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Proteomic Characterization of Eggshell Membranes and Their Effect on Poultry Physiology and Immunity

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Proteomic Characterization of Eggshell Membranes and Their Effect on Poultry Physiology and Immunity

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ABSTRACT

The use of antibiotics in poultry growth and disease control has led to antibiotics resistant problem in human beings, which is a big concern among consumers. With the necessity for judicious use of antibiotics in poultry production, alternative strategies to improve disease resistance in poultry production are necessary. The research is more inclined towards using the natural products available to grow healthier and antibiotic free meat animals. In the context of exploring natural and sustainable resource of alternative to antibiotics, the biochemical milieu of eggshell membranes (ESM) were analyzed by using mass spectrometry techniques including matrix assisted laser desorption ionization and liquid chromatography coupled with tandem mass spectrometry (MALDI-TOF-MS and LC-MS/MS). We found more than 300 proteins and the abundant among them are lysozyme, ovotransferrin, ovocleidin, clusterin, ovokeratin ovodefensin and many more. These proteins are not only antimicrobial in nature, but also many, play a vital role in metabolic and developmental processes. A series of experimental trails were done in which chickens by feeding ESM supplemented diet. Our initial experiments showed that feeding 0.5% levels of eggshell membrane not only improved the body weight of chickens, but also modulated immunoglobulin parameters and stress levels. Further experiments were done to see the effect of ESM under endotoxin challenged conditions in which 5 week old chickens fed with ESM supplemented or control diet were challenged with *Salmonella* lipopolysaccharide. Our results showed significant difference in body weight loss, pro and anti-inflammatory genes, and serum corticosterone levels in control versus ESM fed chickens. ESM supplemented diet not only helped to restore the body weight loss due to LPS injection but it also helped to provides better tolerance to endotoxin challenges as indicated by splenic cytokine profiles of the chickens.

In view of the need for alternatives to antibiotics in meat animal production, exploring the potential of egg byproducts as nutritional modulator of immunity during post hatch period appears logical.

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CHAPTER 2: Published

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CHAPTER 4: Submitted

S. K. Makkar, N. C. Rath B. Packialakshmi; Z. Zhou; G. R. Huff, & A. M. Donoghue. Nutritional supplement of hatchery eggshell membrane improves poultry performance and provides resistance against endotoxin stress

Introduction

The emergence of antibiotics resistant bacteria and their link to prophylactic use of antibiotics as growth promoter in meat producing animals has prompted the search for alternatives to antibiotics. Products such as probiotics, prebiotics, bacteriophages fecal extracts, yolk antibodies, and organic acids have been used to satisfy this need. However, there is no uniformity of the nature and the mechanisms of action of these products. Ideally, a suitable product may be that which would not only protect animals from diseases but also not affect the growth potential and production values of the animals. Deploying the potential of immune system to protect the animals from disease may help. Vaccination against specific pathogens have been the examples of such choice. However, there is no general vaccine that might provide overall resistance against most common health problems of poultry. Besides, nonspecific immune activation is energy expensive which can affect production values. Should it be possible to program the immune system to protect the individual, in the concept of allostatic modulation, may be an option. The immunity of neonates specifically, the newly hatched poultry is not completely developed and plastic hence, it is not only vulnerable to infections but also may be trainable to protect birds against disease without interfering with their growth and wellbeing.. Nutrition modulation is considered one of the effective means to train the immune system and make the animals more immunocompetent. The experiments in this dissertation examines some of these concepts using egg shell membrane, a byproduct of poultry industry that contains a variety of bioactive proteins and peptides, to affect immunity and health outcomes of post hatch poultry measured through selective physiological parameters.

The studies are divided in two parts. The first part deals with the identification of the proteins and peptides of fresh harvested eggshell membranes (ESM) by using the mass spectrometer, Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS/MS), High Pressure Liquid Chromatography (HPLC) and Liquid Chromatography-Tandem Mass Spectrometry (LC/MS/MS) techniques. Based on the qualitative data of protein profiles of the eggshell membranes, the biological effects of the ESM was evaluated on post hatch chickens under both normal and endotoxin challenge conditions.

Chapter 2 discuses about the proteomic aspect of eggshell membranes and all the techniques and procedures used to identify and characterize the protein and peptides present in eggshell membranes by using "top down/bottom up" MALDI and ESI mass spectrometry approaches. Chapter 3 discuses about the nutritional aspect of the eggshell membranes harvested form fresh unfertilized eggs and their immunomodulatory effect on growth and performance of chickens at 3 weeks of age.

Chapter 4 discusses about the proteomic characterization of eggshell membranes obtained from hatchery waste. Chapter 5 is about the ameliorating effect of eggshell membranes in conditions of endotoxin challenge. We explored whether these membranes when give as a supplement to the post hatch chickens can provide resistance and tolerance to the stressful conditions at a later stage.

Chapter 1:

Immunity and antibiotics alternative in the context of poultry health and wellbeing: a literature review

Introduction

Production of healthy livestock is integral to food safety, animal wellbeing, and sustainable agriculture. The emergence of antimicrobial resistant bacteria has been a worldwide concern and squarely blamed on the prophylactic use of antibiotics in meat animal industry where it is used as growth promoter [1-6]. The use of antibiotics is not only implicated in producing antibiotics resistant pathogenic bacteria but it also upsets the regular microflora [7-10]. However, the restriction in the prophylactic use of antibiotics also increases the chance of bacterial diseases and causes food safety problems that could potentially cripple the poultry and meat industry. Hence, there has been increasing research focus on finding alternatives to antibiotics that would provide resistance to microbial disease while maintaining the production values [11]. The quest to improve immunity and disease resistance of meat producing animals and poultry thus raises questions on options to modulate, and assess immunity This review addresses some of these issues particularly in the context of poultry production.

Alternatives to antibiotics

The consumer's concern and demand for antibiotic free food leads to the focus of modulation of the avian immune system particularly using nutritional approaches which not only can increase the production of poultry but also fulfill consumer's demand for antibiotic free food at the same time [12]. But the major concern in adding the alternative to antibiotics is that the product should be equally potent to promote the growth and also keep the animal free from disease. The cost to impact ratio on health status of an animal is another big issue that needs to be addressed when using an alternative to antibiotics [1, 13]. Of a number of methods that have been proposed or are on trial are vaccines, antimicrobial peptides [14-16] (exogenous or induced), bacteriophages, probiotics[17], prebiotics, different phytochemicals (essential oils, saponins) [18] and

recombinant cytokines (recombinant intact and/ or modified synthetically to enhance efficacy) [13]. All these are geared to improve endogenous resistance, modulate immunity directly or indirectly to reduce the burden of harmful agents that affect growth, wellbeing, and food security of meat animals.

Examples of Alternatives:

Vaccines

The first and foremost method, which revolutionized the history of immunomodulation, was vaccination discovered by Edward Jenner that has changed the face of medical research. Vaccine is a preparation from attenuated form of a pathogen, which stimulated the immune system and develops the memory to kill the microorganism encountered later in the life. By exposing the immune system to a harmless form of pathogen it can be made more alert and ready for a vigorous response in times of real pathogen attack. The biggest contribution of vaccine is complete eradication of smallpox [19], and a significant decrease of measles, mumps and rubella worldwide in human medicine. Vaccines can be an easy solution to many challenges faced by poultry industry today [20]. *Salmonella* vaccines along with other preventative strategies are one of the effective measures, which holds a promising future for control of food borne pathogens in poultry products [21]. A greater success is achieved in developing coccidiosis vaccines by injecting the chicken with *Eimeria* oocysts at posthatch stage [22]. Newcastle disease was completely eradicated with the application of a Newcastle virus vaccine, which was initially done by means of slaughtering and sanitary measures [23].

