ovomucoid	P01005	20	416236	SPINK7	86	0	0	1304	0	0	24.78	88.00	0.00	0.00
Ovotransferrin	P02789.2	78	396241	TF	86	2	6	1304	235	41	2204.36	88.00	63.00	28.00
Partia Ig heavy chain variable Region	BAA11105.1	11	N/A	N/A	0	3	0	0	11	0	0.31	0.00	51.00	0.00
Partial Ig lambda chain	BAB47282.1	11	N/A	N/A	0	2	0	0	11	0	0.31	0.00	31.00	0.00
Partial Ig lambda light chain	BAB71934.1	11	N/A	N/A	0	4	0	0	9	0	0.25	0.00	54.00	0.00
Partial Ig light chain variable region	BAE80149.1	12	N/A	N/A	0	2	0	0	10	0	0.28	0.00	29.00	0.00
Pol-like protein	AAA49022.1	131	N/A	N/A	0	0	3	0	0	4	212.00	0.00	0.00	3.60
Polymeric Ig receptor	AAW71994.1	71	419848	PIGR	0	4	0	0	6	0	0.17	0.00	6.50	0.00
6-pyruvoyl tetrahydrobiopterin synthase isoform X7	XP_417928.2	16	374144	PTS	2	0	0	2	0	0	0.04	21.00	0.00	0.00
Radial spoke head protein 4 homolog A	XP_420671.4	73	422717	RSPH4A	0	0	2	0	0	2	106.00	0.00	0.00	6.90
Riboflavin-binding protein	P02752.2	25	396449	RTBDN	6	2	0	28	4	0	0.64	25.00	7.60	0.00

SHC-transforming protein 2 isoform X1	XP_001233682.3	182	770341	SHC2	0	0	2	0	0	2	106.00	0.00	0.00	1.90
Sulfhydryl oxidase 1	Q8JGM4	83	373914	QSOX1	0	14	4	0	26	6	318.73	0.00	29.00	7.90
Tiarin-like	Q25C35	56	418826	OLFM4	0	10	0	0	22	0	0.62	0.00	20.00	0.00
Trinucleotide repeat-containing gene 6A protein isoform X3	XP_004945317.1	187	416570	TNRC6A	2	0	0	2	0	0	0.04	3.50	0.00	0.00
Trypsin inhibitor ClTI-1	P85000.1	9	770729	SPINK2	0	2	0	0	7	0	0.20	0.00	42.00	0.00
Vesicular integral-membrane protein VIP36	XP_003642101.2	38	100859676	LMAN2	0	3	0	0	6	0	0.17	0.00	9.80	0.00
Vitelline membrane outer layer protein 1	NP_001161233.1	18	418974	VMO1	4	0	0	7	0	0	0.13	38.00	0.00	0.00
WAP four-disulfide core domain protein 3 isoform X7	XP_004947252.1	36	419300	WFDC2	0	5	0	0	21	0	0.59	0.00	14.00	0.00
Zinc finger MYM-type protein 2 isoform X2	XP_004938870.1	109	418954	ZMYM2	0	0	2	0	0	6	318.00	0.00	0.00	2.40

[a] Information obtained from Scaffold softwarer, version 4.3.4.
[b] Normalized spectra counts.
[c] Adjusted normalized spectra counts.
[d] Information obtained from PubMed database.

Table S4. List of proteins identified in the eggshell membranes from fertilized eggs at day 7.

Identified Proteins (Day 7 Fertilized)	Acession Number NCBI/UNIPROT	MW (kDa)	Gene ID [d]	Official Gene Symbol [d]	Unique peptides [a]			Normalized spectral counts (NSC) [a,b]			ANSC [a,c]	Percentage Coverage [a] (%)		
					NaCl	Tris	TCEP	NaCl	Tris	TCEP		NaCl	Tris	TCEP
Agrin	AAA48585.1	52	396538	AGRN	0	12	0	0	23	0	0.64	0.00	29.00	0.00
Alpha-1-acid glycoprotein	AAT39530.1	22	395220	ORM1	5	4	0	23	12	0	0.77	30.00	19.00	0.00
Alpha-2-macroglobulin-like 1 isoform X3	XP_416480.4	178	418254	A2ML1	8	0	0	15	0	0	0.29	6.60	0.00	0.00
Alpha-amylase	NP_001001528.1	58	414139	AMY1A	0	4	0	0	4	0	0.11	0.00	9.60	0.00
Avidin	CAC34569.1	17	396260	AVD	6	0	0	16	0	0	0.30	47.00	0.00	0.00
Beta-microseminoprotein-like	XP_004942176.1	12	101750704	LOC101750704	2	0	0	3	0	0	0.06	16.00	0.00	0.00
Chondrogenesis associated Lipocalin	Q8QFM7	21	374110	PTGDS	0	5	0	0	16	0	0.45	0.00	31.00	0.00
Clusterin	AAD17257.1	49	395722	CLU	12	8	3	33	17	6	319.10	34.00	18.00	7.60
Cochlin	O42163	59	395779	COCH	0	2	0	0	3	0	0.08	0.00	6.60	0.00
Collagen alpha-1(X) chain	F1NRH2	66	100858979	LOC100858979	0	0	18	0	0	70	3710.00	0.00	0.00	18.00
Collagen alpha-2(V) chain isoform X2	XP_421846.3	140	423986	COL5A2	0	0	2	0	0	3	159.00	0.00	0.00	1.60
CREMP-like 1	UPI0000ECBE15	33	N/A	N/A	0	0	4	0	0	13	689.00	0.00	0.00	14.00
CREMP-like 3	UPI000044A8C0	34	N/A	N/A	0	4	12	0	10	88	4664.28	0.00	16.00	16.00
Cystatin	P01038	15	396497	CST3	0	2	0	0	12	0	0.34	0.00	20.00	0.00
Diacylglycerol kinase zeta	NP_001026363.1	104	423197	DGKZ	0	2	0	0	2	0	0.06	0.00	3.60	0.00
Dystrophin	NP_990630.1	165	396236	DMD	0	4	0	0	4	0	0.11	0.00	3.60	0.00
EGF-like repeats and discoidin I-like domains 3 isoform X2	XP_424906.3	51	427326	EDIL3	0	0	3	0	0	7	371.00	0.00	0.00	6.10
Extracellular fatty acid binding protein precursor	P21760.2	20	396393	LCN8	2	5	0	3	19	0	0.59	11.00	33.00	0.00
Folate receptor alpha precursor	NP_990165.1	31	395638	FOLR1	0	2	0	0	3	0	0.08	0.00	6.20	0.00
Galactocerebrosidase isoform X3	XP_421304.3	77	423394	GALC	0	2	0	0	3	0	0.08	0.00	3.40	0.00
Gallinacin-11	Q6IV20	12	414876	GAL11	6	4	2	14	6	3	159.43	52.00	33.00	20.00
Gastrokine-2	XP_417666.1	17	419515	GKN2	0	0	4	0	0	14	742.00	0.00	0.00	32.00
Glutathione peroxidase 3 Precursor	NP_001156704.1	19	427638	GPX3	0	0	2	0	0	2	106.00	0.00	0.00	15.00
Hemopexin	XP_417267.3	43	419076	HPX	0	6	0	0	10	0	0.28	0.00	21.00	0.00
Hyaluronan and proteoglycan link protein 1 precursor	NP_990813.1	20	396475	HAPLN1	0	0	2	0	0	2	106.00	0.00	0.00	12.00
la-related protein 4	NP_001012718.1	86	426876	LARP4	0	0	2	0	0	7	371.00	0.00	0.00	4.80
Ig alpha heavy chain	AAB22614.2	62	N/A	N/A	6	19	0	9	37	0	1.21	15.00	35.00	0.00
IgGFc-binding protein-like	XP_426805.4	22	429249	LOC429249	0	0	3	0	0	9	477.00	0.00	0.00	13.00
Ig heavy chain	AAA48833.1	26	N/A	N/A	0	2	0	0	8	0	0.22	0.00	20.00	0.00
Ig heavy chain variable region	CAO79256.1	26	N/A	N/A	0	2	0	0	10	0	0.28	0.00	21.00	0.00
Ig J polypeptide	ENSGALT00000018840	18	N/A	N/A	2	0	0	3	0	0	0.06	18.00	0.00	0.00
Ig lambda chain	BAB47316.1	11	N/A	N/A	0	2	0	0	6	0	0.17	0.00	38.00	0.00
Ig light chain precursor V-J region	A21177	22	N/A	N/A	2	8	0	7	25	0	0.83	17.00	28.00	0.00
Lactadherin isoform 2	NP_001264040.1	59	415494	MFGE8	0	4	0	0	5	0	0.14	0.00	7.30	0.00
Lysozyme C	P00698	14	396218	LYZ	3	2	12	941	345	49	2624.54	92.00	81.00	79.00
Lysyl oxidase homolog 2	E1C3U7	87	419533	LOXL2	0	4	27	0	12	115	6095.34	0.00	6.60	29.00
Meiosis-specific nuclear structural protein 1	XP_423957.4	47	426295	LOC426295	2	0	0	2	0	0	0.04	6.30	0.00	0.00
Mucin-5AC	XP_003641370.2	359	100859916	LOC100859916	0	20	0	0	34	0	0.95	0.00	8.10	0.00
Mucin-5B	Q98UI9	234	395381	LOC395381	9	19	0	13	39	0	1.34	5.80	11.00	0.00
Ovalbumin	P01012	43	396058	OVAL	76	44	12	1500	479	85	4546.91	91.00	88.00	51.00
Ovalbumin-related protein X	P01013	56	420898	OVALX	16	5	0	54	9	0	1.28	44.00	12.00	0.00
Ovalbumin-related protein Y	P01014	44	420897	OVALY	19	8	0	112	21	0	2.72	54.00	31.00	0.00
Ovocalyxin-36	Q53HW8	49	419289	BPIFB8	0	6	11	0	14	33	1749.39	0.00	22.00	28.00

Ovocleidin-17	Q9PRS8	15	100313508	OC-17	3	0	2	4	0	4	212.08	30.00	0.00	15.00
Ovocleidin-116	NP_989900.1	77	395256	MEPE	0	6	5	0	17	8	424.48	0.00	16.00	8.50
OvoglobulinG2 type AA	BAM13272.1	47	395882	BPIFB7	11	2	0	47	2	0	0.95	51.00	8.40	0.00
Ovoinhibitor	XP_004944964.1	101	416235	SPINK5	17	0	0	43	0	0	0.82	24.00	29.00	0.00
Ovomucoid	P01005	20	416236	SPINK7	8	14	0	62	60	0	2.86	59.00	58.00	0.00
Ovostatin	XP_423478.4	166	396151	OVST	9	7	0	16	10	0	0.58	7.30	5.70	0.00
Ovotransferrin	P02789.2	76	396241	TF	60	34	2	559	97	5	278.34	85.00	64.00	3.50
Partial Ig heavy chain variable Region	BAA11105.1	11	N/A	N/A	0	3	0	0	9	0	0.25	0.00	51.00	0.00
Partial Ig light chain variable Region	BAE80149.1	11	N/A	N/A	0	2	0	0	4	0	0.11	0.00	45.00	0.00
Partial ovomucin beta-subunit	BAD22545.1	92	414878	MUC6	2	0	0	4	0	0	0.08	3.30	0.00	0.00
Phosphodiesterase 4D interacting protein	ENSGALT00000019223	245	424382	LOC424382	0	2	0	0	2	0	0.06	0.00	1.90	0.00
Polymeric Ig receptor	AAP69598.1	71	419848	PIGR	0	5	0	0	6	0	0.17	0.00	8.60	0.00
Protein piccolo	XP_001231455.2	594	395319	PCLO	0	4	2	0	4	2	106.11	0.00	1.20	0.53
Riboflavin-binding protein	P02752.2	29	396449	RTBDN	4	3	0	16	6	0	0.47	18.00	14.00	0.00
SNF-related serine/threonine-protein kinase	NP_001186631.1	82	420675	SNRK	0	2	0	0	2	0	0.06	0.00	5.00	0.00
Sulfhydryl oxidase 1	Q8JGM4	83	373914	QSOX1	0	8	2	0	12	2	106.34	0.00	14.00	3.90
Syntaphilin	XP_003643281.2	28	100858058	SNPH	0	0	2	0	0	2	106.00	0.00	0.00	8.70
Tenascin	F1N8F4	199	396440	TNC	0	9	0	0	11	0	0.31	0.00	7.20	0.00
Tiarin-like	Q25C35	56	418826	OLFM4	0	17	0	0	47	0	1.32	0.00	33.00	0.00
Vesicular integral-membrane protein VIP36	XP_003642101.2	38	100859676	LMAN2	0	2	0	0	2	0	0.06	0.00	6.10	0.00
Vitelline membrane outer layer protein 1	NP_001161233.1	21	418974	VMO1	7	0	0	19	0	0	0.36	42.00	0.00	0.00
Vitellogenin	AAA49139.1	211	424533	VTG2	2	0	0	2	0	0	0.04	1.50	0.00	0.00
Vitellogenin-2	NP_001026447.1	205	424533	VTG2	11	0	0	14	0	0	0.27	7.60	0.00	0.00
WAP four-disulfide core domain protein 3 isoform X7	XP_004947252.1	36	419300	WFDC2	0	3	0	0	6	0	0.17	0.00	12.00	0.00
WAP four-disulfide core domain protein 3 isoform X8	XP_004947253.1	36	419300	WFDC2	0	2	0	0	3	0	0.08	0.00	7.20	0.00

[a] Information obtained from Scaffold softwarer, version 4.3.4.

[b] Normalized spectra counts.

[c] Adjusted normalized spectra counts.

[d] Information obtained from PubMed database.

Table S5. List of proteins identified in the eggshell membranes from fertilized eggs at day 11.

Identified Proteins (Day 11 Fertilized)	Acession Number NCBI/UNIPROT	MW (kDa)	Gene ID [d]	Official Gene Symbol [d]	Unique peptides [a]			Normalized spectral counts (NSC) [a,b]			ANSC [a,c]	Percentage Coverage [a] (%)		
					NaCl	Tris	TCEP	NaCl	Tris	TCEP		NaCl	Tris	TCEP
Actin (cytoplasmic type 5)	P53478	42	415296	ACTG1	2	0	0	2	0	0	0.04	7.70	0.00	0.00
Agrin	UPI00003AAAEC	52	396538	AGRN	4	0	0	7	0	0	0.13	7.80	0.00	0.00
Alpha-1-acid glycoprotein	AAT39530.1	22	395220	ORM1	3	5	0	7	17	0	0.61	13.00	30.00	0.00
Alpha-2-macroglobulin-like 1 isoform X3	XP_416480.4	168	418254	A2ML1	6	0	0	10	0	0	0.19	5.60	0.00	0.00
Alpha-amylase	NP_001001528.1	58	414139	AMY1A	0	2	0	0	2	0	0.06	0.00	4.50	0.00
Alternatively spliced tenascin 190, 200 and 230 kd variants	Q90995	189	396440	TNC	0	2	0	0	4	0	0.11	0.00	1.30	0.00
Annexin A2	NP_990682.1	39	396297	ANXA2	5	0	0	10	0	0	0.19	18.00	0.00	0.00
Apolipoprotein A-I	P08250	31	396536	APOA1	0	8	0	0	16	0	0.45	0.00	31.00	0.00
Avidin	CAC34569.1	17	396260	AVD	12	0	0	29	0	0	0.55	53.00	0.00	0.00
Beta-H globin	Q90864	16	428114	HBE1	4	0	0	6	0	0	0.11	31.00	0.00	0.00
Beta-microseminoprotein-like	XP_004942176.1	12	101750704	LOC101750704	0	2	0	0	4	0	0.11	0.00	18.00	0.00
Chondrogenesis associated Lipocalin	AAL99254.1	21	374110	PTGDS	0	4	0	0	7	0	0.20	0.00	28.00	0.00
Clusterin	AAD17257.1	49	395722	CLU	2	6	2	3	8	3	159.28	4.50	14.00	4.70
Collagen alpha-1(X) chain	F1NRH2	66	100858979	LOC100858979	0	3	14	0	5	61	3233.14	0.00	4.40	16.00
Collagen alpha-2(I) chain	P02467.2	133	396243	COL1A2	0	0	2	0	0	3	159.00	0.00	0.00	2.80
CREMP	XP_001236415	33	776923	CREMP	0	0	6	0	0	18	954.00	0.00	0.00	15.00
CREMP-like 2	UPI0000ECBEAB	35	N/A	N/A	0	2	3	0	6	10	530.17	0.00	8.40	7.80
CREMP-like 3	UPI000044A8C0	34	N/A	N/A	0	2	8	0	6	84	4452.17	0.00	8.40	17.00
Cystatin	P01038	15	396497	CST3	3	6	0	7	19	0	0.67	28.00	40.00	0.00
Dystonin isoform X3	XP_419901.4	847	421884	DST	0	0	2	0	0	2	106.00	0.00	0.00	0.44
EGF-like repeats and discoidin I-like domains 3 isoform X3	XP_004949505.1	54	427326	EDIL3	0	5	0	0	10	0	0.28	0.00	10.00	0.00
Extracellular fatty acid-binding Protein	P21760.2	20	396393	LCN8	4	6	0	4	22	0	0.69	17.00	35.00	0.00
Fibronectin	P11722.3	259	396133	FN1	0	3	0	0	6	0	0.17	0.00	1.60	0.00
Galactocerebrosidase isoform X3	XP_421304.3	77	423394	GALC	0	5	0	0	8	0	0.22	0.00	13.00	0.00
Gallin protein	CBE70278.1	5	422031	GALN2	0	0	2	0	0	5	265.00	0.00	0.00	26.00
Gallinacin-11	NP_001001779.1	12	414876	GAL11	3	6	3	5	11	2	106.40	38.00	48.00	32.00
Gastrokine-2	XP_417666.1	17	419515	GKN2	0	0	6	0	0	12	636.00	0.00	0.00	45.00
Gastric intrinsic factor isoform X2	XP_004941337.1	44	770547	GIF	2	0	0	2	0	0	0.04	9.70	0.00	0.00
Glutathione peroxidase 3	NP_001156704.1	19	427638	GPX3	0	2	0	0	3	0	0.08	0.00	12.00	0.00
Hemoglobin subunit alpha-A	P01994	15	416652	HBAA	2	0	0	2	0	0	0.04	25.00	0.00	0.00
Hemoglobin subunit epsilon	NP_001075173.1	17	396485	HBE	2	0	0	7	0	0	0.13	35.00	0.00	0.00
Hemopexin	XP_417267.3	43	419076	HPX	0	7	0	0	11	0	0.31	0.00	23.00	0.00
Hyaluronan and proteoglycan link protein 1 precursor	NP_990813.1	41	396475	HAPLN1	0	0	6	0	0	7	371.00	0.00	0.00	76.00
Hypothetical protein RCJMB04_3p21	CAG31238.1	22	421932	YWHAQ	0	2	0	0	2	0	0.06	0.00	13.00	0.00
Ia-related protein 4	NP_001012718.1	17	426876	LARP4	0	0	6	0	0	12	636.00	0.00	0.00	45.00
Ig alpha heavy chain	AAB22614.2	62	N/A	N/A	4	0	0	12	0	0	0.23	14.00	0.00	0.00
Ig heavy chain	AAA48833.1	26	N/A	N/A	0	2	0	0	10	0	0.28	0.00	18.00	0.00
Ig heavy chain variable region	CAO79256.1	26	N/A	N/A	0	2	0	0	10	0	0.28	0.00	18.00	0.00
IgGFc-binding protein-like	XP_426805.4	22	429249	LOC429249	0	0	2	0	0	3	159.00	0.00	0.00	10.00
Ig lambda light chain VLJ region	UPI00003AB0E1	24	N/A	N/A	2	0	0	9	0	0	0.17	16.00	0.00	0.00
Ig light chain	A21177	25	N/A	N/A	0	6	0	0	37	0	1.04	0.00	46.00	0.00
Ig mu chain C region	P01875	48	101748478	LOC101748478	0	3	0	0	6	0	0.17	0.00	11.00	0.00