Antimicrobial proteins and peptides

Antimicrobial peptides (AMP) are endogenous or exogenous low molecular weight proteins, which can provide protection against a wide range of microbes including bacteria, fungi

and viruses. They are cationic in nature and create pores on the bacterial cell wall and control microbial growth (6). AMP's also known as "natural antibiotics" have numerous applications for therapeutic, nutraceutical, and biotechnological industries [24]. Antimicrobial peptides specially derived from food products are safer for human consumption. Most of them are explored mainly in milk, egg and rice [25, 26]. Lysozymes, defensins and transferrins that are present in milk and egg are also important molecules of our innate immune cells such as neutrophils and macrophages [27, 28]. Lysozyme is well known for its efficacy against gram-positive bacteria and is extensively used in food industry as natural source of food preservative, which increases the shelf life of food [29]. Transferrins, such as lactotransferrin and ovotransferrin present in milk and egg respectively help to fight against infections by chelating iron and thus inhibiting the growth of bacteria by limiting the availability to this essential component needed for bacterial growth [30]. Nissin, an antimicrobial peptide produced by *lactococcus lactis*, is widely used to increase the shelf life of the food by preventing the spoilage done by pathogenic bacteria [31]. The advantage of AMP over the synthetic antibiotics is that the bacteria are less resistant to the them as compared to the latter [24]. Advances in our understanding of the mechanism of action of AMP's will open up new avenues for developing novel and therapeutic applications.

Bacteriophage

A bacteriophage is a virus that lyses the bacterium, invade and kill it by disrupting its metabolic system. Bacteriophage therapy has been reported to be an effective alternative to antibiotic in vancomycin resistant enterococcus infection in the mouse model [32]. In contrast to antibiotics the mode of antimicrobial action by bacteriophage does not lead to the development of resistance mechanisms in bacteria. Because of the specific mode of action, the use of phage against the targeted bacteria is safe for the beneficial microbiota [33]. The use of bacteriophages in the food

industry to eliminate food born pathogens has also gained considerable recognition [34]. The 'phage biocontrol' approach is safe and effective at both the pre-harvest and post-harvest stage of controlling food borne pathogens and has the potential to be considered as the most effective methods for food safety in the future [35].

Probiotic and prebiotic

Changing of the gut microbiota through dietary means has been a subject of much discussion. The gut biology is an important area especially in agriculture animals. The use of probiotic and prebiotic in the treatment of various metabolic disorders is gaining momentum in past few years. The term probiotics is defined "as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" [36]. By the mechanism of competitive exclusion, for colonization sites and the production of compounds, which are toxic for pathogenic bacteria, probiotics inhibit bacterial growth and help to maintain the intestinal flora [37]. Prebiotics, similarly are defined as " a nondigestible food ingredient that beneficially affect the host by selectively stimulating the growth and / or activity of one or limited number of bacteria in the colon" [38]. The use of prebiotics and probiotics not only for increasing the productivity but also for disease prevention in poultry production is deemed an effective alternative to antibiotics to satisfy the consumer's unmet demand of healthy and diseases free meat. [39]

Herbal Products

The extract from various plants such as thyme, eugene, oregano have been shown to inhibit the growth of pathogenic bacteria such as *Salmonella*, *Escherichia coli* and *Clostridium* in *vitro* as well as in birds [40]. Essential oils (EO) extracted from different parts of the plant stimulate the digestive tract by promoting the formation of digestive enzymes in the gut. They also exert their

antimicrobial effect by creating the pores in the lipid bilayer of the cell membrane, which makes the cell leaky and disturbs the metabolism of the bacteria and leads to its death [41]. The applications of antimicrobial activity of EOs are not limited to meat and meat products but also apply to vegetables, rice, and dairy products. However the usage of EOs can sometimes add a flavor and distinct kind of aroma to the meat, which is a limiting factor from the consumer's sensory point. Due to its increasing applications in the food industry, Eos are gaining great attention for future research, that would provide more insights into their mechanism of action and also address safety concerns [42].

However, there is a lack of consistency regarding the findings of the effects these additives have on the various health parameters that are measured to prove their efficacy. The systematic investigation of the effect of additives on the various aspects of the immune system is needed to convince the commercial producers to completely rely on these products. By keeping in mind that the immune system is an integrated system, which cannot be determined by solely measuring one, or few parameters will help us to avoid unintended consequences in the near future [43, 44]. Rather than interpreting for results with individual markers if we try to congregate the related markers together and see their mass effect, and focus on their consistency it will help make a better conclusion [45].

Immunomodulation

Immunomodulation is the manipulation or adjustment of the immune system to improve resistance to disease. It includes all possible means of altering the immune system such as immunosuppression or enhancement based on the necessities of the prevailing health conditions. Immunomodulation helps to alleviate the existing pathological condition and control the damage done by it. In case of autoimmune problems, where the immune system attacks self-components,

the main target is to suppress it or reduce its activity which otherwise can result in immunopathology. In the same token, immunocompromised individuals can be susceptible to infections and become victims of pathogenic attacks more easily, the enhancement of immunity and protection against deadly infections [46]. Hence a well-balanced immunity against pathogen can protect the individual against disease and improve well-being.

Nutrition immunomodulation

Well-balanced nutrition is one of the main factors which can help in optimizing the function of the immune system [47]. Immunonutrition, even though it is an emerging science, roots back to 1880s where the effect of malnutrition was seen on the growth of lymphoid organs [48]. The immune system and nutrition are catalogued in such a way that excess or lack of a nutrient debilitates its function [49-51]. Excess or deficiency of essentials in the diet can make the immune system vulnerable to several infections, which not only worsens the quality of life but also decrease its expectancy. Even though there is a fundamental understanding of how innate and adaptive immune systems interact for the clearance of pathogens, there is a need to investigate further when trying to modulate the immune system to improve the quality of life. The immune system operates under normal conditions for maintenance, but at the time of pathogenic attack its dietary requirements change. It undergoes cell proliferation to increase the number of leukocytes to make its army ready for the defense against the attack. There is also an intensive demand of nutrition for the synthesis of acute phase proteins by the liver [52]. The nutrient requirements of the immune system can also change with the type of infection an individual encounters. By inferring the nutritional cost of the immune system it will be easy to manipulate the essential components in the diet to manage the loss of production during infection [52, 53]. Significant effort is made to improve immunity and disease resistance through

nutritional means. Nutrition based epigenetic programming during the neonatal and perinatal period may increase the efficiency of the immune system to fight against the infection [54, 55].

Nutritional immunomodulation is seen as a panacea to deal with the immune system problems. With the dietary interventions it is possible to fine-tune the immune system and make it better to fight with infectious agents. Since the ancient time the use of many plant products such as green tea, turmeric, fish oil, vitamin D have proven to have a therapeutic and ameliorating effects against sickness [56]. The idea of immunomodulation is not to overwhelm the system by adding or deleting an ingredient in the diet but to provide a means for optimal functioning and analyzing its consequences on the immune system. The main target of the change in dietary elements is to see its beneficial and long lasting effects on the system, which can make it more competent and resistant to infections.

There are several factors, which needs to be carefully considered while designing the experiment for nutritional modulation to avoid study-to-study variation. Age, sex, genetics, eating, stress and many more factors, which vary in different subjects, can bring inconsistency in the results [44].

Interactive factors of Immunity

Immune response is subject to endogenous control such as physiology, age, genetics and even psychosomatic dispositions [57, 58]. Exogenous factors such as environment and nutrition can also affect or permanently modulate the immune system, [59-61]. In the context of food animal production the most relevant ones are genetics and nutrition although a variety of other factors such as housing and hygiene may play roles in immune system function and in the animal's disposition to disease. The genetic variation influences the inflammatory response of an individual to a given challenge. With mutations and single nucleotide polymorphism there is greater probability of having variations in the synthesis of inflammatory mediators.

The relationship between neural, endocrine, and immune system are still unraveled. The neuroendocrine-immune interactions have been studied in the context of stress and inflammation [62]. Stress activates the hypothalamic pituitary (HPA) axis affecting the release of corticotrophin and glucocorticoids [63]. Glucocorticoids produced by adrenal gland have profound effects on the immunological functions and the deregulation in the HPA axis greatly affects the effector mechanism of the immune system. Stress hormones, glucocorticoids and catecholamine influence immunity. The immunosuppressive effects of high levels of corticoids influence the levels of cytokines produced by lymphocytes [64, 65].The nervous system and immune system also cross talk via the HPA axis. The cytokines released by the sentinel cells in the event of inflammation such as interleukins and TNF-a can affect neuroendocrine system and be can be affected by it. Recent studies have also shown that the sympathetic nervous system such as the vagus nerve that innervates the spleen is known to influence immunity [66, 67]. Under inflammatory conditions the vagus nerve stimulates the immune cells in the spleen resulting in the production of acetylcholine that dampens the production of cytokines [68, 69]. Other evidence suggest the mutual influence of nervous and immune system and certain neurotransmitters directly modulate the response of the cells of immune system [70]. T cells and macrophages express β adrenergic receptors, and T cells produce acetylcholine and the stimulation by vagus nerve also causes acetylcholine production that alters the resident immune cell functions of spleen and liver, and their ability to produce specific cytokines [71]. The gut harbors the second largest neural network and several neuropeptides that have been shown to possess antimicrobial, and immunomodulatory activities, and play important roles in the development of self-tolerance [72]. Many of these peptides produced during persistent and chronic stress suppress the immune system and affect the outcome of a disease [73, 74].

The immune system is relatively plastic at the time of birth and is vulnerable to infections that can permanently alter its potential to respond to stressful situations later in life [75]. Early life programming also known as imprinting of the immune system through the neuro-endocrine axis have been speculated to influence immunity over the span of life [76, 77]. The enteric endocrine and nervous system also help chemosensing of nutrients which in turn can influence immunity [78].

The above discussions posit that allostatic modulation of immunity by way of conditioning may be a reasonable option for programming the immune system for a balanced response to protect against infection without the loss of productivity and wellbeing. Epigenetic programming as a concept in physiology is not new. Susceptibility to infection are modulated by epigenetic control of immune cells such as DNA and histone modifications [79]. Wild animals generally show better immune responses to antigens as compared to captive animals because of their exposure to variety of challenges from the environment [80]. In order to enhance the chances of survival, their immune system is differently programmed since birth, thus they are more tolerant to endotoxin challenges [81]. Perinatal malnutrition is known to have profound neuroimmunomodulating effects in mammals to the extent that many metabolic and inflammatory diseases develop as a result [82]. Hence a better understanding of diet and neuro-immuno interactions may help achieve the objective for restricted use of antibiotics.

Conclusion

It is apparent that the postnatal (posthatch) immune system is amenable to modulation. Immune system not only can communicate with the brain and endocrine system, the chemicals such as proteins and peptides produced by those systems also regulate it. The embryonic and fetal factors exert control in training and pruning the system that is retained as immune memory to be

expressed at the time of need. Both resistance and tolerance to microbes may be manifestations of this memory. The programming of the immune system during posthatch period may be critical which brings us to next set of issues such as what are the tools to accomplish such a feat? Could it be the maternal factors such as milk in mammals or wild diets and environmental contaminants including bacteria, viruses, and parasites that can confer broad variety of resistance and immunity, which will benefit survival and wellbeing? Such accomplishments can be engineered to apply to a large-scale scenario for example, poultry production where the birds need to be competent to resist infection without sacrificing their growth potential and performance. There is also more need for research on how do we evaluate the effect of dietary nutrients, to determine its impact on the immune system in terms of not only evading the pathogen but also protecting the tissue from self-destruction

Thus while making a conclusion for the modulating effect of nutrients their sustainability and effects on the host's susceptibility to pathogens should be considered.

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II Chicken egg shell membrane associated proteins and peptides

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ABSTRACT

Eggshells are poultry industry byproducts with potential for use in various biological and agricultural applications. We have been interested in the membranes underlying the calcareous shell, as a feed supplement, which showed potential to improve immunity and performance of post hatch poultry. Therefore, to determine their protein and peptide profiles, we extracted the eggshell membranes (ESM) from fresh unfertilized eggs with methanol and guanidine hydrochloride (GdHCl) to obtain soluble proteins for analysis by mass spectrometry. The methanol extract was subjected to matrix assisted laser desorption ionization (MALDI), electrospray ionization (ESI), high performance reverse phase liquid chromatographic separation (HPLC) and tandem mass spectrometry (MS/MS) to determine its peptide and protein profiles. The GdHCL extract was subjected to ESI-HPLC-MS/MS following trypsin digestion of reduced/alkylated proteins. Nine proteins from the methanol extract and >275 proteins from the GdHCl extract were tentatively identified. The results suggested the presence of several abundant proteins from egg whites, such as, ovoalbumin, ovotransferrin, and lysozyme as well as many others associated with antimicrobial, biomechanical, cytoskeletal organizational, cell signaling, and enzyme activities. Collagens, keratin, agrin, and laminin were some of the structural proteins present in the ESM. The methanol soluble fraction contained several clusterin peptides and defensins particularly, 2 isoforms of gallin. The ratios of the 2 isoforms of gallin differed between the membranes obtained from brown and white eggs. The high abundance of several anti-microbial, immunomodulatory, and other bioactive proteins in the ESM along with its potential to entrap various microbes and antigens may make it a suitable vehicle for oral immunization of post hatch poultry, and improve their disease resistance.

KEYWORDS: *Eggshell, membrane, mass spectrometry, proteins*

INTRODUCTION

With over 90 billion eggs produced annually in the USA(USDA, 2014), the egg shells constitute a significant byproduct of the poultry industry with potential for use in various agricultural and biomedical applications(Anton, et al., 2006; Cordeiro and Hincke, 2011; Kovacs-Nolan, et al., 2005; Mine and Kovacs-Nolan, 2006). The egg shell consists of a calcareous outer crust underlined by two layers of proteinaceous membranes which enclose a composite chemical milieu of egg whites and yolk, and provide both physical and biological protection to embryo (Ahlborn, et al., 2006; Hincke, et al., 2012). Understanding the protein and peptide constituents of the egg shell membrane (ESM) may provide better insight into their roles in embryo development and protection, improve egg quality, and facilitate the utilization of this agricultural waste product. In recent years there has been many studies of the protein components of various avian egg compartments including the ESM as well as their biological significance (Mann, et al., 2006)(Kaweewong, et al., 2013). However, there are very few studies of egg membrane associated peptides. Whereas the proteins have both structural and functional bases within tissues, the peptides also play important roles in many biological processes such as signal transduction, transportation, and host defense(Brown and Hancock, 2006; Hu, et al., 2009; Soloviev and Finch, 2006). Therefore, the objective of this study was to profile the extractable peptide and protein composition of the inner eggshell membranes by using "top down/bottom up" MALDI and ESI mass spectrometry approaches.