Lactadherin	UPI00003AA422	51	415494	MFGE8	0	11	0	0	16	0	0.45	0.00	22.00	0.00
Lysozyme C	P00698	14	396218	LYZ	12	11	11	1130	163	46	2464.03	92.00	40.00	76.00
Lysyl oxidase homolog 2	E1C3U7	87	419533	LOXL2	0	7	0	0	18	0	0.50	0.00	19.00	0.00
Mesothelin	XP_001234087.3	82	770761	MSLNL	0	2	0	0	6	0	0.17	0.00	8.10	0.00
Metalloproteinase inhibitor 3	P26652	25	396483	TIMP3	2	0	0	2	0	0	0.04	15.00	0.00	0.00
Mucin-5AC	XP_003641370.2	383	100859916	LOC100859916	6	21	0	9	46	0	1.46	3.30	8.60	0.00
Mucin-5B	Q98UI9	234	395381	LOC395381	4	8	0	6	14	0	0.51	2.60	4.40	0.00
Ovalbumin	P01012.2	43	396058	OVAL	30	88	15	354	1631	43	2331.39	88.00	96.00	56.00
Ovalbumin-related protein X	P01013.1	45	420898	OVALX	15	7	0	30	17	0	1.05	34.00	16.00	0.00
Ovalbumin-related protein Y	P01014.1	44	420897	OVALY	10	15	0	24	59	0	2.11	36.00	50.00	0.00
Ovocalyxin-32	Q90YI1	31	395209	RARRES1	0	0	3	0	0	4	212.00	0.00	0.00	20.00
Ovocalyxin-36	Q53HW8	49	419289	BPIFB8	0	15	9	0	45	23	1220.26	0.00	36.00	23.00
Ovocleidin-17	Q9PRS8	15	100313508	OC-17	0	0	3	0	0	12	636.00	0.00	0.00	20.00
Ovocleidin-116	NP_989900.1	81	395256	MEPE	0	0	5	0	0	8	424.00	0.00	0.00	26.00
OvoglobulinG2 type AA (TENP)	NP_990357.1	47	395882	BPIFB7	9	8	2	14	15	2	106.69	7.70	26.00	8.60
Ovoinhibitor	P10184.2	52	416235	SPINK5	0	6	0	0	14	0	0.39	0.00	19.00	0.00
Ovomucoid	P01005	23	416236	SPINK7	3	6	0	15	20	0	0.85	35.00	40.00	0.00
Ovostatin	XP_423478.4	166	396151	OVST	0	11	0	0	17	0	0.48	0.00	8.10	0.00
Ovotransferrin	P02789.2	76	396241	TF	33	34	0	68	68	0	3.20	54.00	4.40	0.00
Partial anti-prion protein immunoglobulin light chain variable region	BAE20399.1	11	N/A	N/A	0	2	0	0	6	0	0.17	0.00	4.40	0.00
Partial Ig heavy chain variable region	BAA11105.1	13	N/A	N/A	0	2	0	0	5	0	0.14	0.00	27.00	0.00
Partial Ig lambda chain	BAB47282.1	11	N/A	N/A	0	3	0	0	5	0	0.14	0.00	31.00	0.00
Partial Ig lambda light chain	BAB71907.1	11	N/A	N/A	0	2	0	0	6	0	0.17	0.00	47.00	0.00
Partial Ig light chain variable region	BAE80142.1	13	N/A	N/A	0	2	0	0	14	0	0.39	0.00	37.00	0.00
Polymeric Ig receptor	AAP69598.1	71	419848	PIGR	0	8	0	0	15	0	0.42	0.00	13.00	0.00
Polyubiquitin-C-like isoform X2	XP_004946720.1	41	101747587	LOC101747587	0	2	0	0	3	0	0.08	0.00	8.20	0.00
Protein Jade-3 isoform X2	XP_416870.4	92	418674	PHF16	0	2	0	0	2	0	0.06	0.00	2.80	0.00
Protein S100-A11	P24479	11	396075	S100A11	2	0	0	2	0	0	0.04	23.00	0.00	0.00
Protein Smaug homolog 2 isoform X2	UPI00020276DB	74	N/A	N/A	0	0	2	0	0	2	106.00	0.00	0.00	4.70
Quiescence-specific protein	AAA53371.1	20	396393	LCN8	3	6	0	5	22	0	0.71	67.00	35.00	0.00
Rho guanine nucleotide exchange factor 10 isoform X1	XP_001235643.3	179	421900	ARHGEF10	0	0	3	0	0	3	159.00	0.00	0.00	2.00
Riboflavin-binding protein	P02752.2	25	396449	RTBDN	2	2	0	7	6	0	0.30	9.10	8.00	0.00
Sulfhydryl oxidase 1	Q8JGM4	83	373914	QSOX1	0	16	2	0	37	3	160.04	0.00	29.00	3.90
Tiarin-like	Q25C35	56	418826	OLFM4	0	17	0	0	47	0	1.32	0.00	32.00	0.00
Transmembrane protease serine 9 isoform X3	XP_425880.4	117	428320	TMPRSS9	4	0	0	9	0	0	0.17	6.80	0.00	0.00
Transmembrane protease serine 9 isoform X4	XP_004948709.1	170	428320	TMPRSS9	0	6	0	0	8	0	0.22	0.00	6.20	0.00

Vesicular integral-membrane protein VIP36	XP_003642101.2	38	100859676	LMAN2	0	2	0	0	2	0	0.06	0.00	6.10	0.00
Vitelline membrane outer layer protein 1	NP_001161233.1	20	418974	VMO1	16	0	0	34	0	0	0.65	67.00	0.00	0.00
Vitellogenin	AAA49139.1	31	424533	VTG2	0	2	0	0	4	0	0.11	0.00	7.70	0.00
WAP four dissulfide domain protein 3 isoform 7	XP_004947250.1	36	419300	WFDC2	0	3	0	0	7	0	0.20	0.00	12.00	0.00
Zona pellucida C	AAV35182.1	48	378906	ZP3	6	0	0	9	0	0	0.17	16.00	0.00	0.00
Zona pellucida glycoprotein 1	H9L0C5	100	395418	ZP1	3	2	0	4	4	0	0.19	4.60	3.10	0.00
Zona pellucida sperm-binding protein 3	NP_989720.3	48	378906	ZP3	0	3	0	0	5	0	0.14	0.00	9.90	0.00

[a] Information obtained from Scaffold softwarer, version 4.3.4.

[b] Normalized spectra counts.

[c] Adjusted normalized spectra counts.

[d] Information obtained from PubMed database.

Table S6. List of proteins identified in the eggshell membranes from fertilized eggs at day 15.

Identified Proteins (Day 15 Fertilized)	Acession Number NCBI/UNIPROT	MW (kDa)	Gene ID [d]	Official Gene Symbol [d]	Unique peptides [a]			Normalized spectral counts NSC [a,b]			ANSC [a,c]	Percentage Coverage [a] (%)		
					NaCl	Tris	TCEP	NaCl	Tris	TCEP		NaCl	Tris	TCEP
Alpha-amylase	NP_001001528.1	58	414139	AMY1A	0	6	0	0	7	0	0.20	0.00	15.00	0.00
Alpha-1-acid glycoprotein	AAT39530.1	22	395220	ORM1	3	5	0	4	30	0	0.92	13.00	30.00	0.00
Alpha-2-macroglobulin-like 1 isoform X3	XP_416480.4	165	418254	A2ML1	0	5	0	0	5	0	0.14	0.00	4.70	0.00
Alternatively spliced tenascin 190 200 and 230 kd variants	Q90995	189	396440	TNC	0	6	0	0	9	0	0.25	0.00	4.80	0.00
Annexin A2	NP_990682.1	39	396297	ANXA2	4	0	0	7	0	0	0.13	12.00	0.00	0.00
Apolipoprotein A-I	P08250	31	396536	APOA1	0	3	0	0	6	0	0.17	0.00	13.00	0.00
Avidin	CAC34569.1	17	396260	AVD	5	0	0	17	0	0	0.32	24.00	0.00	0.00
Basement membrane-specific heparan sulfate proteoglycan core protein heparan sulfate proteoglycan core protein	Q6KDZ1	433	429806	HSPG2	0	7	0	0	8	0	0.22	0.00	2.60	0.00
Beta-actin	CAA25004.1	42	396526	ACTB	2	0	0	3	0	0	0.06	7.50	0.00	0.00
Beta-microseminoprotein-like	XP_004942176.1	12	101750704	LOC101750704	0	3	2	0	8	6	318.22	0.00	31.00	16.00
BPI fold-containing family C protein isoform X2	XP_004937862.1	103	771461	BPIFCB	0	5	0	0	7	0	0.20	0.00	5.60	0.00
Cadherin-1	E1C6M9	98	415860	CDH1	0	3	0	0	4	0	0.11	0.00	6.80	0.00
Caldesmon	NP_989489.1	86	373965	CALD1	6	0	0	12	0	0	0.23	12.00	0.00	0.00
Cathepsin B	AAA87075.1	38	396329	CTSB	0	2	0	0	2	0	0.06	0.00	8.50	0.00
Chondrogenesis associated Lipocalin	AAL99254.1	21	374110	PTGDS	0	8	0	0	15	0	0.42	0.00	50.00	0.00
Clusterin	AAD17257.1	49	395722	CLU	0	6	2	0	11	2	106.31	0.00	16.00	4.90
Collagen alpha 1 (X)	F1NRH2	66	100858979	LOC100858979	0	5	15	0	9	61	3233.25	0.00	6.50	17.00
CREMP-like 2	UPI0000ECBEAB	34	N/A	N/A	0	2	0	0	9	0	0.25	0.00	8.40	0.00
CREMP-like 3	UPI000044A8C0	34	N/A	N/A	0	2	7	0	9	84	4452.25	0.00	8.40	18.00
Cystatin	P01038	15	396497	CST3	0	8	0	0	46	0	1.29	0.00	53.00	0.00
Cytochrome c	P67881	12	420624	CYCS	2	0	0	3	0	0	0.06	23.00	0.00	0.00
EGF-like repeats and discoidin I -like domains 3 isoform X3	XP_004949505.1	54	427326	EDIL3	0	8	5	0	11	9	477.31	0.00	18.00	9.60
Extracellular fatty acid-binding Protein	P21760.2	20	396393	LCN8	0	0	2	0	0	3	159.00	0.00	0.00	13.00
Fibronectin	P11722.3	259	396133	FN1	0	16	0	0	30	0	0.84	0.00	9.10	0.00
Galactosylceramidase	UPI00004484A5	80	423394	GALC	0	3	0	0	4	0	0.11	0.00	6.50	0.00
Gallinacin-11	Q6IV20	12	414876	GAL11	0	5	2	0	12	4	212.34	0.00	46.00	20.00
Gastrokine 2	XP_417666.1	17	419515	GKN2	0	2	0	0	4	0	0.11	0.00	19.00	0.00
Glutathione peroxidase 3 Precursor	NP_001156704.1	19	427638	GPX3	0	3	0	0	6	0	0.17	0.00	18.00	0.00
HEMCAM	CAA70079.1	56	448832	MCAM	0	3	0	0	5	0	0.14	0.00	6.50	0.00
Hemoglobin subunit alpha-A	P01994	15	416652	HBAA	0	6	0	0	10	0	0.28	0.00	39.00	0.00
Hemoglobin subunit beta	NP_990820.1	17	396485	HBG2	0	5	0	0	8	0	0.22	0.00	46.00	0.00
Hemopexin	XP_417267.3	43	419076	HPX	0	12	0	0	22	0	0.62	0.00	37.00	0.00
High mobility group protein B1	Q9YH06	25	395724	HMGB1	4	0	0	5	0	0	0.10	17.00	0.00	0.00
Histone H1.11L	P08287	23	427892	HIST1H111L	8	0	0	18	0	0	0.34	27.00	0.00	0.00
Histone H3.2 like	NP_001268409.1	82	769852	LOC769852	4	0	0	5	0	0	0.10	5.00	0.00	0.00
Hyaluronan and proteoglycan link protein 1 precursor	NP_990813.1	41	396475	HAPLN1	0	0	2	0	0	4	212.00	0.00	0.00	6.10
la-related protein 4	NP_001012718.1	17	426876	LARP4	0	2	5	0	4	17	901.11	0.00	19.00	41.00
Ig alpha heavy chain	AAB22614.2	62	N/A	N/A	0	20	0	0	211	0	5.91	0.00	35.00	0.00
IgGFc-binding protein-like	XP_426805.4	22	429249	LOC429249	0	0	2	0	0	6	318.00	0.00	0.00	7.40
Ig heavy chain variable region, partial	UPI0000449E4D	51	N/A	N/A	0	2	0	0	7	0	0.20	0.00	9.50	0.00
Ig light chain	AAA48859.1	22	N/A	N/A	0	11	0	0	156	0	4.37	0.00	62.00	0.00

Ig mu chain C region	P01875	48	101748478	LOC101748478	0	5	0	0	14	0	0.39	0.00	17.00	0.00
Lactadherin	UPI00003AA422	51	415494	MFGE8	0	11	0	0	20	0	0.56	0.00	22.00	0.00
Lamin-A	P13648	73	396224	LOC396224	3	0	0	6	0	0	0.11	5.00	0.00	0.00
Lamin-B2	NP_990616.1	68	396222	LMNB2	3	0	0	6	0	0	0.11	6.20	0.00	0.00
Laminin gamma-1 chain precursor	AAK55397.1	162	424442	LAMC1	0	6	0	0	9	0	0.25	0.00	6.40	0.00
Laminin subunit alpha-5 isoform X7	XP_004947146.1	380	428148	LAMA5	0	3	0	0	4	0	0.11	0.00	0.93	0.00
Laminin subunit beta-1	XP_415943.3	234	396478	LAMB1	0	8	0	0	12	0	0.34	0.00	4.80	0.00
Lysozyme C	P00698	14	396218	LYZ	28	11	3	423	104	41	2183.95	88.00	86.00	74.00
Lysyl oxidase homolog 2	E1C3U7	87	419533	LOXL2	6	11	33	11	34	173	9170.16	12.00	16.00	47.00
Melanotransferrin	Q92062	81	396127	MFI2	0	2	0	0	2	0	0.06	0.00	5.30	0.00
Mesothelin-like protein-like	XP_001234087.3	82	770011	LOC770011	0	5	0	0	19	0	0.53	0.00	7.00	0.00
Mucin-5AC	XP_003641370.2	383	100859916	LOC100859916	0	52	2	0	136	2	109.81	0.00	19.00	1.20
Mucin-5B	Q98UI9	234	395381	LOC395381	0	24	0	0	47	0	1.32	0.00	13.00	0.00
Mucin-6	XP_426405.4	159	414878	MUC6	0	2	0	0	3	0	0.08	0.00	1.30	0.00
Myosin regulatory light chain 2	R0KEE7	20	416874	MYL2	2	0	0	4	0	0	0.08	12.00	0.00	0.00
Neuroserpin	Q90935	47	425002	SERPINI1	0	2	0	0	3	0	0.08	0.00	6.10	0.00
Nidogen-1	XP_419556.3	137	395531	NID1	0	5	0	0	7	0	0.20	0.00	5.60	0.00
Nidogen 2	XP_421471.4	131	423583	NID2	0	3	0	0	4	0	0.11	0.00	3.40	0.00
Ovalbumin	P01012.2	43	396058	OVAL	16	82	15	112	1516	121	6457.58	59.00	90.00	56.00
Ovalbumin-related protein X	P01013.1	45	420898	OVALX	0	12	0	0	26	0	0.73	0.00	30.00	0.00
Ovalbumin-related Y	P01014.1	44	420897	OVALY	0	15	2	0	51	3	160.43	0.00	50.00	6.30
Ovocalyxin-32	Q90YI1	31	395209	RARRES1	0	0	2	0	0	4	212.00	0.00	0.00	9.80
Ovocalyxin-36	Q53HW8	49	419289	BPIFB8	0	10	9	0	32	21	1113.90	0.00	28.00	27.00
Ovocleidin-17	Q9PRS8	15	100313508	OC-17	2	0	2	4	0	0	0.08	13.00	0.00	15.00
Ovocleidin-116	NP_9899001.1	77	395256	MEPE	0	3	4	0	6	10	530.17	0.00	6.20	8.10
OvoglobulinG2 type AA (TENP)	NP_990357.1	47	395882	BPIFB7	0	7	0	0	14	0	0.39	0.00	24.00	0.00
Ovoinhibitor	P10184.2	52	416235	SPINK5	0	17	0	0	36	0	1.01	0.00	37.00	0.00
Ovomucoid	P01005	20	416236	SPINK7	2	11	0	3	48	0	1.40	39.00	58.00	0.00
Ovostatin	XP_423478.4	185	396151	OVST	0	16	0	0	27	0	0.76	0.00	13.00	0.00
Ovotransferrin	P02789.2	78	396241	TF	14	43	0	22	143	0	4.42	28.00	63.00	0.00
Pantetheinase precursor	NP_001034377.1	58	421702	VNN1	0	2	0	0	2	0	0.06	0.00	6.30	0.00
Partial Ig heavy variable region	BAA11105.1	11	N/A	N/A	0	3	0	0	14	0	0.39	0.00	51.00	0.00
Partial Ig lambda chain	BAB47279.1	11	N/A	N/A	0	3	0	0	6	0	0.17	0.00	30.00	0.00
Partial Ig lambda light chain	BAB71903.1	11	N/A	N/A	0	2	0	0	10	0	0.28	0.00	48.00	0.00
Partial Ig light chain variable region	BAE80149.1	12	N/A	N/A	0	2	0	0	17	0	0.48	0.00	29.00	0.00
Peroxiredoxin-1	P0CB50	22	424598	PRDX1	0	7	0	0	11	0	0.31	0.00	48.00	0.00
Pleckstrin homology domain-containing family G member 3	XP_004941317.1	119	101666690	PLEKHG3	2	0	0	2	0	0	0.04	2.40	0.00	0.00
Pleiotrophin	P32760	15	418125	PTN	2	0	0	3	0	0	0.06	13.00	0.00	0.00
Polymeric Ig receptor	AAP69598.1	71	419848	PIGR	0	12	0	0	28	0	0.78	0.00	19.00	0.00
Polyubiquitin-C-like isoform X2	XP_004946720.1	41	101747587	LOC101747587	2	0	0	3	0	0	0.06	9.50	0.00	0.00
Profilin	Q5ZL50	15	771904	PFN2	0	2	0	0	2	0	0.06	0.00	31.00	0.00
14-3-3 Protein theta	CAG31238.1	22	421932	YWHAQ	0	2	0	0	4	0	0.11	0.00	13.00	0.00
Protocadherin Fat 1 isoform X11	XP_004936051.1	32	395168	FAT1	0	0	3	0	0	6	318.00	0.00	0.00	10.00
Quiescence-specific protein	AAA53371.1	20	396393	LCN8	0	12	0	0	59	0	1.65	0.00	60.00	0.00
Receptor-type tyrosine-protein phosphatase F isoform X5	XP_004936750.1	212	424568	PTPRF	0	2	0	0	2	0	0.06	0.00	1.30	0.00
Riboflavin-binding protein	P02752	25	396449	RTBDN	2	2	0	4	6	0	0.24	9.10	8.00	0.00
Sulfhydryl oxidase 1	Q8JGM4	83	373914	QSOX1	0	20	3	0	69	6	319.93	0.00	31.00	5.90
Tiarin-like	Q25C35	56	418826	OLFM4	0	13	0	0	31	0	0.87	0.00	29.00	0.00
Transmembrane protease serine 9 isoform X3	XP_425880.4	114	428320	TMPRSS9	0	19	0	0	30	0	0.84	0.00	25.00	0.00

Transthyretin	P27731	16	396277	TTR	0	2	0	0	3	0	0.08	0.00	23.00	0.00
Tropomyosin alpha-3 chain	NP_001232856.1	29	770103	TPM3	0	2	0	0	3	0	0.08	0.00	9.70	0.00
Vitelline membrane outer layer protein 1	NP_001161233.1	20	418974	VMO1	2	0	0	3	0	0	0.06	18.00	0.00	0.00
Vesicular integral-membrane protein VIP36	XP_003642101.2	38	100859676	LMAN2	0	2	0	0	3	0	0.08	0.00	6.40	0.00
WAP four dissulfide domain protein 3 isoform X7	XP_004947252.1	36	419300	WFDC2	0	5	0	0	13	0	0.36	0.00	18.00	0.00
Zona pellucida C protein	AAV35182.1	48	378906	ZP3	0	8	0	0	16	0	0.45	0.00	21.00	0.00
Zona pellucida glycoprotein 1	Q9DER4	100	395418	ZP1	0	7	0	0	13	0	0.36	0.00	13.00	0.00

[a] Information obtained from Scaffold softwarer, version 4.3.4.

[b] Normalized spectra counts.

[c] Adjusted normalized spectra counts.

[d] Information obtained from PubMed database.

Table S7. List of proteins identified in the eggshell membranes from fertilized eggs at day 19.

Identified Proteins (Day 19 Fertilized)	Acession Number NCBI/UNIPROT	MW (kDa)	Gene ID [d]	Official Gene Symbol [d]	Unique peptides [a]			Normalized spectral counts NSC [a,b]			ANSC [a,c]	Percentage Coverage [a] (%)		
					NaCl	Tris	TCEP	NaCl	Tris	TCEP		NaCl	Tris	TCEP
Actin (cytoplasmic type 5)	NP_001007825.1	42	415296	ACTG1	7	0	0	8	0	0	0.15	29.00	0.00	0.00
Alpha-1-acid glycoprotein	AAT39530.1	22	395220	ORM1	5	3	2	11	27	7	371.97	20.00	13.00	14.00
Alpha-2-antiplasmin isoform X1	XP_003642447.1	52	100857105	SERPINF2	2	0	0	4	0	0	0.08	3.90	2.40	0.00
Alpha-2-macroglobulin-like 1	XP_416480.4	165	418254	A2ML1	30	4	0	37	11	0	1.00	2.50	2.90	0.00
Alpha-fetoprotein	XP_003641248.2	71	422652	AFP	4	0	0	2	0	0	0.04	11.00	0.00	0.00
Anchorin CII	AAB39917.1	37	428767	ANXA5	3	0	0	2	0	0	0.03	9.10	0.00	0.00
Annexin A2	NP_990682.1	39	396297	ANXA2	11	0	0	11	0	0	0.22	40.00	0.00	0.00
Annexin A8	E1C8K3	37	423774	ANXA8	4	0	0	2	0	0	0.04	15.00	0.00	0.00
Apolipoprotein A-I	AAA48597.1	31	396536	APOA1	2	10	0	2	33	0	0.96	9.50	36.00	0.00
Apolipoprotein AIV	CAA76273.1	41	395780	APOA4	8	0	0	4	0	0	0.09	28.00	0.00	0.00
Apolipoprotein B	ABF70173.1	523	396535	APOB	49	0	0	38	0	0	0.72	11.00	0.00	0.00
Astacin-like metalloendopeptidase	P0DJJ2	46	423176	ASTL	4	0	0	2	0	0	0.03	13.00	0.00	0.00
Avidin	P02701	17	396260	AVD	8	0	0	11	0	0	0.21	55.00	0.00	0.00
Beta-H globin	Q90864	16	428114	HBE1	3	0	0	12	0	0	0.24	44.00	0.00	0.00
Clusterin	AAD17257.1	51	395722	CLU	7	10	2	8	68	7	373.05	17.00	23.00	4.90
CREMP	UPI0000E8213B	33	776923	CREMP	0	0	3	0	0	106	5618.00	0.00	0.00	12.00
Cystatin	P01038	15	396497	CST3	2	2	2	3	11	12	636.37	20.00	20.00	14.00
Dickkopf-related protein 3	NP_990456.1	39	396023	DKK3	4	0	0	4	0	0	0.07	11.00	0.00	0.00
EGF-like repeats and discoidin I-like domains 3 isoform X2	XP_424906.3	54	427326	EDIL3	4	2	4	3	11	25	1325.36	9.40	5.40	8.10
Fibronectin	P11722.3	259	396133	FN1	3	0	0	2	0	0	0.03	1.70	0.00	0.00
Gallinacin-11	Q6IV20	12	414876	GAL11	0	0	3	0	0	16.12	854.31	0.00	0.00	22.00
Gastrokine 2 (OCX-21)	XP_417666.1	21	419515	GKN2	0	0	3	0	0	51	2703.00	0.00	0.00	13.00
Hemoglobin subunit alpha-A	P01994	15	416652	HBAA	6	3	0	9	18	0	0.68	46.00	22.00	0.00
Hemoglobin subunit alpha-D	P02001	16	416651	HBAD	4	0	0	4	0	0	0.08	48.00	0.00	0.00
Hemoglobin subunit beta	NP_990820.1	16	396485	HBG2	7	4	0	13	29	0	1.06	52.00	29.00	0.00
Hemopexin	XP_417267.3	45	419076	HPX	2	0	0	4	0	0	0.08	6.40	0.00	0.00
Histone H1.01	NP_001035732.1	22	417954	HIST1H101	3	0	0	2	0	0	0.05	13.00	0.00	0.00
Ig alpha heavy chain	AAB22614.2	62	N/A	N/A	10	8	0	9	60	0	1.86	17.00	16.00	0.00
Ig mu chain C region	P01875	48	101748478	LOC101748478	3	0	0	4	0	0	0.07	12.00	0.00	0.00
Lipocalin 8	P21760.2	20	396393	LCN8	4	5	0	6	35	0	1.09	26.00	19.00	0.00
Lactadherin isoform 2 precursor	NP_001264040.1	59	415494	MFGE8	0	4	2	0	13	5	265.36	0.00	7.70	3.90
Lysozyme C	P00698	16	396218	LYZ	37	4	14	356	64	309	16385.56	82.00	33.00	67.00
Lysyl oxidase homolog 2	E1C3U7	87	419533	LOXL2	0	2	29	0	5	530	28090.14	0.00	3.50	27.00
Mesothelin-like protein-like	XP_001234087.3	78	770761	MSLNL	4	3	0	3	24	0	0.73	5.70	5.50	0.00
Mucin-5AC	XP_003641370.2	383	100859916	LOC100859916	0	2	0	0	9	0	0.25	0.00	0.55	0.00
Mucin-5B	Q98UI9.1	234	395381	LOC395381	32	11	0	50	49	0	2.33	19.00	5.30	0.00
Mucin 6	XP_426405.4	294	414878	MUC6	13	0	0	13	0	0	0.26	5.60	0.00	0.00
Ovalbumin	P01012.2	43	396058	OVAL	44	53	13	231	576	304	16132.52	74.00	66.00	44.00
Ovalbumin-related protein X	P01013.1	45	420898	OVALX	11	6	0	22	31	0	1.28	24.00	20.00	0.00
Ovalbumin-related protein Y	P01014.1	44	420897	OVALY	10	7	0	16	37	0	1.34	29.00	16.00	0.00
Ovocalyxin-32	Q90YI1	31	395209	RARRES1	0	0	3	0	0	62	3295.28	0.00	0.00	16.00
Ovocalyxin-36	Q53HW8	58	419289	BPIFB8	0	3	5	0	15	41	2197.27	0.00	7.10	13.00
Ovocleidin-17	Q9PRS8	15	100313508	OC-17	10	0	5	16	0	64	3392.30	60.00	0.00	32.00
OvoglobulinG2 type AA (TENP)	NP_990357.1	47	395882	BPIFB7	5	0	0	7	0	0	0.13	17.00	0.00	0.00
Ovoinhibitor	P10184.2	52	416235	SPINK5	14	0	0	17	0	0	0.32	37.00	0.00	0.00
Ovomucoid	P01005	23	416236	SPINK7	9	3	0	16	7	0	0.50	52.00	33.00	0.00
Ovostatin	XP_423478.4	165	396151	OVST	21	3	0	19	15	0	0.77	17.00	2.20	0.00
Ovotransferrin	P02789.2	78	396241	TF	63	12	7	134	77	23	1223.70	80.00	19.00	12.00
Peroxiredoxin-1	P0CB50	22	424598	PRDX1	0	2	0	0	5	0	0.15	0.00	9.50	0.00
Phosvitin	P67869.1	196	100873151	CSNK2B	10	0	0	9	0	0	0.16	8.00	0.00	0.00

Plasma protease C1 inhibitor isoform X1	XP_003641424.1	52	423132	SERPING1	4	0	0	2	0	0	0.04	11.00	0.00	0.00
PIT54 protein precursor	NP_997063.1	80	395364	PIT54	3	0	0	3	0	0	0.07	9.10	0.00	0.00
Polymeric Ig receptor	AAP69598.1	71	419848	PIGR	4	0	0	2	0	0	0.04	7.40	0.00	0.00
Polyubiquitin-C-like isoform X2	XP_004946720.1	41	101747587	LOC101747587	3	0	0	3	0	0	0.07	8.60	0.00	0.00
Protein S100-A9-like	XP_004948279.1	14	101747463	LOC101747463	3	0	0	2	0	0	0.05	32.00	0.00	0.00
Prothrombin	NP_989936.1	69	395306	F2	2	0	0	1	0	0	0.02	4.80	0.00	0.00
Riboflavin-binding protein	P02752.2	25	396449	RBP	4	0	0	5	0	0	0.09	16.00	0.00	0.00
Secretoglobin family 1C member 1-like, partial	XP_004941584.1	10	101749303	LOC101749303	5	0	0	3	0	0	0.07	43.00	0.00	0.00
Serum albumin precursor	NP_990592.2	70	396197	ALB	16	0	0	10	0	0	0.20	37.00	0.00	0.00
Spectrin alpha chain non-erythrocytic 1	NP_001036003.1	285	374234	SPTAN1	3	0	0	1	0	0	0.02	1.60	0.00	0.00
Sulfhydryl oxidase 1	Q8JGM4	83	373914	QSOX1	2	3	2	2	20	5	265.60	3.20	4.80	3.40
Tiarin-like	Q25C35	56	418826	OLFM4	0	6	0	0	29	0	0.81	0.00	15.00	0.00
Transmembrane protease serine 9 isoform X3	XP_425880.4	117	428320	TMPRSS9	2	0	0	1	0	0	0.02	2.80	0.00	0.00
Vacuolar protein sorting 13 homolog D	E1BYT6	492	419481	VPS13D	0	2	0	0	3.6574	0	0.10	0.00	0.59	0.00
Vitelline membrane outer layer protein 1	NP_001161233.1	20	418974	VMO1	5	0	0	7	0	0	0.14	35.00	0.00	0.00
Vitellogenin	AAA49139.1	33	424533	VTG2	3	0	0	34	0	0	0.64	77.00	0.00	0.00
Vitellogenin-1	NP_001004408.1	211	424533	VTG2	41	0	0	42	0	0	0.81	27.00	0.00	0.00
Vitellogenin-2	P02845.1	205	424533	VTG2	61	0	0	109	0	0	2.07	39.00	0.00	0.00
Vitronectin	O12945	52	395935	VTN	2	0	0	2	0	0	0.04	5.50	0.00	0.00
Zona pellucida glycoprotein 1	Q9DER4	100	395418	ZP1	7	0	0	4	0	0	0.08	11.00	0.00	0.00
Zona pellucida sperm-binding protein 3	NP_989720.3	47	378906	ZP3	6	0	0	4	0	0	0.08	16.00	0.00	0.00
WAP four-disulfide core domain protein 3 isoform X7	XP_004947252.1	27	419300	WFDC2	0	2	0	0	15	0	0.41	0.00	11.00	0.00

[a] Information obtained from Scaffold softwarer, version 4.3.4.

[b] Normalized spectra counts.

[c] Adjusted normalized spectra counts.

[d] Information obtained from PubMed database.

Table S8. List of proteins identified in the eggshell membranes from unfertilized eggs at day 0.

Identified Proteins (Day 0 Unfertilized)	Acession Number NCBI/UNIPROT	MW (kDa)	Gene ID [d]	Official Gene Symbol [d]	Unique peptides [a]			Normalized spectral counts (NSC) [a,b]			ANSC [a,c]	Percentage Coverage [a] (%)		
					NaCl	Tris	TCEP	NaCl	Tris	TCEP		NaCl	Tris	TCEP
Alpha-1-acid glycoprotein	Q8JIG5	22	395220	ORM1	5	3	0	4	8	0	0.30	20.00	13.00	0.00
Alpha-amylase	NP_001001528.1	58	414139	AMY1A	0	5	0	0	11	0	0.31	0.00	13.00	0.00
Avidin	P02701	17	396260	AVD	10	0	0	26	0	0	0.50	55.00	0.00	0.00
Beta-2-microglobulin	P21611	13	414830	B2M	0	3	0	0	5	0	0.15	0.00	32.00	0.00
Clusterin	AAD17257.1	51	395722	CLU	0	2	0	0	3	0	0.08	0.00	3.80	0.00
Collagen alpha-1(X) chain	F1NRH2	66	100858979	LOC100858979	0	3	10	0	7	621	32901.00	0.00	5.50	11.00
CREMP	XP_001236415.1	33	776923	CREMP	0	0	2	0	0	85	4505.00	0.00	0.00	7.70
CREMP-like 3	UPI000044A8C0	34	N/A	N/A	0	0	2	0	0	170	9010.00	0.00	0.00	11.00
Cystatin	P01038	15	396497	CST3	0	3	0	0	23	0	0.65	0.00	27.00	0.00
EGF-like repeats and discoidin I-like domains 3 isoform X3	XP_004949505.1	54	427326	EDIL3	0	0	3	0	0	36	1908.00	0.00	0.00	5.60
EW135	BAO03774.1	104	N/A	N/A	7	0	0	10	0	0	0.19	9.00	0.00	0.00
Folate receptor alpha precursor	NP_990165.1	31	395638	FOLR1	0	3	0	0	5	0	0.15	0.00	9.10	0.00
Galactocerebrosidase isoform X3	XP_421304.3	77	423394	GALC	0	5	0	0	16	0	0.46	0.00	8.90	0.00
Gallinacin-11	Q6IV20	12	414876	GAL11	3	0	2	3	0	6	321.24	38.00	0.00	24.00
Gastrokine 2 (OCX-21)	XP_417666.1	21	419515	GKN2	0	0	5	0	0	145	7703.55	0.00	0.00	33.00
Hemopexin	H9L385	45	419076	HPX	3	4	0	3	10	0	0.32	8.10	12.00	0.00
Hyaluronan and proteoglycan link protein 3	XP_413868.3	41	415495	HAPLN3	0	0	9	0	0	48	2567.85	0.00	0.00	15.00
Ig alpha heavy chain	AAB22614.2	62	N/A	N/A	7	13	0	12	134	0	3.97	11.00	22.00	0.00
Ig gamma H	1405234A	54	N/A	N/A	5	3	0	6	8	0	0.34	12.00	6.90	0.00
Ig lambda chain V-1 region Precursor	NP_001265474.1	24	416928	IGLL1	0	5	0	0	86	0	2.40	0.00	32.00	0.00
Lactadherin isoform 2	NP_001264040.1	59	415494	MFGE8	0	0	2	0	0	6	318.00	0.00	0.00	3.60
Lipocalin 8	P21760.2	20	396393	LCN8	0	11	0	0	48	0	1.34	0.00	48.00	0.00
Lysozyme C	P00698	16	396218	LYZ	43	4	5	543	31	309	16381.31	84.00	31.00	35.00
Lysyl oxidase homolog 2	E1C3U7	87	419533	LOXL2	0	3	23	0	5	851	45098.38	0.00	4.90	28.00
Mucin-5AC	XP_003641370.2	383	100859916	LOC100859916	0	2	0	0	7	0	0.19	0.00	0.63	0.00
Mucin 6	XP_426405.4	294	414878	MUC6	12	0	0	15	0	0	0.29	4.40	0.00	0.00
Ovalbumin	P01012.2	43	396058	OVAL	13	13	4	33	79	58	3052.99	36.00	40.00	15.00
Ovalbumin related-protein X	P01013.1	45	420898	OVALX	6	0	0	10	0	0	0.18	19.00	0.00	0.00
Ovocalyxin-36	F1P1Y2	58	419289	BPIFB3	0	7	4	0	91	21	1126.15	0.00	19.00	6.40
Ovocleidin-17	Q9PRS8	15	100313508	OC-17	6	0	4	11	0	67	3531.06	54.00	0.00	25.00

Ovocleidin-116	NP_989900.1	77	395256	MEPE	0	17	2	0	89	21	1126.08	0.00	29.00	4.20
Ovoinhibitor	P10184.2	52	416235	SPINK5	0	6	0	0	27	0	0.76	0.00	14.00	0.00
Ovomucoid	P01005	23	416236	SPINK7	6	3	0	11	5	0	0.35	41.00	30.00	0.00
Ovotransferrin	P02789.2	78	396241	TF	42	14	0	64	33	0	2.12	59.00	29.00	0.00
Partial Ig heavy chain variable region	BAA11105.1	13	N/A	N/A	0	2	0	0	4	0	0.11	0.00	14.00	0.00
Polymeric Ig receptor	AAP69598.1	71	419848	PIGR	0	5	0	0	18	0	0.50	0.00	13.00	0.00
Prostaglandin-H2 D-isomerase Precursor	NP_989590.1	21	374110	PTGDS	0	5	0	0	16	0	0.46	0.00	24.00	0.00
Semaphorin-3G isoform X4	XP_414289.3	82	415945	SEMA3G	0	2	0	0	5	0	0.15	0.00	3.30	0.00
Spectrin beta chain non-erythrocytic 5	XP_421149.4	453	N/A	N/A	0	2	0	0	3	0	0.08	0.00	0.57	0.00
Sulfhydryl oxidase 1	Q8JGM4	83	373914	QSOX1	0	4	0	0	10	0	0.27	0.00	8.10	0.00
Tenascin	F1N8F4	199	396440	TNC	0	10	0	0	20	0	0.57	0.00	7.50	0.00
Tiarin-like	Q25C35	56	418826	OLFM4	0	9	0	0	29	0	0.81	0.00	21.00	0.00
Trypsin inhibitor ClTI-1	P85000.1	6	770729	SPINK2	0	2	0	0	19	0	0.53	0.00	40.00	0.00
Ubiquitin	NP_001280103.1	41	396190	UBB	0	4	0	0	16	0	0.46	0.00	11.00	0.00
Vacuolar protein sorting 13 homolog D	E1BYT6	492	419481	VPS13D	0	2	0	0	3	0	0.08	0.00	0.36	0.00
Vesicular integral-membrane protein VIP36	XP_003642101.2	38	100859676	LMAN2	0	2	0	0	10	0	0.27	0.00	6.10	0.00
Vitelline membrane outer layer protein 1	NP_001161233.1	20	418974	VMO1	2	0	0	13	0	0	0.25	51.00	0.00	0.00

[a] Information obtained from Scaffold softwarer, version 4.3.4.
[b] Normalized spectra counts.
[c] Adjusted normalized spectra counts.
[d] Information obtained from PubMed database.

Table S9. List of proteins identified in the eggshell membranes from unfertilized eggs at day 3.

Identified Proteins (Day 3 Unfertilized)	Acession Number NCBI/UNIPROT	MW (kDa)	Gene ID [d]	Official Gene Symbol [d]	Unique peptides [a]			Normalized spectral counts(NSC) [a,b]			ANSC [a,c]	Percentage Coverage [a] (%)		
					NaCl	Tris	TCEP	NaCl	Tris	TCEP		NaCl	Tris	TCEP
Alpha-amylase	NP_001001528.1	58	414139	AMY1A	0	5	0	0	7	0	0.196	0	11	0
Alpha-1-acid glycoprotein	AAT39530.1	22	395220	ORM1	7	3	0	19	4	0	0.473	33	13	0
Avidin	P02701	17	396260	AVD	5	0	0	13	0	0	0.247	53	0	0
Beta actin	AAA48615.1	42	396526	ACTB	3	0	0	5	0	0	0.095	13	0	0
Beta-microseminoprotein-like	XP_004942176.1	12	101750704	LOC101750704	2	0	0	3	0	0	0.057	23	0	0
Chondrogenesis associated Lipocalin	AAL99254.1	21	374110	PTGDS	2	2	0	3	7	0	0.253	17	18	0
Clusterin	AAD17257.1	51	395722	CLU	0	0	2	0	0	3	159	0	0	3.8
Collagen alpha-1(X) chain isoform X1	F1NRH2	66	100858979	LOC100858979	0	0	16	0	0	69	3657	0	0	16
Collagen alpha-3(IV)	XP_001234730.3	163	424797	COL4A3	0	0	2	0	0	7	371	0	0	1.5
CREMP-like 1	UPI0000ECBE15	33	N/A	N/A	0	0	5	0	0	14	742	0	0	14
CREMP-like 2	UPI0000ECBEAB	35	N/A	N/A	0	0	3	0	0	7	371	0	0	7.1
CREMP-like 3	UPI000044A8C0	34	N/A	N/A	2	0	8	4	0	77	4081.08	8.7	0	18
Dystonin isoform X3	XP_419901.4	883	421884	DST	0	0	2	0	0	3	159	0	0	0.48
EGF-like repeats and discoidin I-like domains 3 isoform X3	XP_004949505.1	51	427326	EDIL3	6	0	5	10	0	13	689.19	16	0	10
Enhancer of mRNA-decapping protein 3	NP_001025556.1	55	415294	EDC3	0	0	2	0	0	4	212	0	0	4
Extracellular fatty acid-binding protein	P21760.2	20	396393	LCN8	0	6	0	0	19	0	0.532	0	34	0
Folate receptor alpha Precursor	NP_990165.1	31	395638	FOLR1	0	2	0	0	3	0	0.084	0	6.2	0
Gallin	CBE70278.1	7	422031	GALN2	0	0	3	0	0	7	371	0	0	46
Gallinacin-11	Q6IV20.1	12	414876	GAL11	5	0	3	9	0	6	318.171	52	0	37
Gastrokine-2	XP_417666.1	17	419515	GKN2	2	0	13	4	0	25	1325.08	37	0	45
Glutathione peroxidase 3 Precursor	NP_001156704.1	22	427638	GPX3	0	0	2	0	0	2	106	0	0	13
Heterochromatin-associated protein MENT	NP_990228.1	42	395715	SERPINB10	2	0	0	3	0	0	0.057	6.1	0	0
Homeobox protein cut-like 1-like	XP_004946538.1	441	101749861	CUX1	2	0	0	2	0	0	0.038	0.89	0	0
Hyaluronan and proteoglycan link protein 1	NP_990813.1	20	396475	HAPLN1	0	0	6	0	0	10	530	0	0	21
Ig alpha heavy chain	AAB22614.2	62	N/A	N/A	5	9	0	7	25	0	0.833	7.7	18	0