MATERIALS AND METHODS

Chemicals and reagents. The following reagents and devices including Centricon YM-10 filtration units (EMDMillipore.com), C18 Nu tips (Glysci.com), 1 kDa Dispodialyzer (Harvardapparatus.com), Spectra/Por membranes (Spectrumlabs.com), Biowide Pore C_{18} reverse phase HPLC column (15 cm x 4.6 mm, 5µm particle size, 300 Å pore size, Sigma-Aldrich, St. Louis, MO), C_{18} column (150 x 0.1mm, 3.5 µm particle size, 300 Å pore size, Zorbax SB (Agilent), BCA protein assay kit, Pierce C18 spin columns, MS grade trypsin (Fisher Scientific.com), peptide calibration standard II (*m/z* 500-16000, Bruker Daltonics, Bremen, Germany), and 2-iodoacetamide (IAA) (MP Biomedicals, OH) were purchased from their respective vendors. All other reagents and supplies including 1, 4-dithiothreitol (DTT), 2, 5-dihydroxybenzoic acid (DHB), and 1,5-diaminonaphthalene (DAN), were purchased from Sigma Aldrich (St. Louis, MO).

Egg membrane harvest and extraction. Egg shells from fresh unfertilized brown and white eggs were washed with deionized water inside with mild scrubbing to remove loosely adsorbing egg white proteins, and the membranes were peeled free of calcareous shells. Pooled or individual egg shell membranes (ESM) were again washed with excess deionized water by stirring for 2-3 h, blot dried with Whatman filter papers then chopped into small pieces for further processing. Figure 1 shows a flow chart of the general procedure of membrane extraction and processing. The pooled ESM were extracted by 2 methods (a) with 70% methanol containing 0.1% acetic acid in and (b) with a buffer consisting of 4 M guanidine hydrochloride (GdHCl), 20 mM EDTA, and 50 mM Na-acetate, pH 5.8. The ESM fragments were extracted by stirring with 10 volumes of respective solutions for 24 hours at 4^{o}C. The extracts were centrifuged at 21,000 g for 15 min, and the clear supernatant dialyzed against excess 50 mM ammonium carbonate

solution using 1,000 Da Spectra/Por membranes with 3-4 changes. The membrane retentate of both extracts following dialysis were concentrated by lyophilization and resuspended in a smaller volumes of 50 mM ammonium bicarbonate to measure their protein concentrations by the BCA protein assay using bovine serum albumin (BSA) as the standard. The ESM harvested from individual brown and white ESM were similarly extracted with acidified methanol and screened by direct matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Kannan, et al., 2007; Kannan, et al., 2009). The experiments were carried out in two separate trials to confirm the overall repeatability of the results.

Direct MALDI-TOF MS of methanol extract. The methanol extracts of individual or pooled membrane preparations were screened for their peptide profiles in the mass range of 1-20 kDa by direct MALDI-TOF-MS using 2 dihydroxybenzoic acid (DHB) as the matrix. The standard dry droplet method with 1:1 matrix: analyte ratio was employed to prepare spots on a Bruker ground steel MTP 384 MALDI target plate. To find the effect of reduction and alkylation, aliquots of samples in methanol were diluted with 3 volumes of 70% methanol containing 200 mM ammonium bicarbonate then treated with 10 mM DTT for 10 minutes in a boiling water bath, cooled to room temperature for 1 h followed by alkylation with 40 mM iodoacetamide for an additional 1 h in the dark. The control samples were identically treated except that DTT was omitted from the reaction mixture. Both control and reduced/alkylated samples were spotted on target plates along with calibrating Bruker peptide standard II in adjacent spots. The spectra were acquired using a Bruker Ultraflex II MALDI-TOF mass spectrometer (Bruker Daltonics GMBH, Bremen, Germany), operated in the positive-ion reflectron mode. The ammonium bicarbonate dialysate of the methanol extract was similarly, subjected to reduction/alkylation then dried with

a Centrivap evaporator (Labonco) to reduce the volume, desalted, and spotted for MALDI-TOF-MS. The TOF analyzer was calibrated with peptide standard II. Accurate mono isotopic peptide masses were determined by MALDI-TOF-MS using combinations of external and internal calibration procedures, and spotting with equal volumes of α-cyano-4-hydroxycinnamic acid (HCCA) matrix, prepared in 0.1% FA, 50:50 water/ACN (Kannan, et al., 2013). The LIFT-MS/MS was performed on selected peaks to determine their identity.

Reverse phase HPLC purification of peptides in methanol extract. The dialyzed methanol extract was passed through a 10 kDa Centricon filter to exclude high molecular weight proteins in order to purify some of the peaks observed in MALDI-TOF-MS. The filtrate with ≤10 kDa peptides was evaporated with Centrivap, re-dissolved in 0.1% formic acid, centrifuged at 21,000 g, and the supernatant subjected to reverse phase HPLC purification. The chromatographic separation was done on a BiowideC_{18} reverse phase column attached to an Agilent 1100 HPLC interfaced with an ESI mass spectrometer. Several major peptide fractions based on the ESI-MS multiply charged mass spectra corresponding to *m/z* 4484 and 4597, 2157, 3231, 2878, 2804, 2641, and 1902 peaks, were collected, pooled from replicate runs, and concentrated by evaporative drying for further characterization as described below.

Peptide identification by MALDI-TOF-MS and MS/MS (LIFT-TOF/TOF). The peptide fractions were reconstituted in smaller volumes of 50 mM ammonium bicarbonate and checked for homogeneity by MALDI-TOF-MS then reduced and alkylated with DTT and iodoacetamide as described above followed by trypsin digestion for 24 h at 37^{o}C. The tryptic peptides were desalted with C18 Nu tips, spotted on MALDI target plates with saturated HCCA as described

above, and the spectra collected in both MALDI-TOF (MS) and LIFT-TOF/TOF (MS/MS) modes.

MALDI-ISD (in source decay) analysis. The *m/z* 4597 and 4484 peptide fractions were reconstituted in 0.1% formic acid and spotted mixed with a saturated solution of 1, 5 diaminonaphthalene (DAN) prepared in 50% ACN containing 0.1% formic acid at 1:1 ratio of analyte: matrix then subjected to MALDI-ISD fragmentation (Fukuyama, et al., 2006; Kannan, Liyanage, Lay Jr, Packialakshmi, Anthony and Rath, 2013; Quinton, et al., 2007). ISD spectra were acquired with a Bruker Reflex III MALDI-TOF mass spectrometer. The MALDI-ISD mass spectra were similarly analyzed with Bruker BioTools 3.1 to obtain sequence tags to search the NCBI Gallus data base using protein blast. Both these peptides, m/z 4484 and 4597, were also subjected to LIFT-TOF/TOF fragmentation for further confirmation of their identities.

Comparative differences in selective peptides of brown and white ESM. The MALDI-TOF-mass spectra of methanol extracts of individual brown and white egg membranes were screened to determine the relative spectral intensities of *m/z* 4597 and 4484 peptides in each preparation and the means of the cumulative results were compared using Student's t test.

LC-MS/MS analysis of methanol and guanidine extracted proteins. Following the measurement of protein concentrations of 1 kDa membrane retentate of both methanol and GdHCl extracts as described earlier, approximately 10 µg of methanol extracted and 50 µg of GdHCl extracted proteins were dried by vacuum evaporation and reconstituted in 10 µl of ammonium bicarbonate, subjected to reduction and alkylation, and digestion with trypsin at a protein: trypsin ratio of 50:1 for 24 h at 37^{o}C. The tryptic digest was desalted using Pierce C18

spin columns and chromatographed on a capillary C_{18} column (150 x 0.1mm, 3.5 μm particle size, 300 Å pore size, Zorbax SB) attached to an Agilent 1200 series HPLC interfaced with a Bruker Amazon-SL quadrupole ion trap mass spectrometer and captive spray ion source. Tryptic peptides were separated at a solvent flow rate of 1.6 μL/min with 0 to 40 % gradient of 0.1% FA (solvent A) and ACN in 0.1% FA (solvent B) over a 320 minutes period.

MALDI-TOF-MS data analysis. All MALDI-TOF-MS data were processed using Bruker Flex Analysis 3.3 and Bruker BioTools 3.1 software. Peptides were identified using LIFT-TOF/TOF data by searching the NCBI Gallus database using the MASCOT MS/MS ion search tool with a peptide mass tolerance of 200 ppm and MS/MS tolerance of 0.6 Da. For MS and MS/MS data obtained from tryptic digests of the fractions corresponding to m/z 4597, 4484, 2157, 3231, 2878, 2893, and 1902 were searched in the NCBI Gallus database as above but with trypsin, listed as the digestion enzyme. Accurate monoisotopic peptide masses (± 0.1 Da) were used for peptide identifications.

LC-MS/MS analyzed proteins. Peaks were picked in the LC-MS/MS chromatogram using Bruker default settings. Bruker Proteinscape bioinformatics suite coupled with MASCOT 2.1 was used to search NCBI Gallus protein database for identification of proteins. The parent ion mass tolerance and fragment ion mass tolerance, were both set at 0.6 Da. A MASCOT decoy database search was performed with all the datasets. A score threshold of 45 or above was used as a high probability match for protein identifications. The proteins with only <1% false discovery rate (FDR) and at least 1unique peptide were reported. Functional annotation for these

proteins was performed using the Software Tool for Researching Annotation of Proteins (STRAP) (Bhatia, et al., 2009).

RESULTS

MALDI-TOF-MS identification of methanol extracted proteins and peptides. Figure 2 shows a MALDI-TOF-MS of the methanol extract of ESM shown in the range between *m/z* 1000-6000 range with peaks corresponding to *m/z* 1616, 1902, 2001, 2157, 2641, 2797, 2878, 2894, 3231, 4484, 4597, and 4778. Some of the other peaks that occurred beyond 10,000 m/z was 14302 matching to the corresponding MW of chicken lysozyme. The methods used for identification of some of the peaks and their identities are summarized in Table 1. The peaks corresponding to *m/z* 4484, 4597, and 4778 showed a 348 Da mass difference upon reduction and alkylation suggestive of the presence of 3 disulfide bonds while several other peaks did not show any mass shifts (Figure 3). Reduction and alkylation, particularly under complete aqueous conditions, rendered the *m/z* 4484, 4597, and some other peptides insoluble with 0.1% FA indicated by the disappearance or observance of low intensities in MALDI signals. Figures 4 and 5 show the MALDI-ISD and MALDI-LIFT-TOF/TOF results for peptides *m/z* 4597 and 4484. MALDI-ISD yielded a high confident sequence tag "YCSNTCSKTQI" based on observed c ions (N-terminus protected) from *m/z* 4597. MASCOT sequence query and MS/MS search using MALDI-LIFT-TOF/TOF data and blast search against NCBI Gallus data base all, resulted in significant hit against the protein precursor named "gallin' with a sequence "LVLKYCPKIGYCSNTCSKTQIWATSHGCKMYCCLPASWKW", matching to m/z 4597. Almost same sequence, but without the N-terminal leucine (L) is a perfect match to m/z 4484. The peak at *m/z* 4778 although showed to have 3 disulfide bonds from MALDI-TOF-MS results

(Figure 3), its identification was not possible through these means most likely due to insufficient amounts of material. The LC-MS/MS data from the methanol extracted proteins however, suggested a high possible identity for this peptide to be gallinacin 10 as will be described later with LC-MS-MS results. The peaks at m/z 1902, 2001, 2157, and 3231, observed in direct MALDI-TOF-MS, were all identified as fragments of clusterin having the common sequence tag "TPPFGGFREAFVPPVQRVR" by MASCOT MS/MS ion search of the LIFT-TOF/TOF data using NCBI Gallus database (Figures 6-8 and S1 and S2). The results were also supported by bottom up identification that showed the presence of two common tryptic fragments corresponding to their respective protonated monoisotopic masses at *m/z* 878.4 (TPPFGGFR) and m/z 1042.5 (EAFVPPVQR), for each of those peptides, both derived from the same domain of clusterin (Figures 7 and S3-S4). Thus, the m/z 1902, 2001, 2157 and 3231 peaks were identified as "TPPFGGFREAFVPPVQR", "TPPFGGFREAFVPPVQRV", "TPPFGGFREAFVPPVQRVR" and "TPPFGGFREAFVPPVQRVRLVPPRRRLS," respectively (Table 1). The peptides corresponding to peaks at m/z 2878 and 2894 were both identified by MALDI LIFT-TOF/TOF fragmentation as one phosphatase and actin regulator protein (PHACTR) with a sequence of "PPKRGLLPTNPPEAALPSKPPGDRTVTA" and a sporozoite surface protein 2-like with the sequence of "PNPIGLIGPIGPNVSNPIGLLGPNGPNAFS" (Figures 9 and 10, Table 1).

LC-MS/MS identification of methanol and GdHCl extracted peptides/proteins. Major proteins identified in methanol and GdHCl extracts are listed in Table 2 and Table S1. There were 9 proteins identified in the methanol extract and over 275 in GdHCl extract. Six of the methanol extracted proteins were identified in the GdHCl extract which included the proteins,

lysozyme, clusterin, gallin, and ovocleidin. Since gallinacin 10 propeptide that contains 3 disulfide bonds (Lynn, et al., 2004), was identified in the methanol extract, we presumed that this LC-MS/MS identified tryptic fragment "AGACPPTFTISGQCHGGLLNCCAK" could relate to the *m/z* 4778 peak, observed in MALDI-TOF mass spectrum. The accurate protonated mono isotopic mass for the m/z 4778 peak was determined to be 4772.9 ± 0.3 Da using replicate MALDI-TOF-MS measurements. Combining the mass information with the "AGACPPTFTISGQCHGGLLNCCAK" sequence tag and MASCOT query lead to a significant match with the sequence corresponding to "DPLFPDTVACRTQGNFCRAGACPPTFTISGQCHGGLLNCCAKIPAQ" belonging to gallinacin 10(Lynn, Higgs, Gaines, Tierney, James, Lloyd, Fares, Mulcahy and O'Farrelly, 2004) with a score of 308, and an expect value 8.2^{e-027}. The sequence corresponding to the accurate mass for m/z 4778 peptide appeared to be 5 amino acids longer N terminally than the predicted sequence of the mature gallinacin 10 peptide(Lynn, Higgs, Gaines, Tierney, James, Lloyd, Fares, Mulcahy and O'Farrelly, 2004; Xiao, et al., 2004). The 2 other proteins namely, an angiotensinogen isoform X7, and an uncharacterized protein LOC771972 isoformX1 though were also identified from LC/MS-MS analyses of the methanol extract (Table 2) could not be identified elsewhere.