IgGFc-binding protein-like	XP_426805.4	22	429249	LOC429249	0	0	5	0	0	9	477	0	0	19
Lactadherin isoform 2	NP_001264040.1	50	415494	MFGE8	5	0	0	11	0	0	0.209	14	0	0
Lipocalin 8	P21760.2	20	396393	LCN8	0	6	0	0	19	0	0.532	0	34	0
Lysosomal-trafficking regulator	XP_419558.4	428	421514	LYST	0	0	3	0	0	3	159	0	0	0.89
Lysozyme C	P00698	14	396218	LYZ	35	2	12	817	911	44	2373.03	92	67	74
Lysyl oxidase homolog 2	E1C3U7	82	419533	LOXL2	0	0	36	0	0	168	8904	0	0	36
Mucin-5B	Q98UI9	234	395381	LOC395381	17	0	0	28	0	0	0.532	10	0	0
Mucin-6	XP_426405.4	159	414878	MUC6	34	0	0	72	0	0	1.368	17	0	0
Ovalbumin	P01012.2	43	396058	OVAL	57	27	18	1637	288	106	5657.17	94	91	67
Ovalbumin-related protein X	P01013.1	56	420898	OVALX	0	2	0	0	11	0	0.308	0	19	0
Ovalbumin-related protein Y	P01014.1	44	420897	OVALY	19	6	0	104	9	0	2.228	53	26	0
Ovocalyxin-32	Q90YI1	31	395209	RARRES1	2	0	0	3	0	0	0.057	9.5	0	0
Ovocalyxin-36	F1P1Y2	56	419289	BPIFB8	7	0	9	16	0	23	1219.3	7.5	0	23
Ovocleidin-116	F1NSM7	77	395256	MEPE	21	2	8	55	4	17	902.157	37	4.7	14
Ovocleidin-17	Q9PRS8	15	100313508	OC-17	6	0	4	12	0	8	424.228	51	0	21
OvoglobulinG2 type AA (TENP)	NP_990357.1	47	395882	BPIFB7	12	0	0	38	0	0	0.722	53	0	0
Ovoinhibitor	P10184.2	57	416235	SPINK5	18	0	0	44	0	0	0.836	51	0	0
Ovomucoid	P01005	20	416236	SPINK7	16	6	0	137	18	0	3.107	58	44	0
Ovostatin	XP_423478.4	165	396151	OVST	19	0	0	36	0	0	0.684	17	0	0
Ovotransferrin	P02789.2	78	396241	TF	84	26	4	808	131	7	390.02	89	51	6.7
Partial Ig heavy chain variable region	BAA11105.1	11	N/A	N/A	2	2	0	3	4	0	0.169	31	31	0
Phosphofurin acidic cluster sorting protein 1 isoform X5	XP_004940210.1	90	421254	PACS1	2	0	0	2	0	0	0.038	2	0	0
Pol-like protein ENS-3	NP_989963.1	157	395341	ENS-3	2	0	0	2	0	0	0.038	1.6	0	0
Protocadherin beta-15-like Partial	XP_004944803.1	86	100858025	LOC100858025	0	0	2	0	0	3	159	0	0	3
Protocadherin Fat 2	XP_414584.4	40	416266	FAT2	2	0	0	6	0	0	0.114	6.6	0	0
Riboflavin-binding protein	P02752	25	396449	RTBDN	4	0	0	13	0	0	0.247	17	0	0
Serpin B6	NP_001006377.1	42	420895	SERPINB6	2	0	0	3	0	0	0.057	6.1	0	0
Serine/arginine repetitive matrix protein 2-like	XP_004946268.1	39	101751537	LOC101751537	0	0	2	0	0	2	106	0	0	3.3
Sulfhydryl oxidase 1	Q8JGM4	83	373914	QSOX1	0	2	3	0	3	5	265.084	0	3.5	5.4
Titin isoform X2	XP_421979.4	3834	424126	TTN	0	0	3	0	0	3	159	0	0	0.09
Titin isoform X3	XP_004942758.1	2461	424126	TTN	2	0	0	2	0	0	0.038	0.14	0	0
Vitelline membrane outer layer protein 1	NP_001161233.1	18	418974	VMO1	2	0	0	4	0	0	0.076	20	0	0
WAP four-disulfide core domain protein 3 isoform X7	XP_004947252.1	21	419300	WFDC2	5	0	0	17	0	0	0.323	18	0	0

[a] Information obtained from Scaffold softwarer, version 4.3.4.

[b] Normalized spectra counts.

[c] Adjusted normalized spectra counts.

[d] Information obtained from PubMed database.

Table S10. List of proteins identified in the eggshell membranes from unfertilized eggs at day 7.

Identified Proteins (Day 7 Unfertilized)	Acession Number NCBI/UNIPROT	MW (kDa)	Gene ID [d]	Official Gene Symbol [d]	Unique peptides [a]			Normalized spectral counts (NSC) [a,b]			ANSC [a,c]	Percentage Coverage [a] (%)		
					NaCl	Tris	TCEP	NaCl	Tris	TCEP		NaCl	Tris	TCEP
Alpha-1-acid glycoprotein	AAT39530.1	22	395220	ORM1	5	2	0	15	4	0	0.40	30.00	13.00	0.00
Alpha-amylase	NP_001001528.1	58	414139	AMY1A	0	4	0	0	7	0	0.20	0.00	8.60	0.00
Apolipoprotein L domain-containing protein 1	XP_004937733.1	51	769889	APOLD1	0	2	0	0	2	0	0.06	0.00	6.20	0.00
Avidin	P02701	17	396260	AVD	2	0	0	3	0	0	0.06	15.00	0.00	0.00
Beta-microseminoprotein-like	XP_004942176.1	12	101750704	LOC101750704	0	0	2	0	0	5	265.00	0.00	0.00	11.00
Chondrogenesis associated Lipocalin	Q8QFM7	21	374110	PTGDS	0	5	0	0	11	0	0.31	0.00	41.00	0.00
Clusterin	AAD17257.1	49	395722	CLU	0	2	2	0	2	4	212.06	0.00	4.50	4.90
Collagen alpha-1(X) chain	F1NRH2	66	100858979	LOC100858979	0	0	14	0	0	43	2279.00	0.00	0.00	18.00
Collagen alpha-2(V) chain isoform X2	XP_421846.3	140	423986	COL5A2	0	0	2	0	0	3	159.00	0.00	0.00	1.40
CREMP-like 1	UPI0000ECBE15	33	N/A	N/A	0	0	2	0	0	6	318.00	0.00	0.00	7.30
CREMP-like 3	UPI000044A8C0	34	N/A	N/A	2	0	7	3	0	31	1643.06	8.70	0.00	14.00
Cystatin	P01038	15	396497	CST3	0	2	0	0	5	0	0.14	0.00	20.00	0.00
Dedicator of cytokinesis protein 4 isoform X4	XP_416024.3	219	417779	DOCK4	0	2	0	0	2	0	0.06	0.00	1.80	0.00
EGF-like repeats and discoidin I-like domains 3 isoform X2	XP_424906.3	93	427326	EDIL3	2	2	0	2	3	0	0.12	2.80	3.00	0.00
ELKS/Rab6-interacting/ CAST family member 1 isoform X8	XP_004937993.1	110	418153	ERC1	0	2	0	0	2	0	0.06	0.00	1.90	0.00
Erythroid-specific folate receptor	Q9PW81	31	395638	FOLR1	0	2	0	0	4	0	0.11	0.00	6.20	0.00
Extracellular fatty acid-binding protein precursor	P21760.2	20	396393	LCN8	0	9	0	0	25	0	0.70	0.00	39.00	0.00
Gallin	CBE70278.1	7	422031	GALN2	3	0	0	5	0	0	0.10	49.00	0.00	0.00
Gallinacin-11	Q6IV20	12	414876	GAL11	0	2	0	0	2	0	0.06	0.00	13.00	0.00
Gastrokine-2	XP_417666.1	17	419515	GKN2	0	0	5	0	0	14	742.00	0.00	0.00	41.00
la-related protein 1 isoform X4	XP_414577.4	121	416258	LARP1	0	2	0	0	3	0	0.08	0.00	3.10	0.00
Ig alpha heavy chain	AAB22614.2	62	N/A	N/A	0	11	0	0	37	0	1.04	0.00	22.00	0.00
Ig gamma chain (clone 36)	S00390	54	N/A	N/A	2	0	0	2	0	0	0.04	6.30	0.00	0.00

Ig heavy chain variable region	CAO79256.1	26	N/A	N/A	0	2	0	0	5	0	0.14	0.00	4.90	0.00
Ig light chain precursor V-J region	A21177	22	N/A	N/A	0	4	0	0	23	0	0.64	0.00	28.00	0.00
Inactive heparanase-2 isoform X10	XP_004942232.1	177	423834	HPSE2	0	2	0	0	2	0	0.06	0.00	1.40	0.00
Lysozyme	630460A	14	396218	LYZ	4	2	9	989	259	29	1563.04	88.00	81.00	67.00
Lysyl oxidase homolog 2	E1C3U7	87	419533	LOXL2	0	7	28	0	13	110	5830.36	0.00	37.00	33.00
Mucin-5AC	XP_003641370.2	359	100859916	LOC100859916	0	5	0	0	9	0	0.25	0.00	2.40	0.00
Olfactomedin-4 precursor	NP_001035553.1	51	418826	OLFM4	0	2	0	0	2	0	0.06	0.00	4.70	0.00
Ovalbumin	P01012.2	43	396058	OVAL	63	22	13	1322	225	67	3582.42	95.00	81.00	50.00
Ovalbumin-related protein X	P01013.1	56	420898	OVALX	10	0	0	22	0	0	0.42	26.00	0.00	0.00
Ovalbumin-related protein Y	P01014.1	44	420897	OVALY	16	4	0	75	7	0	1.62	52.00	16.00	0.00
Ovocalyxin-36	Q53HW8	49	419289	BPIFB3	3	0	8	5	0	14	742.10	10.00	0.00	20.00
Ovocleidin-17	Q9PRS8	15	100313508	OC-17	0	0	2	0	0	5	265.00	0.00	0.00	15.00
Ovocleidin-116	F1NSM7	77	395256	MEPE	0	5	4	0	9	6	318.25	0.00	14.00	7.40
OvoglobulinG2 type AA (TENP)	NP_990357.1	47	395882	BPIFB7	6	0	0	14	0	0	0.27	26.00	0.00	0.00
Ovomucoid	P01005	20	416236	SPINK7	2	9	0	63	64	0	2.99	59.00	55.00	0.00
Ovotransferrin	P02789.2	76	396241	TF	2	17	0	371	41	0	8.20	84.00	38.00	0.00
Partial Ig lambda chain	BAA09984.1	11	N/A	N/A	0	9	0	0	64	0	1.79	0.00	42.00	0.00
Partial Ig light chain variable region	BAE80149.1	11	N/A	N/A	0	2	0	0	8	0	0.22	0.00	1.90	0.00
Riboflavin-binding protein	P02752.2	25	396449	RTBDN	0	3	0	0	6	0	0.17	0.00	12.00	0.00
Secretory trypsin inhibitor	1708290A	8	N/A	N/A	0	2	0	0	2	0	0.06	0.00	64.00	0.00
Sulfhydryl oxidase 1	Q8JGM4	83	373914	QSOX1	2	0	0	3	0	0	0.06	3.20	0.00	0.00
Trypsin inhibitor CITI-1	P85000.1	9	770729	SPINK2	0	2	0	0	7	0	0.20	0.00	42.00	0.00
Vitelline membrane outer layer protein 1 precursor	NP_001161233.1	21	418974	VMO1	3	0	0	5	0	0	0.10	28.00	0.00	0.00
WAP four-disulfide core domain protein 3 isoform X7	XP_004947252.1	36	419300	WFDC2	0	2	0	0	6	0	0.17	0.00	12.00	0.00

[a] Information obtained from Scaffold softwarer, version 4.3.4.

[b] Normalized spectra counts.

[c] Adjusted normalized spectra counts.

[d] Information obtained from PubMed database.

Table S11. List of proteins identified in the eggshell membranes from unfertilized eggs at day 11.

Identified Proteins (Day 11 unfertilized)	Acession Number NCBI/UNIPROT	MW (kDa)	Gene ID [d]	Official Gene Symbol [d]	Unique peptides [a]			Normalized spectral counts (NSC) [a,b]			ANSC [a,c]	Percentage Coverage [a] (%)		
					NaCl	Tris	TCEP	NaCl	Tris	TCEP		NaCl	Tris	TCEP
Adenomatous polyposis coli protein 2	NP_001165242.1	235	429363	APC2	0	2	0	0	2	0	0.06	0.00	2.50	0.00
Agrin	AAA48585.1	52	396538	AGRN	0	17	0	0	48	0	1.34	0.00	46.00	0.00
Alpha-1-acid glycoprotein	A7UEB0	22	395220	ORM1	5	5	0	21	21	0	0.99	30.00	30.00	0.00
Alpha-amylase	NP_001001528.1	58	414139	AMY1A	0	11	0	0	19	0	0.53	0.00	25.00	0.00
Alternatively spliced tenascin 190, 200 and 230 kd variants	Q90995	189	396440	TNC	0	2	0	0	2	0	0.06	0.00	1.80	0.00
Avidin	P02701	17	396260	AVD	2	0	0	4	0	0	0.08	15.00	0.00	0.00
Beta-microseminoprotein-like	XP_004942176.1	12	101750704	LOC101750704	0	0	2	0	0	10	530.00	0.00	0.00	18.00
Chondrogenesis associated Lipocalin	AAL99254.1	21	374110	PTGDS	0	7	0	0	17	0	0.48	0.00	48.00	0.00
Clusterin	AAD17257.1	49	395722	CLU	0	6	0	0	12	0	0.34	0.00	16.00	0.00
Collagen alpha-1(X) chain	F1NRH2	66	100858979	LOC100858979	0	0	14	0	0	60	3180.00	0.00	0.00	13.00
Collagen alpha-2(I) chain	P02467.2	133	396243	COL1A2	0	4	0	0	5	0	0.14	0.00	8.10	0.00
CREMP	XP_001236415	33	776923	CREMP	0	0	4	0	0	16	848.00	0.00	0.00	10.00
CREMP-like 2	UPI0000ECBEAB	35	N/A	N/A	0	0	3	0	0	4	212.00	0.00	0.00	7.80
CREMP like 3	UPI000044A8C0	34	N/A	N/A	0	7	14	0	18	107	5671.50	0.00	32.00	19.00
Cystatin	P01038	15	396497	CST3	0	2	0	0	7	0	0.20	0.00	20.00	0.00
EGF-like repeats and discoidin I-like domains 3 isoform X3	XP_004949505.1	54	427326	EDIL3	0	0	4	0	0	10	530.00	0.00	0.00	7.90
EW135	BAO03774.1	80	N/A	N/A	0	4	0	0	6	0	0.17	0.00	7.00	0.00
Fibronectin	P11722.3	259	396133	FN1	0	4	0	0	9	0	0.25	0.00	3.10	0.00
Gallin protein	CBE70278.1	5	422031	GALN2	0	0	4	0	0	7	371.00	0.00	0.00	71.00
Gallinacin-9	Q6QLR1	7	414343	GAL9	0	2	0	0	3	0	0.08	0.00	30.00	0.00
Gallinacin-11	Q6IV20	12	414876	GAL11	0	6	2	0	12	2	106.34	0.00	12.00	18.00
Gastrokine-2	XP_417666.1	17	419515	GKN2	0	0	4	0	0	15	795.00	0.00	0.00	27.00
Glutathione peroxidase 3	NP_001156704.1	19	427638	GPX3	0	3	0	0	5	0	0.14	0.00	18.00	0.00
Hemopexin	XP_417267.3	43	419076	HPX	0	5	0	0	6	0	0.17	0.00	23.00	0.00
Hyaluronan and proteoglycan link protein1	NP_990813.1	41	396475	HAPLN1	0	0	4	0	0	8	424.00	0.00	0.00	13.00
la-related protein 4	NP_001012718.1	17	426876	LARP4	0	0	4	0	0	15	795.00	0.00	0.00	27.00
Ig alpha heavy chain	AAB22614.2	62	N/A	N/A	0	13	0	0	34	0	0.95	0.00	21.00	0.00
IgGFc-binding protein-like	XP_426805.4	22	429249	LOC429249	0	0	2	0	0	9	477.00	0.00	0.00	7.40
Ig light chain	AAA48859.1	25	N/A	N/A	0	2	0	0	23	0	0.64	0.00	37.00	0.00
Insulin-like growth factor binding protein 7 precursor	XP_420577.3	29	422620	IGFBP7	0	2	0	0	3	0	0.08	0.00	6.10	0.00
Lactadherin	NP_001264040.1	51	415494	MFGE8	0	0	2	0	0	2	106.00	0.00	0.00	3.60
Lysozyme C	P00698	14	396218	LYZ	2	10	11	761	69	55	2931.39	92.00	81.00	79.00
Lysyl oxidase homolog 2	E1C3U7	87	419533	LOXL2	0	14	37	0	41	196	10389.15	0.00	28.00	42.00
Mucin-5AC	XP_003641370.2	383	100859916	LOC100859916	0	10	0	0	24	0	0.67	0.00	3.90	0.00

Na(+)/H(+) exchange regulatory cofactor NHE-RF2	XP_414851.3	60	SLC9A3R2	416550	2	0	0	2	0	0	0.04	2.60	0.00	0.00
Ovalbumin	P01012.2	43	396058	OVAL	65	40	0	1166	406	0	33.52	92.00	90.00	0.00
Ovalbumin-related protein X	P01013.1	45	420898	OVALX	7	0	0	17	0	0	0.32	22.00	0.00	0.00
Ovalbumin-related protein Y	P01014.1	44	420897	OVALY	17	12	0	64	33	0	2.14	54.00	34.00	0.00
Ovocalyxin-32	Q90YI1	31	395209	RARRES1	0	0	3	0	0	4	212.00	0.00	0.00	27.00
Ovocalyxin-36	Q53HW8	49	419289	BPIFB8	0	0	9	0	0	26	1378.00	0.00	0.00	26.00
Ovocleidin-17	Q9PRS8	15	100313508	OC-17	0	0	2	0	0	5	265.00	0.00	0.00	15.00
Ovocleidin-116	NP_989900.1	81	395256	MEPE	0	0	5	0	0	8	424.00	0.00	0.00	8.90
OvoglobulinG2 type AA (TENP)	NP_990357.1	47	395882	BPIFB7	3	0	0	7	0	0	0.13	11.00	0.00	0.00
Ovoinhibitor	P10184.2	52	416235	SPINK5	10	0	0	17	0	0	0.32	24.00	0.00	0.00
Ovomucoid	P01005	23	416236	SPINK7	0	20	0	0	151	0	4.23	0.00	70.00	0.00
Ovotransferrin	P02789.2	76	396241	TF	63	22	0	389	38	0	8.46	73.00	44.00	0.00
Partial anti-prion protein Ig light chain variable region	BAE20399.1	11	N/A	N/A	0	2	0	0	6	0	0.17	0.00	48.00	0.00
Partial Ig lambda chain	BAB47282.1	11	N/A	N/A	0	2	0	0	6	0	0.17	0.00	38.00	0.00
Partial Ig light chain variable region	BAE80142.1	13	N/A	N/A	0	4	0	0	10	0	0.28	0.00	44.00	0.00
Polymeric Ig receptor	AAP69598.1	71	419848	PIGR	0	2	0	0	3	0	0.08	0.00	4.20	0.00
Prostate stem cell antigen	XP_418414.2	44	420302	PSCA	0	2	0	0	3	0	0.08	0.00	6.10	0.00
Quiescence-specific protein	AAA53371.1	20	396393	LCN8	0	9	0	0	27	0	0.76	0.00	58.00	0.00
Riboflavin-binding protein	P02752.2	25	396449	RTBDN	3	2	0	8	9	0	0.40	14.00	9.10	0.00
Sulfhydryl oxidase 1	Q8JGM4	83	373914	QSOX1	0	3	0	0	5	0	0.14	0.00	5.90	0.00
Tiarin-like	Q25C35	56	418826	OLFM4	0	4	0	0	5	0	0.14	0.00	10.00	0.00
Trypsin inhibitor ClTI-1	P85000.1	6	N/A	N/A	0	2	0	0	7	0	0.20	0.00	15.00	0.00
Vesicular integral-membrane protein VIP36	XP_003642101.2	38	100859676	LMAN2	0	5	0	0	7	0	0.20	0.00	20.00	0.00
Vitellogenin	AAA49139.1	31	424533	VTG2	3	0	0	6	0	0	0.11	11.00	0.00	0.00
WAP four-disulfide core domain protein 3 isoform X6	XP_004947251.1	39	419300	WFDC2	0	2	0	0	3	0	0.08	0.00	7.20	0.00
WAP four-disulfide core domain protein 3 isoform X7	XP_004947252.1	36	419300	WFDC2	0	3	0	0	4	0	0.11	0.00	12.00	0.00

[a] Information obtained from Scaffold softwarer, version 4.3.4.

[b] Normalized spectra counts.

[c] Adjusted normalized spectra counts.

[d] Information obtained from PubMed database.

Table S12. List of proteins identified in the eggshell membranes from unfertilized eggs at day 15.