The guanidine extract containing 276 proteins with Mascot scores 45 or above were identified with one_or more unique peptides. When the identification was done on the basis of a single unique peptide, the fragmentation score was sufficient to identify with 95% confidence (supplementary Table S1). GO classification done using STRAP used 103 IDs (Figure 11) to access the likely function of the proteins based on the annotations in the database. Several high

abundant egg white associated proteins such as ovalbumin, ovotransferrin, lysozyme, ovomucoid, and ovoglobulin were present in ESM. A large repertoire of proteins associated with muscle associated and motor functions such as, titin, dynein, obscurin, myosin, and nebulin, and others with cytoskeletal organizational and anchoring functions (xin, golgin, spectrin, ninein), enzymes (kinases, helicase, protein ligase), enzyme inhibitors (ovomucoid), and signaling functions were identified in ESM. Proteins such as collagens, keratins, laminins, agrin, and chondroitin sulfate that are structural components of the membrane were present in GdHCl extract. Similarly several antimicrobial proteins such as lysozyme, gallinacin, mucin, ovocalyxin, proteases and protease inhibitors were also identified.

Differential expression of m/z 4597 and 4484 in brown and white ESM. Figure 12 shows comparative profiles of *m/z* 4484 and 4597 peaks in ESM from brown and white eggs. Calculated by their peak intensities, the brown ESM had lower levels of m/z 4597 peptide relative to m/z 4484 isoform of gallin than the white ESM which had higher levels of m/z 4597 and lower level of m/z 4484 peptide (brown, 0.42±0.04; white,0.72±0.08, $p<0.05$, n=7).

DISCUSSION

Egg is a large haploid cell and fertilized eggs can give rise to a young organism. Hence, analysis of proteins in avian egg membrane is expected to reveal their role in nourishment, development, immune protection and structural strength. Our results show that the eggshell membranes contain many extractable proteins and peptides notwithstanding the fact that much of the membrane material remains insoluble even, under chaotropic extraction condition. Many proteins identified in the ESM have previously been shown to be present in other compartments of the egg

(Gautron, et al., 2001; Kaweewong, Garnjanagoonchorn, Jirapakkul and Roytrakul, 2013; Miksík, et al., 2007). Some of these included egg white proteins such as ovalbumin, ovotransferrin, lysozyme, clusterin, ovocleidin, ovoglycoprotein, ovomucoid, and ovoinhibitors that are considered to be highly abundant (Boschetti and Righetti, 2008; Mann, 2007a; Rose and Hincke, 2009). The methanol extraction led to the recovery of several peptides some of which turned out to be the fragments of clusterin, a secretory multifunctional glycoprotein associated with cytoprotective, and chaperon-like function(Jones and Jomary, 2002). It has been reported that clusterin protects against a wide range of environmental, microbial, and oxidative stress which the egg may naturally be exposed to. However, the significance of different clusterin peptides, most of which appeared to be derived from one domain, is not understood. Two of the peptides identified in the methanol extract by MALDI-TOF-MS were derived from, a phosphatase and actin regulator (PHAR) protein and another, a sporozoite surface protein 2-like protein. PHAR is involved in actin binding cytoskeletal organizing function associated with neuronal development of embryo (Allen, et al., 2004)although the significance of its presence along with many other signaling proteins in ESM, is not understood. However, there were also many cytoskeletal organizational proteins identified in guanidine extracts of ESM. The sporozoite surface protein 2-like (SSP2-like) protein is an orthologue of a protein present on the surface of several unicellular parasites (Tewari, et al., 2002). It is an adhesive protein that can bind to extracellular matrix based on its function in malarial parasites (Behet, et al., 2014). Whether SSP2-like protein acts as a decoy protein protective against parasite invasion of egg is not known. Other major peptides of note identified in the methanol soluble fraction, were lysozyme, a cationic, antibacterial protein which is one of the most abundant proteins present in all compartments of the egg and 3 other defensin-like peptides corresponding to m/z 4484, 4597,

and 4778 all of which showed to contain 3 disulfide bonds common to most avian beta defensins (AvBD)(Lynn, Higgs, Gaines, Tierney, James, Lloyd, Fares, Mulcahy and O'Farrelly, 2004; Zhang and Sunkara, 2014). Two of these peptides, m/z 4484 and 4597, were of interest because they occurred at different proportions in white and brown ESM both of which were identified as gallins with the former being shorter by a single N-terminal amino acid leucine (L). The gallin, also known as ovodefensin, was identified by Mann(Mann, 2007b) as a meleagrin-like peptide in chickens although similar homologous peptides were identified in many other species of birds(Naknukool, et al., 2011; Odani, et al., 1989). Gong et al.(Gong, et al., 2010a) identified 3 isoforms of gallin in chicken oviduct suggesting the polymorphism resulted from gene duplication. However, in ESM we detected only 2 isoforms of the same gallin in both white and brown eggs although they occurred in differential proportions. The peptide corresponding to m/z 4778 was provisionally identified as gallinacin 10 containing 3 disulfide bonds(Lynn, et al., 2007; Xiao, Hughes, Ando, Matsuda, Cheng, Skinner-Noble and Zhang, 2004; Zhang and Sunkara, 2014) that we deduced to match to the stretch of sequence corresponding to "DPLFPDTVACRTQGNFCRAGACPPTFTISGQCHGGLLNCCAKIPAQ". This sequence nested the predicted, mature sequence of gallinacin 10 suggesting that the m/z 4778 peptide may be the mature peptide sequence of gallinacin 10 that is 5 amino acid longer than the predicted sequence(Lynn, Higgs, Gaines, Tierney, James, Lloyd, Fares, Mulcahy and O'Farrelly, 2004; Xiao, Hughes, Ando, Matsuda, Cheng, Skinner-Noble and Zhang, 2004; Zhang and Sunkara, 2014). The occurrence of gallinacin 10 in other egg compartments and uterine secretion have been reported(Mann, Macek and Olsen, 2006; Marie, et al., 2015).

The GdHCl extract included most of the proteins and peptides in the methanol extract. A functional annotation of them using the STRAP(Bhatia, Perlman, Costello and McComb, 2009), showed these proteins being largely associated with metabolic, regulatory, developmental, and binding activities. Collagens, keratin, laminin, agrin, ovoglycan, and chondroitin sulfate are most likely associated with structural ecomponents of the membrane whereas proteins such as titin, obscurin, and nebulin, that are associated with muscle biomechanical function(Meyer and Wright, 2013), presumably, provide biomechanical support and resilience to the membrane protecting the egg against drop damage. There were numerous cytoskeletal organizational, anchoring, scaffolding, and tethering proteins (dynein, filamin, nesprin, ninein, xin, golgin, and aczonin), and glycoproteins related to adhesion and differentiation functions (protocadherin), metal and vitamin binding proteins (ovotransferrin, riboflavin-binding), enzyme proteins (kinases, helicase, ligase), and regulatory proteins, the functional significance of which in ESM are not understood. Many of these molecules although may have been acquired during the passage of egg (Sun, et al., 2013) in the reproductive tract, they could very likely be responsible for providing molecular coordination for the development of embryo.

Many proteins identified in the ESM such as defensin, ovotransferrin, ovocalyxin, and lysozyme including some keratin peptides which have been shown to be antimicrobial conceivably provide protection against microbial invasion (Gautron, et al., 2011; Superti, et al., 2007; Tam, et al., 2012; Zhang and Sunkara, 2014). Protease inhibitors and anti-proteases such as ovalbumin Y, ovomacroglobulin (ovostatin), ovomucoid, ovoglycan, also possess antimicrobial activities that are associated with defensive functions (Gautron, et al., 2007; Huopalahti, 2007; Mann and Mann, 2011; Mann and Mann, 2013). Mucoid substance such as ovomucin and mucin similarly,

provide defense against virus(Lieleg, et al., 2012). 222Likewise, there are serine proteases which possess microbiocidal activities (Heutinck, et al., 2010) present in GdHCl extracts of ESM. The shell membrane is an antimicrobial protein rich matrix that not only provides protection to the egg but also harbors other proteins associated with cellular development that can provide external cues to embryo development.

Mann et al.(Mann, et al., 2007), using decalcified egg shell membrane, identified the presence of several phosphoproteins such as osteopontin and phosvitin which are implicated in eggshell calcification(Hincke, et al., 2010). The conspicuous absence of these 2 proteins in the inner eggshell membrane in our study suggests that either the shell membrane proximal to egg white, is naturally low or deficient in these proteins since it does not undergo calcification or our search parameters precluded the identification of these phosphoproteins. However, both ovocleidin-116 (OC-116) and ovocalyxin, both of which are phosphoproteins and implicated in mineralization process were identified (Hincke, Nys and Gautron, 2010; Horvat-Gordon, et al., 2008) that suggests that there was no problem related to our methodology to identify osteopontin and phosvitin.

In conclusion, our results show that the ESM is rich in a variety of proteins and peptides many of which are associated with different protective and supportive functions for embryo. Whereas the presence of many abundant proteins in the ESM are consistent with the literature, the differences in identification of some minor abundance proteins can also be attributed to other related issues such as extraction conditions, and post translational modifications as well as search parameters (Aebersold, 2009; Ahmed and Rice, 2005). Overall, the natural abundance of such a large

repertoire of bioactive proteins and peptides in ESM suggests that it can be a potent nutritional supplement to improve health and performance of post-hatch poultry(Makkar, et al., 2015b) in the same paradigm of mammalian milk.

ACKNOWLEDGMENT

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ASSOCIATED CONTENT

Supporting Information

Table S1, List of Proteins identified in guandine HCl extract of eggshell membranes, Figures S1-S4, fragmentation spectra and peptide mass fingerprints of m/z 2001 and 3231.

AUTHOR INFORMATION

NCR and RL conceived and designed the experiment. SM, LK and RL performed the experiments and analyzed the data. NCR, SM, LK, and BP wrote the manuscript. NCR, RL, and JL made critical revision and final approval of the manuscript.

ABBREVIATIONS

AvBD, avian beta defensin; ESI, electrospray ionization; ESM, egg shell membrane; FA, formic acid; ISD, in source decay; GdHCl, guanidine hydrochloride; LC-MS, liquid chromatography mass spectrometry; MALDI-TOF, matrix assisted laser desorption ionization time-of-flight

Table 1: Identification of peptides or proteins corresponding to mass and the analytical methods

m/z	Sequence	Protein	Method of identification
1902	TPPFGGFREAFVPPVQR	clusterin	MALDI LIFT-TOF/TOF, PMF-
2001	TPPFGGFREAFVPPVQRV	clusterin	MALDI LIFT-TOF/TOF, PMF-
2157	TPPFGGFREAFVPPVQRVR	clusterin	MALDI LIFT-TOF/TOF, PMF-
3231	TPPFGGFREAFVPPVQRVRLVPPRRRLS	clusterin	MALDI LIFT-TOF/TOF, PMF-
2878	PPKRGLLPTNPPEAALPSKPPGDRTVTA	phosphatase and actin regulator	MALDI-TOF-MS, LIFT-MS-MS
2894	PNPIGLIGPIGPNVSNPIGLLGPNGPNAFS	sporozoite surface protein 2-like	MALDI-TOF-MS, LIFT-MS-MS
4484	VLKYCPKIGYCSNTCSKTQIWATSHGCKMYCCLPA	gallin protein precursor	MALDI-ISD, LIFT-TOF/TOF
4597	LVLKYCPKIGYCSNTCSKTQIWATSHGCKMYCCLP	gallin protein precursor	MALDI-ISD, LIFT-TOF/TOF
4778	DPLFPDTVACRTQGNFCRAGACPPTFTISGQCHGGLLNCCAKIPAQ KIPAQ	predicted gallinacin 10	MALDI-TOF-MS, LC-MS/MS

Table 2. List of proteins/peptides identified from methanol extract of eggshell membrane

	Accession	Protein	MW [kDa]	Scores	#Peptides
1	gi|345100466	Chain A, Hen Egg White Lysozyme with A Isoaspartate Residue	14.3	801.6 (M:801.6)	14
2	gi|342165190	Ovocleidin-116; Short=OC-116; Flags: Precursor	76.8	285.4 (M:285.4)	7
3	gi|4325105	clusterin [Gallus gallus]	51.3	109.6 (M:109.6)	3
4	gi|293321591	Gallin protein precursor [Gallus gallus]	4.9	90.8 (M:90.8)	2
5	gi|212485	ovoinhibitor [Gallus gallus]	51.9	90.6 (M:90.6)	2
6	gi|513218610	PREDICTED: uncharacterized protein LOC771972 isoformX1 [Gallus gallus]	27.1	71.5 (M:71.5)	2
7	gi|513175885	PREDICTED: angiotensinogen isoform X7 [Gallus gallus]	51.3	45.3 (M:45.3)	2
8	gi|46487955	gallinacin 10 prepropeptide [Gallus gallus]	7.1	35.0 (M:35.0)	1
9	gi|295982528	Chain P, Tcr 21.30 in complex With MHC Class II-ag (11-27)	2.0	33.4 (M:33.4)	1

Figure 1. Flow chart of eggshell membrane (ESM) sample processing.

Figure 1

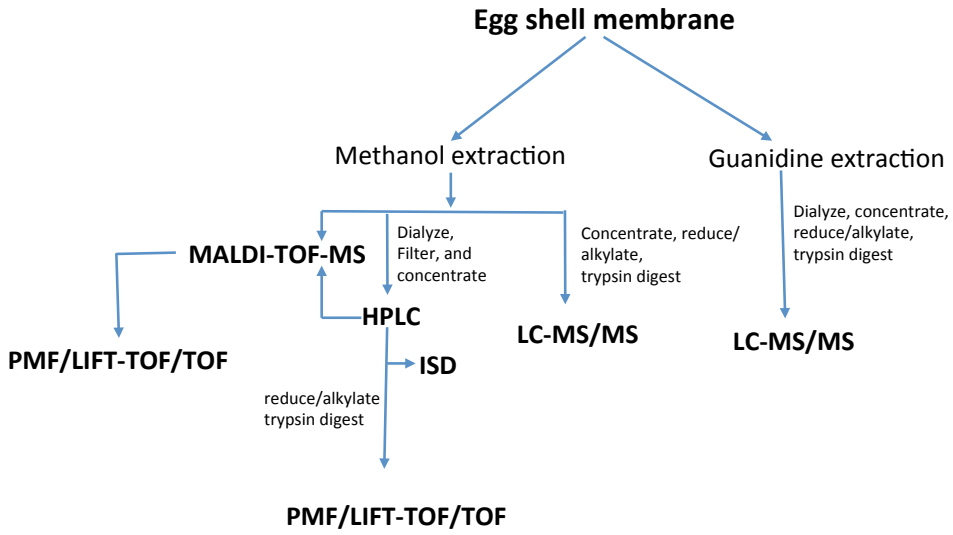


Figure 2. Direct MALDI-TOF mass spectrum of 70% methanol extract of ESM spotted with dihydroxybenzoic acid (DHB) as the MALDI matrix; m/z values of all the annotated peaks shown between m/z 1,000-6,000 represent values closer to average masses rather than monoisotopic masses.