Identified Proteins (Day 15 Unfertilized)	Acession Number NCBI/UNIPROT	MW (kDa)	Gene ID [d]	Official Gene Symbol [d]	Unique peptides [a]			Normalized spectral counts (NSC) [a,b]			ANSC [a,c]	Percentage Coverage [a] (%)		
					NaCl	Tris	TCEP	NaCl	Tris	TCEP		NaCl	Tris	TCEP
Alpha-amylase	NP_001001528.1	58	414139	AMY1A	0	2	0	0	2	0	0.06	0.00	4.50	0.00
Alpha-1-acid glycoprotein	AAT39530.1	22	395220	ORM1	2	0	0	3	0	0	0.06	13.00	0.00	0.00
Alternatively spliced tenascin 190, 200 and 230 kd variants	Q90995	189	396440	TNC	0	25	0	0	45	0	1.26	0.00	17.00	0.00
Avidin	P02701	17	396260	AVD	2	0	0	4	0	0	0.08	13.00	0.00	0.00
Basement membrane-specific heparan sulfate proteoglycan core protein	Q6KDZ1	433	429806	HSPG2	0	5	0	0	7	0	0.20	0.00	1.50	0.00
Chondrogenesis associated Lipocalin	AAL99254.1	21	374110	PTGDS	0	6	0	0	13	0	0.36	0.00	41.00	0.00
Collagen alpha 1 (X)	F1NRH2	66	100858979	LOC100858979	0	15	14	0	79	47	2493.21	0.00	12.00	15.00
CREMP-like 3	UPI000044A8C0	34	N/A	N/A	0	3	8	0	8	81	4293.22	0.00	13.00	18.00
Cystatin	P01038	15	396497	CST3	0	3	0	0	8	0	0.22	0.00	20.00	0.00
EGF-like repeats and discoidin I-like domains 3 isoform X3	XP_004949505.1	54	427326	EDIL3	0	3	2	0	3	3	159.08	0.00	7.70	4.40
Extracellular fatty acid-binding Protein	P21760.2	20	396393	LCN8	0	7	2	0	22	3	159.62	0.00	34.00	15.00
Fibronectin	P11722.3	259	396133	FN1	0	5	0	0	7	0	0.20	0.00	2.60	0.00
Gastrokine 2	XP_417666.1	17	419515	GKN2	0	2	0	0	3	0	0.08	0.00	19.00	0.00
Hemopexin	XP_417267.3	43	419076	HPX	0	6	0	0	7	0	0.20	0.00	24.00	0.00
Hyaluronan and proteoglycan link protein 1 precursor	NP_990813.1	41	396475	HAPLN1	0	0	2	0	0	2	106.00	0.00	0.00	6.10
la-related protein 4	NP_001012718.1	17	426876	LARP4	0	2	6	0	3	12	636.08	0.00	19.00	33.00
IgGFc-binding protein-like	XP_426805.4	22	429249	LOC429249	0	0	3	0	0	10	530.00	0.00	0.00	10.00
Ig alpha heavy chain	AAB22614.2	62	N/A	N/A	0	20	0	0	69	0	1.93	0.00	32.00	0.00
Lactadherin	UPI00003AA422	51	415494	MFGE8	0	6	0	0	8	0	0.22	0.00	12.00	0.00
Lysozyme C	P00698	14	396218	LYZ	31	9	6	527	38	33	1760.08	92.00	81.00	38.00
Lysyl oxidase homolog 2	E1C3U7	87	419533	LOXL2	2	4	22	5	6	173	9169.26	4.40	7.90	30.00
Mucin-5AC	XP_003641370.2	383	100859916	LOC100859916	0	8	0	0	14	0	0.39	0.00	3.20	0.00
Ovalbumin	P01012.2	43	396058	OVAL	12	36	12	33	359	74	3932.68	52.00	85.00	59.00
Ovalbumin-related protein X	P01013.1	45	420898	OVALX	0	4	0	0	8	0	0.22	0.00	11.00	0.00
Ovalbumin-related protein Y	P01014.1	44	420897	OVALY	0	11	0	0	24	0	0.67	0.00	34.00	0.00
Ovocalyxin-36	Q53HW8	49	419289	BPIFB8	0	8	5	0	14	14	742.39	0.00	25.00	18.00
Ovocleidin-17	Q9PRS8	15	100313508	OC-17	0	2	2	0	2	4	212.06	0.00	13.00	15.00
Ovocleidin-116	NP_989900.1	77	395256	MEPE	0	0	3	0	0	8	424.00	0.00	0.00	6.10
Ovomucoid	P01005	23	416236	SPINK7	3	4	0	6	10	0	0.39	29.00	46.00	0.00
Ovotransferrin	P02789.2	78	396241	TF	2	17	0	3	35	0	1.04	15.00	36.00	0.00
Polymeric Ig receptor	AAP69598.1	71	419848	PIGR	0	6	0	0	10	0	0.28	0.00	14.00	0.00
Protocadherin Fat 1 isoform X11	XP_004936051.1	32	395168	FAT1	0	0	5	0	0	14	742.00	0.00	0.00	10.00
Quiescence-specific protein	AAA53371.1	20	396393	LCN8	0	7	0	0	22	0	0.62	0.00	34.00	0.00
Sulfhydryl oxidase 1	Q8JGM4	83	373914	QSOX1	0	8	0	0	11	0	0.31	0.00	16.00	0.00
Tiarin-like	Q25C35	56	418826	OLFM4	0	3	0	0	3	0	0.08	0.00	7.00	0.00

[a] Information obtained from Scaffold softwarer, version 4.3.4.
[b] Normalized spectra counts.
[c] Adjusted normalized spectra counts.
[d] Information obtained from PubMed database.

Table S13. List of proteins identified in the eggshell membranes from unfertilized eggs at day 19.

Identified Proteins (Day 19 Unfertilized)	Acession Number NCBI/UNIPROT	MW (kDa)	Gene ID [d]	Official Gene Symbol [d]	Unique peptides [a]			Normalized spectral counts (NSC) [a,b]			ANSC [a,c]	Percentage Coverage [a] (%)		
					NaCl	Tris	TCEP	NaCl	Tris	TCEP		NaCl	Tris	TCEP
Alpha-1-acid glycoprotein	AAT39530.1	22	395220	ORM1	5	4	0	13	30	0	1.09	20.00	20.00	0.00
Avidin	P02701	17	396260	AVD	3	0	0	3	0	0	0.05	22.00	0.00	0.00
Clusterin	AAD17257.1	51	395722	CLU	0	10	3	0	8	10	530.21	0.00	5.60	6.70
CREMP	UPI0000E8213B	33	776923	CREMP	0	0	2	0	0	34	1802.00	0.00	0.00	4.00
Cystatin	P01038	15	396497	CST3	0	2	0	0	18	0	0.50	0.00	20.00	0.00
EGF-like repeats and discoidin I-like domains 3 isoform X2	XP_424906.3	54	427326	EDIL3	0	0	4	0	0	18	954.00	0.00	0.00	5.40
Gallinacin-11	Q6IV20	12	414876	GAL11	0	3	2	0	8	10	556.41	0.00	38.00	20.00
Gastrokine 2 (OCX-21)	XP_417666.1	21	419515	GKN2	3	0	0	34	0	0	0.65	19.00	0.00	0.00
Ig alpha heavy chain	AAB22614.2	62	N/A	N/A	0	9	0	0	47	0	1.32	0.00	18.00	0.00
Ig lambda chain V-1 region precursor	UPI0003505F71	24	N/A	N/A	0	2	0	0	43	0	1.20	0.00	8.80	0.00
Lactadherin isoform 2 Precursor	NP_001264040.1	59	415494	MFGE8	0	0	2	0	0	5	265.00	0.00	0.00	3.00
Lipocalin 8	P21760.2	20	396393	LCN8	0	11	0	0	59	0	1.65	0.00	48.00	0.00
Lysozyme C	P00698	16	396218	LYZ	36	9	13	351	144	375	19885.70	82.00	52.00	46.00
Lysyl oxidase homolog 2	E1C3U7	87	419533	LOXL2	0	12	36	0	87	624	33074.44	0.00	19.00	30.00
Mucin-5B	Q98UI9.1	234	395381	LOC395381	4	0	0	3	0	0	0.06	2.30	0.00	0.00
Mucin 6	XP_426405.4	294	414878	MUC6	6	0	0	5	0	0	0.10	2.60	0.00	0.00
Ovalbumin	P01012.2	43	396058	OVAL	50	15	8	441	123	144	7643.82	89.00	50.00	32.00
Ovalbumin-related protein X	P01013.1	45	420898	OVALX	8	0	0	14	0	0	0.26	22.00	0.00	0.00
Ovalbumin-related protein Y	P01014.1	44	420897	OVALY	15	7	0	31	30	0	1.43	45.00	16.00	0.00
Ovocalyxin-36	F1P1Y2	58	419289	BPIFB8	0	3	3	0	29	18	974.10	0.00	8.00	5.60
Ovocleidin-17	Q9PRS8	15	100313508	OC-17	0	0	4	0	0	58	3074.00	0.00	0.00	17.00
OvoglobulinG2 type AA (TENP)	NP_990357.1	47	395882	BPIFB7	2	0	0	3	0	0	0.06	7.70	0.00	0.00
Ovoinhibitor	P10184.2	52	416235	SPINK5	7	5	0	5	32	0	0.99	20.00	16.00	0.00
Ovomucoid	P01005	23	416236	SPINK7	9	7	0	23	44	0	1.67	44.00	43.00	0.00
Ovostatin	XP_423478.4	165	396151	OVST	12	0	0	8	0	0	0.16	9.20	0.00	0.00
Ovotransferrin	P02789.2	78	396241	TF	77	5	4	273	26	16	853.91	82.00	8.20	7.00
Polymeric Ig receptor	AAP69598.1	71	419848	PIGR	0	2	0	0	6	0	0.17	0.00	4.20	0.00
Serum albumin precursor	F2Z4L6	70	396197	ALB	4	0	0	3	0	0	0.05	12.00	0.00	0.00
Tiarin-like	Q25C35	56	418826	OLFM4	0	5	0	0	21	0	0.59	0.00	12.00	0.00
Vitellogenin-1	NP_001004408.1	211	424533	VTG2	4	0	0	4	0	0	0.08	2.90	0.00	0.00
Vitellogenin-2	NP_001026447.1	205	424533	VTG2	4	4	0	3	11	0	0.37	2.90	2.80	0.00

[a] Information obtained from Scaffold softwarer, version 4.3.4.
[b] Normalized spectra counts.
[c] Adjusted normalized spectra counts.
[d] Information obtained from PubMed database.

VIII. CHAPTER 5. Discussion

General Discussion

Chicken eggs are a foodstuff with high nutritional value for humans. The high level of production and consumption of eggs throughout the world requires a strict surveillance of hygienic quality to prevent the occurrence of egg food-borne diseases that may be transmitted by table eggs (Bedrani et al., 2013). The chicken egg is sterile when laid in non-pathogenic conditions but afterwards it can become contaminated by microbial pathogens despite its physical and chemical barriers (De Reu et al., 2006; Gantois et al., 2009). Eggs contain the necessary nutrients for chick embryo development and to protect from pathogens in order to ensure survival. The chick embryo is primarily protected physically by the eggshell and associated ESM. In addition, an innate immune chemical protection is present which consists of a large number of proteins and peptides with antimicrobial activities throughout the different egg compartments (cuticle, shell, eggshell membranes (ESM), egg white and vitelline membrane (VM)) (Rose et al., 1974; Rehault-Godbert et al., 2011).

The ESM comprises polypeptides that offer protection during chick embryonic development. Many of the proteins and peptides associated with ESM have antimicrobial and immune-modulatory activities that are important for innate immune protection of the chick embryo (Cordeiro et al., 2013; Kovacs-Nolan et al., 2014; Cordeiro and Hincke, 2015; Makkar et al., 2015).

We have used proteomics analysis to identify proteins associated with ESM that are likely to modulate the innate immune responses of the developing embryo as reflected by antimicrobial and anti-endotoxin functionality of the ESM proteins. The study of these antimicrobial proteins is extremely valuable to improve our understanding of the mechanism of avian eggs protection from bacterial invasion and to find new strategies to combat pathogen contamination.

1. Avian innate host defense

The chick embryo is threatened by pathogens during embryonic development. Pathogens may come from hen reproductive organs which lead to contamination of the egg before oviposition, or through penetration of the shell after laying (De Buck et al., 2004). Prior to maturation of adaptive immunity, the innate immune system is the first line of defense against pathogens and functions as the main protection for the chick embryo (Abbas et al., 2006; Kannaki et al., 2015).

Antimicrobial proteins have been identified by proteomic studies and are present in different compartments of the chicken egg (Mann et al., 2006; Mann 2007; Mann, 2008). These antimicrobial proteins participate in the protection of the embryo during its development and contribute to the production of pathogen-free eggs (Hervé-Grépinet et al., 2010). Avian antimicrobial proteins and peptides modulate the chemical protection of eggs and the chick embryo by three main mechanisms: (1) sequestration of essential nutrients from bacteria by the chelation

of minerals or vitamins by egg proteins such as ovotransferrin and avidin, respectively (Shawkey et al., 2008); (2) inactivation of exogenous proteases necessary for microbial metabolism and invasion of host tissues such as egg white protease inhibitors including cystatin, ovomucoid and ovoinhibitor (Schafer et al., 1999); and (3) direct lytic activity on microorganisms by lysozyme or peptides belonging to the β-defensin family that lead to the disruption of the bacterial cell wall (van Dijk et al., 2008).

Another way that the host innate immune system responds to the invasion of pathogens is through recognition of their pathogen-associted molecular patterns (PAMPs) by inducing pattern-recogniton receptors (PRRs) (Alexander and Rietschel, 2001).

TLRs are a multi-gene family in vertebrates whose members have diversified functionally as PRRs to recognize pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS), lipoteichoic acids (LTA), bacterial flagellin, lipoproteins, peptidoglycans, bacterial DNA, as well as single-stranded and double-stranded viral RNA (Janeway and Medzhitov, 2002; Takeda and Akira, 2005; Brownlie et al., 2009; Kannaki et al., 2010). For example, the bacterial cell wall constituents LPS and LTA are PAMPs that are recognized by TLR4 and TLR2, respectively (Han et al., 2003; O'Neill et al., 2007).

Proteins with a BPI and LBP-like domain are also PRRs that recognize and respond to the bacterial cell wall constituents. These proteins mediate signals from bacterial endotoxins such as LPS and modulate cellular signals from LPS (Beamer, 2003; Bingle and Craven, 2004). Both BPI and LBP bind the lipid A

component of LPS via their N-terminal domain (Lamping et al., 1996). BPI is involved in the recognition and neutralization of bacteria by the host innate immune system (Akin et al., 2011). However, LBP is able to increase the LPS-mediated effects on immune cells. The invading pathogen activates a host response that is initiated by LPS binding to LBP in the serum, followed by interaction with soluble or membrane CD14 that can be localized in the serum or on the cell surface (Fig.1). The LPS-CD14-LBP complex is transferred to the TLR4–MD-2 complex on target cells such as macrophages and polymorphonuclear leukocytes in the host (Shimazu et al., 1999). LPS binds to MD-2 and induces the dimerization of the TLR4–MD-2 complex to activate the MyD88 (myeloid differentiation factor)-dependent pathways that trigger the signalling cascade, leading to the expression of pro-inflammatory mediators such as TNF-α (Akira and Takeda, 2004; Gyorfy et al., 2013).

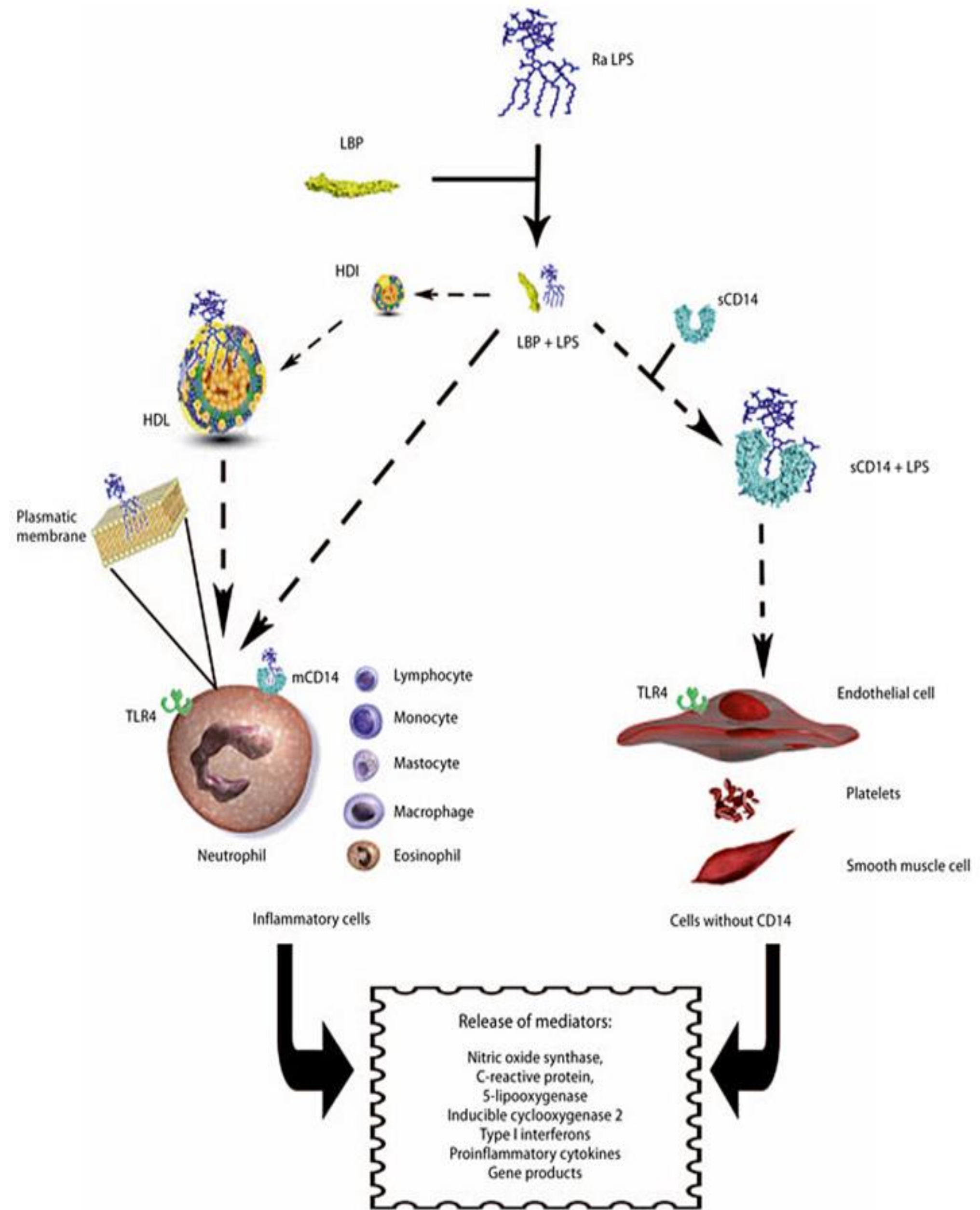

Ra LPS
LBP
HDl
LBP + LPS
sCD14
HDL
sCD14 + LPS
Plasmatic membrane
mCD14
Lymphocyte
Monocyte
Mastocyte
Macrophage
Eosinophil
TLR4
Neutrophil
TLR4
Endothelial cell
Platelets
Smooth muscle cell
Inflammatory cells
Cells without CD14
Release of mediators:
Nitric oxide synthase,
C-reactive protein,
5-lipooxygenase
Inducible cyclooxygenase 2
Type I interferons
Proinflammatory cytokines
Gene products

Fig.1. Recognition and neutralization of LPS in the human body. LPS molecules can be recognized and detoxified by diverse elements in the human body such as LBP,CD14 and AMPs. Moreover, LBP can also transfer LPS to sCD14 or mCD14 that can be recognized byTLR4, initiating the immune response by releasing different mediators by diverse cell types. Protein structures were obtained from the protein data bank (www.pdb.org). When human structures were not available (LBP), a homology model was constructed using Swiss Model (www.swissmodel.expasy.org). Source: Pulido et al., 2012. Lipopolysaccharide neutralization by antimicrobial peptides: a gambit in the innate host defense strategy. *Journal of Innate Immunity*. 4: 327-336, with permission from Karger.

The avian innate immune system has a somewhat different repertoire of PRRs than mammals, including avian TLRs such TLR15 and TLR 21, but the repertoire of potential pathogen-associated molecular patterns (PAMPs) recognized by these PRR is thought to be similar (Kaiser, 2010). For instance, PRRs such as TLR15 and TLR 21 are only detected in the chicken immune system (Keestra et al., 2010; de Zoete et al., 2011). TLR15, which is specific to birds, might recognize LTA and peptidoglycan (Nerren et al., 2010). The presence of TLR15 which is restricted to avian species might be a result of gene gain over evolutionary time to compensate for the absence of certain TLRs in chickens. TLR15 shows similarity (30% identity) with to chicken TLR2 (Roach et al., 2005; Higgs et al., 2006). Indeed, sequence analysis showed that chicken TLR21 is the functional homolog of humanTLR9 (Brownlie et al., 2009)

1.1. Proteins involved in innate immune protection of chicken eggs and developing embryo

In chapter 4, we report our proteomic study that shows the identification of a variety of proteins present in the eggshell membranes (ESMs), extracted from fertilized and unfertilized eggs during incubation using a novel procedure based on (Tris (2-carboxyethyl) phosphine hydrochloride) (TCEP). TCEP is a reducing agent stable and effective at an acidic condition (Winther and Thorpe 2014). TCEP-HCl in the presence of acetic acid at 80°C solubilized the membranes and yielded a material suitable for proteomics analysis (Cordeiro et al., 2015).

Many of the ESM proteins are associated with innate immunity and would provide antimicrobial protection against a broad range of microbes. Some examples of these proteins are lysozyme, avian beta-defensin-11 (AvBD-11), bactericidal/permeability-increasing protein (BPI) proteins, transiently expressed in neural precursor (TENP) and ovocalyxin-36 (OCX-36) (Cordeiro et al., 2013; Cordeiro et al., 2015; Rose-Martel et al., 2015).

Lysozyme shows an antimicrobial activity against Gram-positive bacteria and to a lesser extent Gram-negative bacteria (Wellman-Labadie et al., 2007; Cuperus et al., 2013).

AvBD-9 and AvBD-11 are members of the AvBD family that is expressed in many tissues and plays a significant role in the antimicrobial defense of the chicken hen genital tract. This antimicrobial protection is essential for chick embryonic development and also ensures that table eggs are pathogen-free (Hervé-Grépinet et al., 2010). My study identified AvBD-9, AvBD-11 and gallin in the ESM (Cordeiro et al., 2015); which are also found in the eggshell, egg white and VM (Mann et al., 2006; Mann, 2008; Mann, 2007; Rose-Martel et al., 2015). Recombinant forms of AvBD9 and AvBD11 exhibited antimicrobial effect towards Gram-negative and Gram-positive bacteria (van Dijk et al., 2007; Herve-Grepinet et al., 2010). Gallin (Ovodefensin) is a cationic peptide related to the AvBD family. The gene localization of gallin is on chromosome 3, close to the genes of the other AvBDs. Recombinant gallin possesses antimicrobial activity against *E. coli* (Gong et al., 2010).

LBP/BPI/PLUNC-like proteins are involved in innate immune protection and have been previously identified in chickens (Bingle and Craven, 2004; Chiang et al., 2011). The LPS binding domains of TENP suggest an innate immune protection of eggs against pathogens (Whenham et al., 2014). Purified TENP possesses antibacterial activity specificity against Gram-positive bacteria including *Micrococcus luteus* and *Bacillus subtilis* (Maehashi et al., 2014).

In my work, a number of members of this protein family were identified in the proteomic analysis of eggshell membranes from fertilized eggs, including BPI fold containing family B, member 3 precursor (BPIFB3), BPI fold containing family C protein isoform X2 (BPIFCB), OCX-36 (BPIFB8) and TENP (BPIFB7).

1.2. OCX-36 as pattern recognition protein in innate immune protection of chicken eggs

OCX-36 (BPIFB8), a 36 kDa chicken egg protein was considered to be related to the BPI/LBP/PLUNC superfamily due to the strong similarities between the exon/intron organization of the mammalian *LBP/BPI* and the avian *OCX-36* genes (Gautron et al., 2007). OCX-36 is a highly abundant protein in the uterine fluid during calcification of the eggshell (Gautron et al., 2007). OCX-36 is also present in the ESMs, vitelline membrane and gut tissues (Mann et al., 2006; Gautron et al., 2007; Mann et al., 2007; Mann, 2008; Chiang et al., 2011; Cordeiro et al., 2013).

The functional biological characterization of proteins with LBP/BPI/PLUNC domains is important to characterize their putative LPS-binding properties and antimicrobial activity would be essential to play a role in the innate immune protection. In chapter 2, we reported the characterization of purified OCX-36 protein extracted from the ESM of unfertilized eggs.

Purified OCX-36 protein bound lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 in a dose-dependent manner. Purified OCX-36 bound to LTA from *S. aureus* ATCC 6538 and inhibited its growth. The OCX-36 ability to bind LTA suggested that its inhibition of *S. aureus* is dependent on interaction with bacterial cell wall LTA (Cordeiro et al., 2013). BPI and LBP are known to interact with Gram-positive bacteria through binding to the bacterial cell wall and LTA (Weber et al., 2003).

In addition, some genetic variations of OCX-36 non-synonymous single nucleotide polymorphisms (SNPs) were detected at cDNA 211 position and the corresponding proteins (proline or serine at position 71) were purified from eggs collected from genotyped hens. Genetic variations such as SNPs account for host susceptibility and resistance during the course of infectious diseases (Sorensen et al., 1988; Huebner et al., 2010). Certain LBP SNPs are related to susceptibility to severe infection and multiple organ dysfunction (Zeng et al., 2012). Polymorphisms in the human LBP gene are correlated with the risk of development of bacterial infection (Hubacek et al., 2001; Chien et al., 2008). A recent study reported that the recognition and response to plasma endotoxin may be changed by SNP-derived variability in the levels and/or activity of plasma LBP. In addition, BPI SNPs

were significantly associated with differences in LPS-induced TNF-α release ex vivo and infection frequency (Guinan et al., 2014).

We evaluated the changes in the biological function of OCX-36 SNPs polymorphic forms (Proline-71, Serine-71) by testing the antimicrobial activity of these polymorphic forms against *S. aureus* and comparing their ability to bind to endotoxins LPS and LTA. No difference in binding to *E.coli* O111:B4 LPS binding was detected. No significant differences between OCX-36 polymorphic forms were detected with respect to antimicrobial activity against *S. aureus*. However, it was observed the Proline-71 form binds to LTA significantly greater than the Serine-71 form (Cordeiro et al., 2013).

Our results provide evidence for our hypothesis that OCX-36 shares similar biological function with LBP/BPI/PLUNC protein members. LBP/BPI/PLUNC proteins bind LPS, have antimicrobial activity and participate in the induction of cytokine production (Tobias et al., 1988; Wiesner and Vilcinskas, 2010; Krasity et al., 2011). The PLUNC superfamily and family members (LBP and BPI) are well known PRRs that are activated by PAMPs and contribute to host defense (Barlett et al., 2008).

These findings, described in chapter 2, confirmed our first hypothesis that OCX-36 has a role as A pattern recognition molecule in innate immune protection and would be involved in the endogenous defense of eggs against pathogens.

Investigating the biological function of OCX-36 offered a new insight into the avian chemical innate immune barriers that protect the chick embryo from invading pathogens during its development. The understanding of these proteins involved

in the innate immune protection of avian eggs will lead to improved selection strategies to enhance the egg's innate antimicrobial activity.

1.3. Enhancing innate immune defenses using proteins that modulate the production of proinflammatory mediators.

Bacterial endotoxins such as LPS are amongst the most potent stimuli for the innate immune system (Beutler and Rietschel, 2003). LPS is recognized by TLR4 which is expressed on the surface of macrophages. LPS stimulates the host cells to produce proinflammatory mediators and cytokines such as nitric oxide (NO), tumor necrosis factor (TNF)-α, and interleukins (ILs) (Kumar et al., 2009).

In our *in vitro* and *in vivo* studies (chapter 3), we investigated the impact of purified OCX-36 and digested OCX-36 on the modulation of the effect of LPS at the production of proinflammatory mediators (Kovacs-Nolan et al., 2014). The *in vitro* results from this study revealed that OCX-36 and OCX-36 derived peptides showed potent anti-endotoxin properties. OCX-36 also augmented NO and TNF-α production by macrophages that are important cytotoxic mediators contributing to the bactericidal activity of macrophages. OCX-36-derived peptides showed an inhibitory effect on the production of LPS-induced pro-inflammatory mediators associated with endotoxemia *in vivo*.

The toxic effect of LPS is also neutralized by members of the LBP/BPI/PLUNC protein family. LBP at low concentrations transfers LPS to CD14 molecules and then increases the inflammatory response induced by LPS. On the

other hand, high levels of LBP reduce LPS activation of macrophages (Lamping et al., 1998). Some studies have reported that LBP/BPI/PLUNC proteins neutralize proinflammatory activities of LPS in macrophages, such as induction of cytokine secretion, stimulation of neutrophil oxidase enzymes and NO formation (Schumann, 2001, Lukinskiene et al., 2011). Synthetic peptides based on the LBP sequence have been designed and are able to inhibit LPS-induced septicemia *in vitro* and in a mouse model (Wu et al., 2005; Pulido et al., 2012). For instance, a synthetic peptide LBP-14 enhances the neutralization of LPS effects *in vitro* (Wu et al., 2005).

The findings from our study described in chapter 3 support our second hypothesis that OCX-36 and OCX-36 derived peptides have immune stimulating and anti-endotoxin effects (Kovacs-Nolan et al., 2014).

In unfertilized eggs and freshly laid fertilized eggs, the direct antimicrobial protection by proteins found in the chicken egg compartments such as AvBDs, lysozyme, OCX-36 and TENP are extremely important to protect the egg and developing embryo from the invasion of bacterial pathogens via pores or through the possible micro fractures present in the calcified shell. This is necessary since the unfertilized eggs and laid fertilized eggs initially cannot rely on the innate immune protection of avian TLRs to recognize the presence of pathogens.

Future studies may determine the interaction of OCX-36 with Toll-like receptors (TLR-2 and TLR-4) in order to understand the mechanism of proposed OCX-36 function as a pattern recognition receptor. The identification and isolation of the anti-inflammatory OCX-36 peptides may lead to the development of novel

endotoxin-neutralizing therapeutic agents or conceivably to utilize OCX-36 as a nutraceutical which can be delivered by oral ingestion. Future approaches may lead to the application of OCX-36 in the treatment of sepsis, a fatal disease characterized by an uncontrolled and harmful host reaction to LPS or LTA (Weber et al., 2015). Sepsis is a leading cause of mortality worldwide and thus is a major health concern (Vincent, 2008; Deutschman and Tracey, 2014).

IX. Conclusions

The objectives proposed in this project have been addressed during this thesis research and provided important insights regarding the eggshell membranes contributing to the natural chemical defenses of avian eggs. One of the objectives was to extract and purify OCX-36 from the chicken eggshell membrane to characterize the biological function of OCX-36, which could have applications to mitigate the risk of food-borne disease for egg consumers. Another objective of the project was to assess the potential of OCX-36 for therapeutic and nutraceutical applications through the comparison of native and enzymatically digested OCX-36 with respect to their immune-stimulating and anti-endotoxin properties *in vivo* and *in vitro*. The quantification of the changes in abundance of proteins associated with eggshell membranes during chick embryonic development using proteomics analysis allowed the identification of proteins with antimicrobial and innate immune protection that are essential to protect the chicken embryo from the earliest stages of development until hatching. The project

provides significant contributions to enhancing the knowledge of proteins associated with control of pathogens in chicken eggs, or as therapeutic agents to increase innate immune protection in humans.

X. References

Abbas KA, Lichtman AH, Pillai S (2006) Cellular and Molecular Immunology. Saunders, Elsevier

Abdullah IN (2010) Isolation and identification of some bacterial isolates from table egg. J Vet Sci 3: 59–67

Aguilera O, Quiros LM, Fierro JF (2003) Transferrins selectively cause ion efflux through bacterial and artificial membranes FEBS letters 548: 5-10

Akın H, Tahan G, Türe F, Eren F, Atuğ Ö, Tahan V, Hamzaoglu I, Ture F, Eren F, Imeryuz N, Hamzaoglu HO (2011) Association between bactericidal/permeability increasing protein (BPI) gene polymorphism (Lys216Glu) and inflammatory bowel disease. J Crohns and Colitis 5: 14-18

Akira S, Takeda K (2004) Toll-like receptor signaling. Nat Rev Immunol 7: 499-511

Alabdeh M, Lechevalier V, Nau F, Gautier M, Cochet MF, Jan S and Baron F (2011) Role of incubation conditions and protein fraction on the antimicrobial activity of egg white against *Salmonella enteritidis* and *Escherichia coli*. J Food Prot 74:24–31

Alderton G, Ward WH,Fevold HL (1946) Identification of the bacteria-inhibiting, iron-binding protein of egg white as conalbumin. Arch Biochem 11: 9-13

Alexander C, Rietschel ET (2001) Bacterial lipopolysaccharides and innate immunity. J Endotoxin Res 7: 167–202

Arias JL, Fernandez MS, Dennis JE, Caplan AI (1991) Collagens of the chicken eggshell membranes. Connect Tissue Res 26:37–45

Arias JL, Fink DJ, Xiao SQ, Heuer AH, Caplan AI (1993) Biomineralization and eggshells: cell-mediated acellular compartments of mineralized extracellular matrix. Int Rev Cytol145:217–50

Back JF, Bain JM, Vadehra DV, Burley RW (1982) Proteins of the outer layer of the vitelline membrane. Biochim Biophys Acta 705: 12–19

Bain J, Hall JM (1969) Observations on the development and structure of the vitelline membrane of the hen's egg: An electron microscope study. Aust J Biol Sci 22: 653–665

Bain MM (1992) Eggshell strength: a relationship between the mechanism of failure and the ultrastructural organization of the mammillary layer. Brit Poultry Sci 33: 303–319

Bain MM, McDade K, Burchmore R, Law A, Wilson PW, Schmutz M, Preisinger R, Dunn IC (2013) Enhancing the egg's natural defence against bacterial penetration by increasing cuticle deposition. Anim Genet 44: 661-668

Baláž M (2014) Eggshell membrane biomaterial as a platform for applications in materials science. Acta Biomater 10: 3827-3843

Baron F, Gautier M, Brulé G (1999) Rapid growth of Salmonella enteritidis in egg white reconstituted from industrial egg white powder. J Food Prot 62: 585-591

Baron F, Jan S, Nys Y, Bain M, Immerseel FV (2011). Egg and egg product microbiology. Improving the safety and quality of eggs and egg products. Volume 1: Egg chemistry, production and consumption, 330-350

Bartlett JA, Hicks BJ, Schlomann JM, Ramachandran S, Nauseef WM, McCray PB (2008) PLUNC is a secreted product of neutrophil granules. J Leukocyte Biol 83: 1201-1206

Bausek N, Waclawek M, Schneider WJ, Wohlrab F (2000) The major chicken envelope protein ZP1 is different from ZPB and is synthesized in the liver. J Biol Chem 275:28866–28872

Beamer LJ (2003) Structure of human BPI (bactericidal/permeability-increasing protein) and implications for related proteins. Biochem Soc T 31: 791-794

Bedrani L, Helloin E, Guyot N, Réhault-Godbert S, Nys Y (2013) Passive maternal exposure to environmental microbes selectively modulates the innate defences of chicken egg white by increasing some of its antibacterial activities. BMC Microbiol 13: 128

Bellairs R, Boyde A (1969) Scanning electron microscopy of the shell membranes of the hen’s egg. Z Zellforsch Mikrosk Anat 96: 237–49

Bellairs R, Osmond M (2014) Chapter 13 - Extra-Embryonic Membranes. Atlas of Chick Development (Third Edition), Pages 127-129. Academic Press

Benarafa C, Remold-O'Donnell E (2005) The ovalbumin serpins revisited: perspective from the chicken genome of clade B serpin evolution in vertebrates. Proc Natl Acad Sci USA 102: 11367-11372

Beutler B, Rietschel ET (2003) Innate immune sensing and its roots: the story of endotoxin. Nat Rev Immunol 3: 169-176

Bingle CD, Craven CJ (2002) PLUNC: a novel family of candidate host defence proteins expressed in the upper airways and nasopharynx. Hum Mol Genet 11: 937–943

Bingle CD, Craven CJ (2004) Meet the relatives: a family of BPI-and LBP-related proteins Trends Immunol 25: 53-55

Birkhead TR, Sheldon BC, Fletcher F (1994) A comparative study of sperm-egg interactions in birds. J Reprod Fertil 101: 353-361

Board RG, Tranter HS (1995) The microbiology of eggs. In: W. J. Stadelman and Coterill O. J. (eds). Egg Science and technology. 4th ed. Haworth Press Inc. New York

Brownlie R, Zhu J, Allan B, Mutwiri GK, Babiuk LA, Potter A, Griebel P (2009) Chicken TLR21 acts as a functional homologue to mammalian TLR9 in the recognition of CpG oligodeoxynucleotides. Mol Immunol 46: 3163-3170

Burley RW, Vadehra DV (1989) The Avian Egg – Chemistry and Biology, John Wiley and Sons, New York

Byrne BM, Gruber MABG, Ab G (1989) The evolution of egg yolk proteins. Prog Biophys Mol Bio 53: 33-69

Cain CJ, Heyn ANJ (1964) X-ray diffraction studies of the crystalline structure of the avian egg shell. Biophys J 4: 23-29

Cason JA, Cox NA and Bailey JS (1994) Transmission of Salmonella typhimurium during hatching of broiler chicks. Avian Dis 583-588

Chiang SC, Veldhuizen EJ, Barnes FA, Craven CJ, Haagsman HP, Bingle CD (2011) Identification and characterisation of the BPI/LBP/PLUNC-like gene repertoire in chickens reveals the absence of a LBP gene. Dev Comp Immunol 35: 285-295

Chien JW, Boeckh MJ, Hansen JA, Clark JG (2008) Lipopolysaccharide binding protein promoter variants influence the risk for Gram-negative bacteremia and mortality after allogeneic hematopoietic cell transplantation. Blood 111:2462–2469

Chousalkar KK, Flynn P, Sutherland M, Roberts JR, Cheetham BF (2010) Recovery of *Salmonella* and *Escherichia coli* from commercial shell eggs and effect of translucency on bacterial penetration in eggs. Int J Food Microbiol 142: 207-213

Cook Sr MI, Toranzos B, Arendt GAWJ (2005) Incubation reduces microbial growth on eggs shells and the opportunity for trans-shell infection. Ecol Lett 8:532–7

Cordeiro CM, Hincke MT (2011) Recent patents on eggshell: shell and membrane applications. Recent Pat Food Nutr Agric 3: 1-8

Cordeiro CMM, Esmaili H, Ansah G, Hincke MT (2013) Ovocalyxin-36 is a pattern recognition protein in chicken eggshell membranes. PloS One 8: e84112

Cordeiro CMM, Hincke MT (2015) Quantitative proteomics analysis of eggshell membrane proteins during chick embryonic development. J Proteomics 130:11-25.

Cox NA, Berrang ME and Cason JA (2000) Salmonella penetration of egg shells and proliferation in broiler hatching eggs--a review. Poultry Sci 79: 1571-1574

Cuperus T, Coorens M, van Dijk A, Haagsman HP (2013) Avian host defense peptides. Dev Comp Immunol 41: 352-369

D'Ambrosio C, Arena S, Scaloni A, Guerrier L, Boschetti E, Mendieta ME, Citterio A, Righetti PG (2008) Exploring the chicken egg white proteome with combinatorial peptide ligand libraries. J Proteome Res 7: 3461-3474

De Buck J, Van Immerseel F, Haesebrouck F, Ducatelle R (2004) Colonization of the chicken reproductive tract and egg contamination by Salmonella. J Appl Microbiol 97:233–245

De Oliveira JE, Uni Z, Ferket PR (2008) Important metabolic pathways in poultry embryos prior to hatch. World Poultry Sci J 64:488-499

De Reu K, Grijspeerdt K, Messens W, Heyndrickx M, Uyttendaele M, Debevere J, Herman L (2006) Eggshell factors influencing eggshell penetration and whole egg contamination by different bacteria, including Salmonella enteritidis. Int J Food Microbiol 112: 253-260

De Zoete MR, Bouwman LI, Keestra, AM, van Putten JP (2011) Cleavage and activation of a toll-like receptor by microbial proteases. Proc Natl Acad Sci USA 108: 4968–4973

Debruyne I, Stockx J (1982) A macromolecular sialoglycoprotein of hen's ovovitelline membrane. Arch Int Physiol Biophys 90: B104–B105

Deeley RG, Mullinix DP, Wetekam W, Kronenberg HM, Meyers M, Eldridge JD, Goldberger RF (1975) Vitellogenin synthesis in the avian liver. Vitellogenin is the precursor of the egg yolk phosphoproteins. J Biol Chem 250: 9060-9066

Deignan T, Alwan A, Malone L, Kelly J, O'Farrelly C (2001) Hen egg yolk preventsbacterial adherence: a novel function for a familiar food. J Food Sci 66:158–61

Dennis JE, Xiao SQ, Agarwal M, Fink DJ, Heuer AH, Caplan AI (1996) Microstructure of matrix and mineral components of eggshells from white leghorn chickens (Gallus gallus). J Morphol 228: 287-306

DeReu K, Grijspeerdt K, Messens W, Heyndrickx M, Uyttendaele M, Debevere J, Herman L (2006) Eggshell factors influencing eggshell penetration and whole egg contamination by different bacteria, including *Salmonella enteritidis*. Int J Food Microbiol 112: 253–260

Deutschman CS, Tracey KJ (2014) Sepsis: current dogma and new perspectives. Immunity 4: 463-475

EFSA (2009) Community summary report, food-borne outbreaks in the European Union in 2007.The EFSA Journal 271: 1–128

Evans EW, Beach FG, Moore KM, Jackwood MW, Glisson JR, Harmon BG (1995) Antimicrobial activity of chicken and turkey heterophil peptides CHP1, CHP2, THP1, and THP3.Vet Microbiol 47: 295–303

Faostat (Food and Agriculture Organization of the United Nations Statistics) (2014)(http://faostat3.fao.org.proxy.bib.uottawa.ca/faostat-gateway/go/to/download/FB/CL/E)

Farinazzo A, Restuccia U, Bachi A, Guerrier L, Fortis F, Boschetti E, Fasoli E, Citterio A, Righetti PG (2009) Chicken egg yolk cytoplasmic proteome, mined via combinatorial peptide ligand libraries. J Chromatogr A 1216:1241–52

Fernandez MS, Araya M, Arias JL (1997) Eggshells are shaped by a precise spatio-temporal arrangement of sequentially deposited macromolecules. Matrix Biol 16:13-20

Fernandez MS, Moya A, Lopez L, Arias JL (2001) Secretion pattern,ultrastructural localization and function of extracellular matrix molecules involved in eggshell formation. Matrix Biol 19: 793–803

Fromm D (1966) The influence of ambient pH on moisture content and yolk index of the hen's yolk. Poultry Sci 45: 374-379

Gabrielli MG, Accili D (2010) The chick chorioallantoic membrane: a model of molecular, structural, and functional adaptation to transepithelial ion transport and barrier function during embryonic development. J Biomed Biotechnol: 1-13

Gabrielli MG, Cox JV, Materazzi G, Menghi G (2004) Cell type-specific and developmentally regulated expression of the AE1 anion exchanger in the chicken chorioallantoic membrane. Histochem Cell Biol 121:189-199

Gally F, Di YP, Smith SK, Minor MN, Liu Y, Bratton DL, Frasch SC, Michels NM, Case SR, Chu HW (2011) SPLUNC1 promotes lung innate defense against Mycoplasma pneumoniae infection in mice Am J Pathol 178:2159-67

Gantois I, Ducatelle R, Pasmans F, Haesebrouck F, Gast R, Humphrey TJ, Immerseel FV (2009) Mechanisms of egg contamination by *Salmonella* enteritidis. FEMS Microbiol Rev 33: 718–738

Garibaldi JA (1970) Role of microbial iron transport compounds in bacterial spoilage of eggs. Appl Microbiol 20:558–60

Gautron J, Hincke MT, Nys Y (1997) Precursor matrix proteins in the uterine fluid change with stages of eggshell formation in hens. Connect Tissue Res 36: 195-210

Gautron J, Hincke MT, Panheleux M, Garcia-Ruiz JM, Boldicke T, Nys Y (2001) Ovotransferrin is a matrix protein of the hen eggshell membranes and basal calcified layer. Connect Tissue Res 42: 255-267

Gautron J, Murayama E, Vignal A, Morisson M, McKee MD, Rehault S, Labas V, Belghazi M, Vidal ML, Nys Y, Hincke MT (2007) Cloning of ovocalyxin-36, a novel chicken eggshell protein related to lipopolysaccharide-binding proteins, bactericidal permeability-increasing proteins, and plunc family proteins. J Biol Chem 282: 5273–5286

Gazzano-Santoro H, Parent JB, Grinna L, Horwitz A, ParsonsT, Theofan G, Elsbach P, Weiss J, Conlon PJ (1992) High-affinity binding of the bactericidal/permeability-increasing protein and a recombinant amino terminal fragment to the lipid a region of lipopolysaccharide. Infect Immun 60: 4754–4761

Giansanti F, Massucc MT, Giardi MF, Nozza F, Pulsinelli E,Nicolini C, Botti D, Antonini G (2005) Antiviral activity of ovotransferrin derived peptides. Biochem Biophys Res Commun 331: 69–73

Gioannini TL, Teghanemt A, Zarember KA, Weiss JP (2003) Regulation of interactions of endotoxin with host cells. J. Endotoxin Res 9: 401–408

Gong D, Wilson PW, Bain MM, McDade K, Kalina J, Herve-Grepinet V, Nys Y, Dunn IC (2010) Gallin; an antimicrobial peptide member of a new avian defensin family, the ovodefensins, has been subject to recent gene duplication. BMC Immunol 11:12

Gorr SU, Abdolhosseini M, Shelar A, Sotsky J (2011) Dual host defence functions of splunc2/psp and synthetic peptides derived from the protein. Biochem Soc Trans 39: 1028–1032

Griffin HD, Perry MM, Gilbert AB (1984) Yolk formation. Physiol Biochem Domest Fowl 5: 345-380

Guard-Petter J (2001) The chicken, the egg and Salmonella enteritidis. Environ Microbiol 3: 421–30

Guérin-Dubiard C, Pasco M, Hietanen A, del Bosque AQ, Nau F, Croguennec T (2005) Hen egg white fractionation by ion-exchange chromatography. J Chromatogr A 1090: 58-67

Guérin-Dubiard C, Pasco M, Mollé D, Désert C, Croguennec T, Nau F (2006) Proteomic analysis of hen egg white. J Agri Food Chem 54: 3901-3910

Guinan EC, Palmer CD, Mancuso CJ, Brennan L, Stoler-Barak L, Kalish LA, Suter EE, Gallington LC, Huhtelin DP, Mansilla M, Schumann RR, Murray JC, Weiss J, Levy O (2014). Identification of single nucleotide polymorphisms in hematopoietic cell transplant patients affecting early recognition of, and response to, endotoxin. Innate Immun 20: 697-711

Guru PS, Dash S (2014) Sorption on eggshell waste—A review on ultrastructure, biomineralization and other applications. Adv Colloid Interfac 209: 49-67

Gyorfy Z, Duda E, Vizler C (2013) Interactions between LPS moieties and macrophage pattern recognition receptors. Vet Immunol Immunop 152: 28-36

Han SH, Kim JK, Martin M, Michalek SM, Nahm MH (2003) Pneumococcal Lipoteichoic Acid (LTA) Is Not as Potent as Staphylococcal LTA in Stimulating Toll-Like Receptor 2. Infect Immun 71: 5541-5548

Hancock RE (1997) Peptide antibiotics. The Lancet 349: 418-422

Handelman GJ, Nightingale ZD, Lichtenstein AH, Schaefer EJ, Blumberg JB (1999) Lutein and zeaxanthin concentrations in plasma after dietary supplementation with egg yolk. Am J Clin Nutr 70: 247-251

Harris ED, Blount JE, Leach RM (1980) Localization of lysyl oxidase in hen oviduct: implications in egg shell membrane formation and composition. Science 208: 55-56

Hellgren O, Ekblom R (2010) Evolution of a cluster of innate immune genes (betadefensins) along the ancestral lines of chicken and zebra finch. Immunome Res 6: 3

Herve-Grepinet V, Rehault-Godbert S, Labas V, Magallon T, Derache C, Lavergne M, Gautron J, Lalmanach AC, Nys Y (2010) Purification and characterization of avian beta-defensin 11, an antimicrobial peptide of the hen egg. Antimicrob Agents Chemother 54: 4401–4409

Higgs R, Cormican P, Cahalane S, Allan B, Lloyd AT, Meade K, James T, Lynn DJ, Babiuk LA, O'Farrelly C (2006) Induction of a novel chicken Toll-like receptor following Salmonella enterica serovar Typhimurium infection Infect Immun 74: 1692–1698

Higgs R, Lynn DJ, Gaines S, McMahon J, Tierney J, James T, Lloyd AT, Mulcahy G, O'Farrelly C (2005) The synthetic form of a novel chicken betadefensin identified in silico is predominantly active against intestinal pathogens. Immunogenetics 57: 90–98

Hincke M, Gautron, J, Nys Y, Rodriguez-Navarro AB, McKee MD, Bain M, Immerseel F V (2011)The eggshell: structure and protective function.

Improving the safety and quality of eggs and egg products. Volume 1: Egg chemistry, production and consumption, 151-182

Hincke MT, Gautron J, Panheleux M, Garcia-Ruiz J, McKee MD, Nys Y(2000) Identification and localization of lysozyme as a component of eggshell membranes and eggshell matrix. Matrix Biol19:443–53

Hincke MT, Nys Y, Gautron J (2010) The role of matrix proteins in eggshell formation. The Journal of Poultry Science 47: 208-219

Hincke MT, Nys Y, Gautron J, Mann K, Rodriguez-Navarro AB, McKee MD (2012)The eggshell: structure, composition and mineralization. Front Biosci 17: 1266-80

Hincke MT, Wellman-Labadie O, McKee MD, Gautron J, Nys Y, Mann K (2008) Biosynthesis and structural assembly of eggshell components. Egg bioscience and biotechnology, 97-128

Holen E, Bolann B, Elsayed S (2001) Novel B and T cell epitopes of chicken ovomucoid (Gal d 1) induce T cell secretion of IL-6, IL-13, and IFN-γ. Clin Exp Allergy 31: 952-964

Howarth BJr, Digby ST (1973) Evidence for the penetration of the vitelline membrane of the hen's ovum by a trypsin-like acrosomal enzyme. J Reprod Fertil 33: 123-125

Hubacek JA, Stüber F, Fröhlich D, Book M, Wetegrove S, Ritter M, Rothe G, Schmitz G (2001) Gene variants of the bactericidal/permeability increasing protein and lipopolysaccharide binding protein in sepsis

patients: gender-specific genetic predisposition to sepsis. Crit Care Med 29: 557-561

Huebner C, Petermann I, Lam WJ, Shelling AN, Ferguson LR (2010) Characterization of single-nucleotide polymorphisms relevant to inflammatory bowel disease in commonly used gastrointestinal cell lines. Inflamm Bowel Dis 2:282-95

Hughey VL, Johnson EA (1987) Antimicrobial activity of lysozyme against bacteria involved in food spoilage and food-born disease. Appl Environ Microbiol 53:2165–70

Ibrahim HR, Iwamori E, Sugimoto Y, Aoki T (1998) Identification of a distinct antibacterial domain within the N-lobe of ovotransferrin. Biochim Biophys Acta (BBA)-Mol Cell Res 1401: 289-303

Ibrahim HR, Sugimoto Y, Aoki T (2000) Ovotransferrin antimicrobial peptide (OTAP-92) kills bacteria through a membrane damage mechanism. Biochim Biophys Acta (BBA)-General Subj 1523: 196-205

Ibrahim HR, Thomas U, Pellegrini A (2001) A helix-loop-helix peptide at the upper lip of the active site cleft of lysozyme confers potent antimicrobial activity with membrane permeabilization action. J Biol Chem 276: 43767-43774

Itoh T, Munakata K, Adachi S, Hatta H, Nakamura T, Kato T, Kim M (1990) Chalaza and egg yolk membrane as excellent sources of sialic acid (N-acetylneuraminic acid) for an industrial-scale preparation. Japanese Journal of Zootechnical Science 61: 277-282

Janeway Jr CA, Medzhitov R (2002) Innate immune recognition. Annu Rev Immunol 20:197–216

Jensen C (1969) Ultrastructural changes in the avian vitelline membrane during embryonic development. J Embryol Exp Morph 21: 467-484

Kaiser P (2010) Advances in avian immunology—prospects for disease control: a review. Avian Pathol 39: 309-324

Kannaki TR, Reddy MR, Shanmugam M, Verma PC, Sharma RP (2010) Chicken Toll-like receptors and their role in immunity. Worlds Poult Sci J 66:727–738

Kannaki TR, Reddy MR, Verma PC, Shanmugam M (2015) Differential Toll-Like Receptor (TLR) mRNA Expression Patterns during Chicken Embryological Development. Anim Biotechnol 26: 130-135

Kaweewong K, Garnjanagoonchorn W, Jirapakkul W, Roytrakul S (2013) Solubilization and identification of hen eggshell membrane proteins during different times of chicken embryo development using the proteomic approach. Protein J 32: 297-308

Keestra AM, de Zoete, MR, Bouwman LI, van Putten JP (2010) Chicken TLR21 is an innate CpG DNA receptor distinct from mammalian TLR9. J Immunol 185: 460–467

Keller LH, Benson CE, Krotec K, Eckroade RJ (1995) Salmonella enteritidis colonization of the reproductive tract and forming and freshly laid eggs of chickens. Infect Immun 63: 2443–9

Kido S, Morimoto A, Kim F, Doi Y (1992) Isolation of a novel protein from the outer layer of the vitelline membrane. Biochem J 286: 17–22

Klotman ME, Chang TL (2006) Defensins in innate antiviral immunity. Nat Rev Immunol 6: 447–456

Kodali VK, Gannon SA, Paramasivam S, Raje S, Polenova T, Thorpe C (2011) A novel disulfide-rich protein motif from avian eggshell membranes. PloS one 6: e18187

Kovacs-Nolan J, Cordeiro C, Young D, Mine Y, Hincke M (2014) Ovocalyxin-36 is an effector protein modulating the production of proinflammatory mediators. Vet Immunol Immunop 160: 1-11

Krasity BC, Troll JV, Weiss JP, McFallngai MJ (2011) LBP/BPI proteins and their relatives: Conservation over evolution and roles in mutualism. Biochem Soc Trans 39: 1039-1044

Kumar H, Kawai T, Akira S (2009) Pathogen recognition in the innate immune response. Biochem J 420: 1-16

Lamping N, Dettmer R, Schroder NWJ, Pfeil D, Hallatschek W, Burger R, Schumann RR (1998) LPS-binding protein protects mice from septic shock caused by LPS and gram negative bacteria. J Clin Invest 101: 2065–71

Lamping N, Hoess A, Yu B, Park TC, Kirschning CJ, Pfeil D, Reuter D, Wright SD, Herrmann F, Schumann RR (1996). Effects of site-directed mutagenesis of basic residues (Arg 94, Lys 95, Lys 99) of lipopolysaccharide (LPS)-binding protein on binding and transfer of

LPS and subsequent immune cell activation. J Immunol 157: 4648-4656

Leach RM (1982) Biochemistry of the organic matrix of the eggshell. Poult Sci

Lee-Huang S, Huang PL, Sun Y, Huang PL, Kung HF, Blithe DL, Chen H C (1999) Lysozyme and RNases as anti-HIV components in β-core preparations of human chorionic gonadotropin. Proc Natl Acad Sci USA 96: 2678-2681

Lehrer RI, Ganz T (2002) Defensins of vertebrate animals. Curr Opin Immunol 14:96–102

Levy O, Canny G, Serhan CN, Colgan SP (2003) Expression of BPI (bactericidal/permeability-increasing protein) in human mucosal epithelia. Biochem Soc T 31: 795-800

Liong JWW, Frank JF, Bailey S (1997) Visualization of eggshell membranes and their interaction with Salmonella enteritidis using confocal scanning laser microscopy. J Food Prot 60: 1022–8

Losso JN, Nakai S, Charter EA (2000) Lysozyme. Natural food antimicrobial systems 185: 210

Lukinskiene L, Yang L, Susan D, Reynolds CS, Barry R S, George D L, Jay K K, Peter YD (2011) Antimicrobial activity of PLUNC protects against Pseudomonas aeruginosa infection. J Immunol 187: 382-390

Lunam CA, Ruiz J (2000) Ultrastructural analysis of the eggshell: contribution of the individual calcified layers and the cuticle to hatchability and egg viability in broiler breeders. Brit Poultry Sci 41: 584-592

Lynn DJ, Higgs R, Lloyd AT, O'Farrelly C, Herve-Grepinet V, Nys Y, Brinkman FS, Yu PL, Soulier A, Kaiser P, Zhang G, Lehrer RI (2007) Avian beta-defensin nomenclature: a community proposed update. Immunol Lett 110: 86–89

MacNeil JH (2005) Hatchery eggshell waste processing method and device. US6899294

MacNeil JH (2006) Method and apparatus for separating a protein membrane and shell material in waste egg shells. US7007806

Maehashi K, Ueda M, Matano M, Takeuchi J, Uchino M, Kashiwagi Y, Watanabe T (2014) Biochemical and Functional Characterization of Transiently Expressed in Neural Precursor (TENP) Protein in Emu Egg White. J Agri Food Chem 62: 5156-5162

Mageed AMA, Isobe N, Yoshimura, Y (2009) Immunolocalization of avian beta-defensins in the hen oviduct and their changes in the uterus during eggshell formation. Reproduction 138: 971–978

Makkar S, Rath NC, Packialakshmi B, Huff WE, Huff GR (2015) Nutritional effects of egg shell membrane supplements on chicken performance and immunity. Poultry Sci 94: 1184-1189

Mann K (2007) The chicken egg white proteome. Proteomics 7: 3558-3568

Mann K (2008) Proteomic analysis of the chicken egg vitelline membrane. Proteomics 8: 2322–32

Mann K, Maček B, Olsen JV (2006) Proteomic analysis of the acid-soluble organic matrix of the chicken calcified eggshell layer. Proteomics 6: 3801-3810

Mann K, Mann M (2008) The chicken egg yolk plasma and granule proteomes. Proteomics 8: 178-191

Mannion BA, Weiss J, Elsbach P (1990) Separation of sublethal and lethal effects of the bactericidal/permeability increasing protein on Escherichia coli. J Clin Invest 85: 853–860

Mayes FJ, Takeballi MA (1983) Microbial contamination of the hen's egg: a review. J Food Prot 46:1092–8

McCully K, Mok C, Common R (1962) Paper electrophoresis characterization of proteins and lipoproteins of hen’s yolk. Can J Biochem Physiol 40: 937–952

Medzhitov R, Janeway C (2000) Innate immune recognition: mechanisms and pathways. Immunol Rev 173: 89-97

Mikšík I, Sedláková P, Lacinová K, Pataridis S, Eckhardt A (2010) Determination of insoluble avian eggshell matrix proteins. Anal Bioanal Chem 397: 205-214

Mine Y, Ma F, Lauriau S (2004) Antimicrobial peptides released by enzymatic hydrolysis of hen egg white lysozyme. J Agri Food Chem 5: 1088-1094

Miyagawa S, Nishino N, Kamata R, Okamura R, Maeda H (1991) Effects of protease inhibitors on growth of Serratia marcescens and Pseudomonas aeruginosa. Microb Pathog 11:137–41

Miyake M, Utsuno E, Noda M (2000) Binding of avian ovomucoid to shiga-like toxin type 1 and its utilization for receptor analog affinity chromatography. Anal Biochem 281:202–8

Miyamoto T, Baba E, Tanaka T, Sasai K, Fukata T, Arakawa A (1997) Salmonella enteritidis contamination of eggs from hens inoculated by vaginal, cloacal, and intravenous routes. Avian Dis 41: 296–303

Moffatt CRM, Musto J (2013) *Salmonella* and egg-related outbreaks. Microbiol Aust 34: 94–98

Molla A, Matsumura Y, Yamamoto T, Okamura R, Maeda H (1987) Pathogenic capacity of proteases from Serratia marcescens and Pseudomonas aeruginosa and their suppression by chicken egg white ovomacroglobulin. Infect Immun 55:2509–17

Moran ET (2007) Nutrition of the developing embryo and hatchling. Poultry Sci 86:1043-1049

Muramatsu T, Hiramoto K, Koshi N, Okumura J, Miyoshi S and Mitsumoto T (1990) Importance of albumen content in whole-body protein synthesis of the chicken embryo during incubation. Br Poult Sci 31:101–6

Nakano T, Ikawa NI, Ozimek L (2003) Chemical composition of chicken eggshell and shell membranes. Poultry Sci 82: 510-514

Nash JA, Ballard TNS, Weaver TE, Akinbi HT (2006) The peptidoglycan-degrading property of lysozyme is not required for bactericidal activity in vivo. J Immunol 177: 519-526

Nerren JR, Haiqi HE, Genovese K, Kogut MH (2010) Expression of the avian-specific toll-like receptor 15 in chicken heterophils is mediated by Gram-negative and Gram-positive bacteria, but not TLR agonists. Vet Immunol Immunop 136: 151–156

Noble RC, Cocchi M (1990) Lipid metabolism and the neonatal chicken. Prog Lipid Res 29:107–140

Nys Y, Gautron J (2007). Structure and formation of the eggshell. In Bioactive Egg Compounds (pp. 99-102). Springer Berlin Heidelberg.

Nys Y, Gautron J, Garcia-Ruiz JM, Hincke MT (2004) Avian eggshell mineralization: biochemical and functional characterization of matrix proteins. Comptes Rendus Palevol 3: 549-562

Nys Y, Guyot N, Bain M, Immerseel FV (2011) Egg formation and chemistry. Improving the safety and quality of eggs and egg products. Volume 1: Egg chemistry, production and consumption 83-132

Nys Y, Hincke MT, Arias JL, Garcia-Ruiz JM, Solomon SE (1999) Avian eggshell mineralization. Poult Avian Biol Rev 10: 143-166

Nys Y, Zawadzki J, Gautron J, Mills AD (1991) Whitening of brown-shelled eggs: mineral composition of uterine fluid and rate of protoporphyrin deposition. Poultry Sci 70: 1236-1245

O'Farrelly C, Branton D, Wanke CA (1992) Oral ingestion of egg yolk immunoglobulin from hens immunized with an enterotoxigenic Escherichia coli strain prevents diarrhea in rabbits challenged with the same strain. Infect Immun 60:2593–7

Olsen MW, Fraps RM (1944) Maturation, fertilization and early cleavage of the egg of the domestic turkey. J Morphol 74: 297-309

O'Neill LA, Bowie AG (2007) The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol 7: 353-364

Ooi CE, Weiss J, Doerfler ME, Elsbach P (1991) Endotoxin- neutralizingproperties of the 25 kd n-terminal fragment and a newly isolated 30 kd c terminal fragment of the 55–60 kd bactericidal/permeability-increasing protein of human neutrophils. J Exp Med 174: 649–655

Oratore A, D'Andrea G, D'Alessandro AM, Moreton K, Williams J (1990). Binding and iron delivering of monoferric ovotransferrins to chick-embryo red blood cells (CERBC). Biochem Int 22:111–118

Parsons AH (1982) Structure of the eggshell. Poult Sci 61:2013

Pellegrini A, Thomas U, Wild P, Schraner E, Von Fellenberg R (2000) Effect of lysozyme or modified lysozyme fragments on DNA and RNA synthesis and membrane permeability of Escherichia coli. Microbiol Res 155: 69-77

Predki PF, Harford C, Brar P, Sarkar B (1992) Further characterization of the N-terminal copper (II)-and nickel (II)-binding motif of proteins. Studies of metal binding to chicken serum albumin and the native sequence peptide. Biochem J 287: 211-215

Pulido D, Nogués MV, Boix E, Torrent M (2012) Lipopolysaccharide neutralization by antimicrobial peptides: a gambit in the innate host defense strategy. J Innate Immun 4: 327-336

Rehault-Godbert S, Herve-Grepinet V, Gautron J, Cabau C, Nys Y and Hincke M (2011) Molecules involved in chemical defence of the chicken egg. Improving the safety and quality of eggs and egg products 1: 183-208

Réhault-Godbert S, Labas V, Helloin E, Hervé-Grépinet V, Slugocki C, Berges M, Bourin MC, Brionne A, Poirier, Gautron J, Coste F, Nys Y (2013) Ovalbumin-related protein X is a heparin-binding ov-serpin exhibiting antimicrobial activities. J Biol Chem 288: 17285-17295

Rehault-Godbert S, Mann K, Bourin M, Brionne A, Nys Y (2014) Effect of Embryonic Development on the Chicken Egg Yolk Plasma Proteome after 12 Days of Incubation. J Agr Food Chem 62: 2531-2540

Retzek H, Steyrer E, Sanders EJ, Nimpf J, Schneider WJ (1992) Molecular cloning and functional characterization of chicken cathepsin D, a key enzyme for yolk formation. DNA Cell Biol 11: 661672

Ribatti D, Nico B, Vacca A, Roncali L, Burri PH and Djonov V (2001). Chorioallantoic membrane capillary bed: A useful target for studying angiogenesis and anti-angiogenesis in vivo. Anat Rec 264: 317-324

Ricke SC, Jones DR, Gast RK (2001) Eggs and egg products. Compendium of methods for the microbiological examinations of foods, 5th ed, in press. American Public Health Association, Washington, DC

Roach JC, Glusman G, Rowen L, Kaur A, Purcell MK, Smith KD, Hood LE, Aderem A (2005) The evolution of vertebrate Toll-like receptors. Proc Natl Acad Sci USA 102: 9577–9582

Rodriguez-Navarro A, Kalin O, Nys Y and Garcia-Ruiz JM (2002) Influence of the microstructure on the shell strength of eggs laid by hens of different ages. Brit Poultry Sci 43: 395-403

Rodríguez-Navarro AB, Marie P, Nys Y, Hincke MT, Gautron J (2015) Amorphous calcium carbonate controls avian eggshell mineralization: a new paradigm for understanding rapid eggshell calcification. J Struct Biol 190: 291-303

Romanoff AI, Romanoff AJ (1949) The Avian Egg, Wiley, New York

Romanoff AL (1960) The avian embryo. Structural and functional development. The Macmillan Company, New York.

Romanoff AL (1967) Biochemistry of the Avian embryo. John Wiley & Sons Inc

Rose ME, Orlans E, Buttress N (1974) Immunoglobulin classes in hens Egg – their segregation in yolk and white. Eur J Immunol 4: 521–523

Rose-Martel M, Du J, Hincke MT (2012) Proteomic analysis provides new insight into the chicken eggshell cuticle. J Proteomics 75: 2697-2706

Rose-Martel M, Hincke M (2013) Eggshell as a Source of Novel Bioactive Molecules. J Food Sci Eng 3: 219

Rose-Martel M, Smiley S, Hincke MT (2015) Novel identification of matrix proteins involved in calcitic biomineralization. J Proteomics 116: 81-96

Sava G (1995) Pharmacological aspects and therapeutic applications of lysozymes. Exs 75: 433-449

Schade K, Calzado EG, Sarmiento R, Chacana PA, Porankiewicz-Asplund J, Terzolo HR (2005) Chicken egg yolk antibodies (IgY-technology): A review of progress in production and use in research and human and veterinary medicine. Altern Lab Anim 33: 129–154

Schafer A, Drewes W, Schwagele F (1999) Effect of storage temperature and time on egg white protein. Nahrung-Food 43:86–89

Schalabach MR and Bates GW (1975) The synergistic binding of anions and Fe^{3+} by transferrin. J Biol Chem 250: 2182–2188

Schmidt W (1992) The amniotic fluid compartment: the fetal habitat. Anat Embryol Cell Biol 124:1–100

Schneider WJ (2009) Receptor-mediated mechanisms in ovarian follicle and oocyte development. Gen Comp Endrocrinol 163:18-23

Schultz H, Weiss J, Carroll SF, Gross WL (2001) The endotoxin-binding bactericidal/permeability-increasing protein (BPI): a target antigen of autoantibodies. J Leukocyte Biol 69: 505-512

Schumann RR (2001) High concentrations of lipopolysaccharide-binding protein in serum of patients with severe sepsis or septic shock inhibit the lipopolysaccharide response in human monocytes. Blood 98: 3800–3808

Schumann RR, Leong SR, Flaggs GW, Gray PW, Wright SD, Mathison JC, Tobias PS, Ulevitch RJ (1990) Structure and function of lipopolysaccharide binding protein. Science 249:1429–1431

Scott MG, Dullaghan E, Mookherjee N, Glavas N, Waldbrook M, Thompson A, Wang A, Lee K, Doria S, Hamill P, Yu JJ, Li Y, Domini O, Guarna MM, Finlay BB, North JR, Hancock EW (2007) An anti-infective peptide that selectively modulates the innate immune response. Nat Biotechnol 25: 465–472

Seuss-baum I (2007) Nutritional evaluation of egg compounds. In Bioactive egg compounds (pp. 117-144). Springer Berlin Heidelberg

Shai Y(1999) Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides. Biochim Biophys Acta 1462:55e70

Shawkey MD, Firestone MK, Brodie EL, Beissinger SR (2009) Avian incubation inhibits growth and diversification of bacterial assemblages on eggs. PloS One: e4522

Shebuski JR, Freier TA (2010) Microbiological spoilage of eggs and egg products. In Compendium of the Microbiological Spoilage of Foods and Beverages (pp. 121-134). Springer New York

Sheng G (2010) Primitive and definitive erythropoiesis in the yolk sac: a bird's eye view. Int J Dev Biol 54: 1033

Shenstone FS (1968) The gross composition, chemistry, and physical-chemical basis organization of the yolk and white. Pages 89–103 in Egg Quality: A Study of the Hen’s Egg. T. C. Carter, ed. Oliver and Boyd, Edinburgh, UK

Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, Kimoto M (1999) MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J Exp Med 189:1777–1782

Simkiss K (1961) Calcium metabolism and avian reproduction. Biol Rev 36:321-367

Solomon SE (1991) Egg and eggshell quality. London: Wolfe;149

Sorensen TI, Nielsen GG, Andersen PK, Teasdale TW (1988) Genetic and environmental influences on premature death in adult adoptees. N Engl J Med 318: 727–732

Sparks NHC, Board RG (1984) Cuticle, shell porosity and wate intake through hen's eggshells. Br Poult Sci 25:267–76

Stadelman WJ (2000) Eggs and egg products. In: Francis, F.J. (Ed.), Encyclopedia of Food Science and Technology, second ed. John Wiley and Sons, New York, pp593–599

Sugiarto H, Yu PL (2004) Avian antimicrobial peptides: the defense role of β-defensins. Biochem Bioph Res Co 323: 721-727

Sugino H, Nitoda T and Juneja LR (1997) General chemical composition of hen eggs. *In:* Hen Eggs, Their Basic and Applied Science; Yamamoto, T.; Juneja, L. R.; Hatta, H.; Kim, M., Eds.; CRC Press,Inc.: New York, New York, pp. 13-24

Sunwoo HH, Gujral N (2014) Chemical Composition of Egg and Egg Products. Handbook of Food Chemistry, pp 1-27.Springer Berlin Heidilberg

Takahashi K, Shirai K, Kitamura M, Hattori M (1996) Soluble egg shell membrane protein as a regulating material for collagen matrix reconstruction. Biosci Biotechnol Biochem 60:1299–302

Takeda K, Akira S (2005) Toll-like receptors in innate immunity. Int Immunol 17:1–14

Takeuchi Y, Nishimura K, Aoki N, Adachi SC, Kitajima K, Matsuda T (1999) A 42 kDa glycoprotein from chicken egg-envelope, an avian homolog of the ZPC gamily glycoproteins in mammalian zona pellucida. Its first identification, cDNA cloning and granulosa cell-specific expression. Eur J Biochem 260: 736–742

Tanizaki H, Tanaka H, Iwata H, Kato A (1997) Activation of macrophages by sulfated glycopeptides in ovomucin, yolk membrane, and chalazae in chicken eggs. Biosci Biotechnol Biochem 61: 1883-1889

Tankrathok A, Daduang S, Patramanon R, Araki T, Thammasirirak S (2009) Purification process for the preparation and characterizations of hen egg white ovalbumin, lysozyme, ovotransferrin, and ovomucoid. Prep Biochem Biotech 39: 380-399

Terepka A, Coleman J, Armbrecht H and Gunther T (1976)Transcellular transport of calcium. Calcium in Biological Systems. In “Symposia of the Society for Experimental Biology” (C. Duncan, Ed.), pp. 117-140. Cambridge Univ. Press, Cambridge

Thoroski JH (2004) Processing of eggshells involves drying, separating membrane portions by sieving, impacting remaining eggshell component in processing chamber impacting zone, and separating remaining membrane from outer eggshell particles. US166213

Threlfall EJ, Wain J, Peters T, Lane C, De Pinna E, Little CL, Wales AD, Davies RH (2014) Egg-borne infections of humans with salmonella: not only an S. enteritidis problem. Worlds Poult Sci J 70: 15-26

Tobias PS, Mathison J, Ulevitch R (1988) A family of lipopolysaccharide binding proteins involved in responses to gram-negative sepsis. J Biol Chem 263: 13479-13481

Tobias PS, Soldau K, Ulevitch RJ (1989) Identification of a lipid A binding site in the acute phase reactant lipopolysaccharide binding protein. J Biol Chem 264:10867–10871

Tokarski C, Martin E, Rolando C, Cren-Olivé C (2006) Identification of proteins in renaissance paintings by proteomics. Anal Chem 78: 1494-1502

Tong Q, Romanini CE, Exadaktylos V, Bahr C, Berckmans D, Bergoug H, Eterradossi N, Roulston N, Verhelst R, McGonnell IM and Demmers T (2013) Embryonic development and the physiological factors that coordinate hatching in domestic chickens. Poultry Sci 92: 620-628

Tranter HS and Board RG (1982) The influence of incubation temperature and pH on the antimicrobial properties of hen egg albumen. J Appl Bacteriol 56: 53–61

Tsai WT, Yang JM, Lai CW, Cheng YH, Lin CC, Yeh CW (2006) Characterization and adsorption properties of eggshells and eggshell membrane. Bioresource Technol 97: 488-493

Tsou YA, Chen CM, Lin TC, Hu FW, Tai CJ, Chen HC, Lin TC, Hu FW, Tai CJ, Chen HC, Yeh TH, Harn HJ,Tsai MH, Jan CI (2013) Decreased SPLUNC1 expression is associated with Pseudomonas infection in surgically treated chronic rhinosinusitis patients who may require repeated sinus surgery. Laryngoscope 123: 845-851

Tyler C (1961) Shell strength. Its measurement and relationship to other factors. Br Poultry Sci J 2: 3–18

Valenti P, Antonini G, Von Hunolstein C, Visca P, Orsi N, Antonini E (1982) Studies of the antimicrobial activity of ovotransferrin. Int J Tissue React 5: 97-105

Valenti P, Visca P, Antonini G, Orsi N, Antonini E (1987) The effect of saturation with Zn 2+ and other metal ions on the antibacterial activity of ovotransferrin. Med Microbiol Immun 176: 123-130

Van Dijk A, Veldhuizen EJ, Kalkhove SI, Tjeerdsma-van Bokhoven JL, Romijn R A, Haagsman HP (2007) The β-defensin gallinacin-6 is expressed in the chicken digestive tract and has antimicrobial activity against food-borne pathogens. Antimicrob Agents Ch 51: 912-922

Van Dijk A, Veldhuizen EJA, Haagsman HP (2008) Avian defensins. Vet Immunol Immunopathol 124:1–18

Varon O, Allen KJ, Bennett DC, Mesak LR, Scaman CH (2013) Purification and characterization of tinamou egg white ovotransferrin as an antimicrobial agent against foodborne pathogenic bacteria. Food Res Int 54: 1836-1842

Vincent JL (2008) EPIC II: Sepsis around the world. Minerva Anestesiol 74:2936

Waclawek M, Foisner R, Nimpf J, Schneider WJ (1998) The chicken homologue of zona pellucida protein 3 is synthesized by granulosa cells. Biol Reprod 59: 1230–1239

Weber GF, Chousterman BG, He S, Fenn AM, Nairz M, Anzai A, Brenner T, Uhle F, Iwamoto Y, Robbins CS, Maier SL, Zonnchen T, Rahbari NN, Scholch S, Ameln AK, Chavakis T, Weitz J, Hofer S, Weigand MA, Nahrendorf M, Weissleder R, Swirski F K (2015) Interleukin-3 amplifies acute inflammation and is a potential therapeutic target in sepsis. Science 347:1260-1265

Weber JR, Freyer D, Alexander C, Schroder NWJ, Reiss A, Kuster C, Pfeil D, Tuomanen EI, Schumann RR (2003) Recognition of pneumococcal peptidoglycan: an expanded, pivotal role for LPS binding protein. Immunity 19: 269-279

Wellman-Labadie O, Picman J, Hincke MT (2007) Avian antimicrobial proteins:structure, distribution and activity. Worlds Poult Sci J 63: 421–438

Wellman-Labadie O, Lakshminarayanan R, Hincke MT (2008) Antimicrobial properties of avian eggshell-specific C-type lectin-like proteins. FEBS Lett 582 :699–704

Wesierska E, Saleh Y, Trziszka T, Kopec W, Siewinski M, Korzekwa K (2005) Antimicrobial activity of chicken egg white cystatin. World J Microbiol Biotechnol 21:59–64

Whenham N, Wilson PW, Bain MM, Stevenson L, Dunn IC (2014) Comparative biology and expression of TENP, an egg protein related to the bacterial permeability-increasing family of proteins. Gene 538: 99-108

Whittow GC (1999) Sturkie's avian physiology. Fifth Edition. Academic Press

Wiesner J, Vilcinskas A (2010) Antimicrobial peptides. The ancient arm of the human immune system. Virulence 1: 440–464

Williams J (1962) Serum proteins and the livetins of hen’s-egg yolk. Biochem J 83: 346–355

Winther JR, Thorpe C (2014) Quantification of thiols and disulfides. (BBA) Gen Subjects 1840: 838-846

Wong M, Hendrix MJ, von der Mark K, Little C and Stern R (1984) Collagen in the eggshell membranes of the hen. Dev Biol 104:28–36

Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 249: 1431-1433

Wu KC, Streicher J, Lee ML, Hall BK and Muller GB (2001) Role of motility in embryonic development I: Embryo movements and amnion contractions in the chick and the influence of illumination. J Exp Zool 291: 186194

Wu X, Qian G, Zhao Y and Xu D (2005) LBP inhibitory peptide reduces endotoxin-induced macrophage activation and mortality. Inflamm Res 54: 451-457

Yan RT, Wang SZ (1998) Identification and characterization of tenp, a gene transiently expressed before overt cell differentiation during neurogenesis. J Neurobiol 34:319-328

Zeng L, Gu W, Zhang AQ, Zhang M, Zhang LY, Du DY, Huang SN, Jiang JX (2012) A functional variant of lipopolysaccharide binding protein predisposes to sepsis and organ dysfunction in patients with major trauma. Ann Surg 255: 147–157

Zhao YH, Chi YJ (2009) Characterization of collagen from eggshelll membrane. Biotechnology 8:254–8

Zweigner J, Schumann RR, Weber JR (2006) The role of lipopolysaccharide-binding protein in modulating the innate immune response. Microbes Infect 8:946-952

XI. Authorizations

Frontiers in Bioscience

FBS <fbs@bioscience.org> 29 de julho de 2015

Use is educational and does not require a license. Figures of high quality require license fee. If you are interested in paying the fee ($95 per figure) let me know.

Cordially

Dave

Frontiers in Bioscience
E-mail: fbs@bioscience.org

Hindawi Publishing Corporatton Right and Permissions

Sara Ammar <sara.ammar@hindawi.com> 22 de julho de 2015

Dear Dr. Monteiro,

Thank you for your email. All articles published in **Hindawi** journals are released under a "Creative Commons Attribution License," enabling the unrestricted use, distribution, and reproduction of an article in any medium, provided that the original work is properly cited which means the authors of the article, along with any interested reader, are free to view, print, and download any articles published in the journal.

Since the figure that you want to use was previously published in an Open Access journal as Hindawi, you do not need any permission to use it. However, you must cite the original source of this figure to ensure proper attribution.

The following is the proper citation to the article:

Maria Gabriella Gabrielli and Daniela Accili, "The Chick Chorioallantoic Membrane: A Model of Molecular, Structural, and Functional Adaptation to Transepithelial Ion Transport and Barrier Function during Embryonic Development," Journal of Biomedicine and Biotechnology, vol. 2010, Article ID 940741, 12 pages, 2010. doi:10.1155/2010/940741

Please let me know if I can be of further help.

Best regards,

Sara

--
****

Sara Ammar
Editorial Office
Hindawi Publishing Corporation
http://www.hindawi.com

ELSEVIER LICENSE
TERMS AND CONDITIONS

Jul 28, 2015

This is a License Agreement between Cristianne MM Cordeiro ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Cristianne MM Cordeiro
Customer address	451 Smyth Road
	Ottawa, ON K1H 8M5
License number	3673680446099
License date	Jul 21, 2015
Licensed content publisher	Elsevier
Licensed content publication	Matrix Biology
Licensed content title	Identification and localization of lysozyme as a component of eggshell membranes and eggshell matrix
Licensed content author	M.T Hincke,J Gautron,M Panheleux,J Garcia-Ruiz,M.D McKee,Y Nys
Licensed content date	September 2000
Licensed content volume number	19
Licensed content issue number	5
Number of pages	11
Start Page	443
End Page	453
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations

Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Original figure numbers	figure 4
Title of your thesis/dissertation	Eggshell Membrane Proteins provide Innate Immune Protection
Expected completion date	Oct 2015
Estimated size (number of pages)	250
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 CAD
VAT/Local Sales Tax	0.00 CAD / 0.00 GBP
Total	0.00 CAD

Terms and Conditions

David Publishing Right and Permissions

order <order@**davidpublishing**.com> 21 de julho de 2015

Dear Cristianne,

Glad to hear from you. You can use the figure directly but please cite where it is from. Also hope to publish some papers from you. You can send your paper to us at any time.

Best regards,

Shelly

Karger Right and Permissions

22 de julho de 2015

Dear Dr. Cordeiro,

Thank you for your email. As to your request, I am pleased to inform you that permission is granted herewith to use Figure 2 from the article

Lipopolysaccharide Neutralization by Antimicrobial Peptides: A Gambit in the Innate Host Defense Strategy
Pulido D.a · Nogués M.V.a · Boix E.a · Torrent M.a, b
J Innate Immun 2012;4:327-336 (DOI:10.1159/000336713)

to be reproduced in your PhD thesis.

Please note that this is a non-exclusive permission, hence any further use, edition, translation or distribution, either in print or electronically, requires written permission again as this permission is valid for the above mentioned purpose only.

This permission applies only to copyrighted content that S. Karger AG owns, and not to copyrighted content from other sources. If any material in our work appears with credit to another source, you must also obtain permission from the original source cited in our work. All content reproduced from copyrighted material owned by S. Karger AG remains the sole and exclusive property of S. Karger AG. The right to grant permission to a third party is reserved solely by S. Karger AG.

Thank you for your understanding and cooperation.

Hopefully, I have been of assistance to you with the above.

Best regards,

Silvia Meier
Rights Manager
permission@karger.com