Figure 2

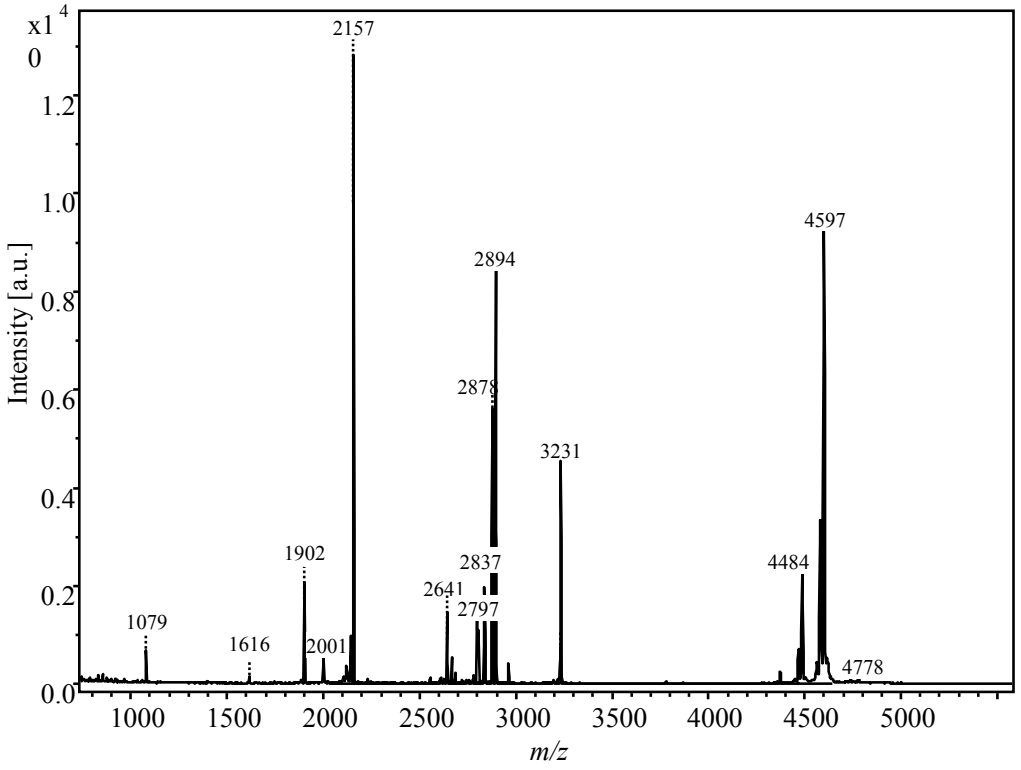


Figure 3. MALDI-TOF mass spectra of methanol extract of ESM without (a) and with reduction and alkylation with DTT/ iodoacetamide (b). Arrows show peaks that were modified by carbamidomethylation and the m/z values of all annotated peaks represent values closer to average masses rather than monoisotopic masses.

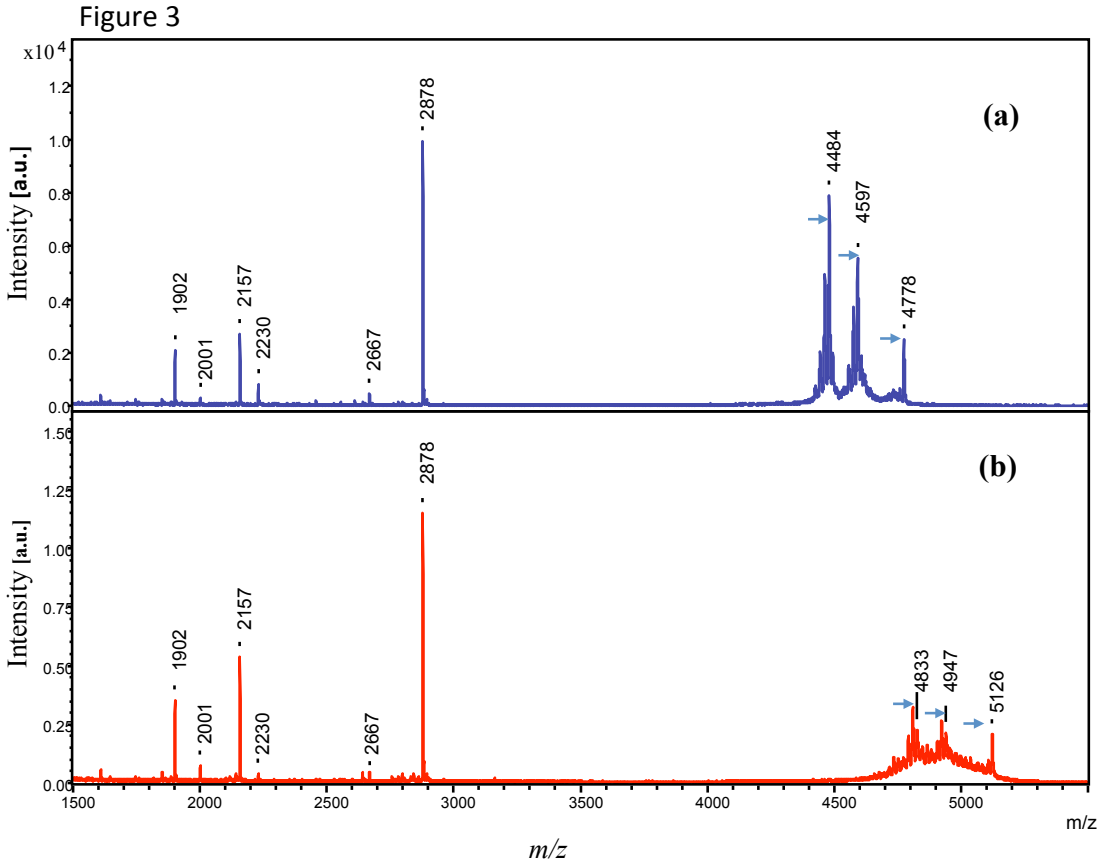
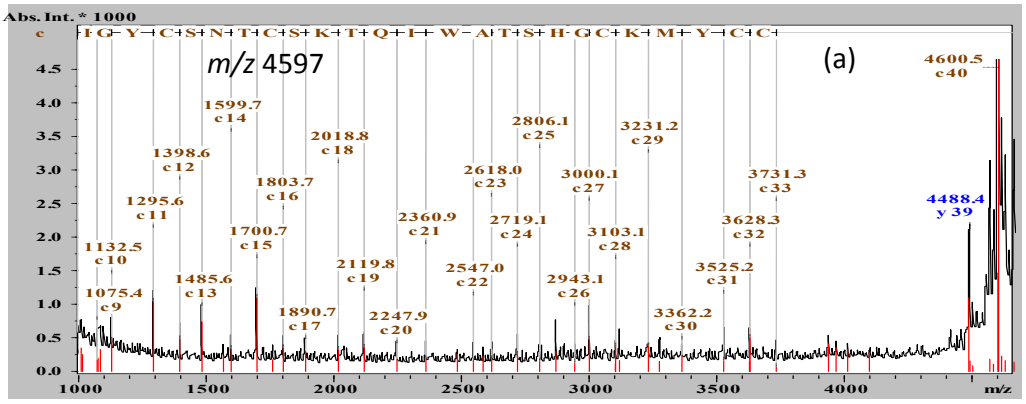


Figure 4. MALDI-ISD-TOF-MS of LC purified *m/z* 4597 (a) and 4484 (b) peaks in Figure 2 showing the N-terminus sequence tag obtained from the corresponding c fragment ions. MASCOT sequence query identified them as gallin precursors.



Match to : gi|293321607 Score : 132 Expect: 3.3e-009; Gallin protein precursor [Gallus gallus]

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
5 - 47	4595.0000	4593.9926	4596.4988	-2.5062	0	L.CLVFAVLLLVSLAAPGYGLVLKYCPKIGYCSNTCSKTQIWATS

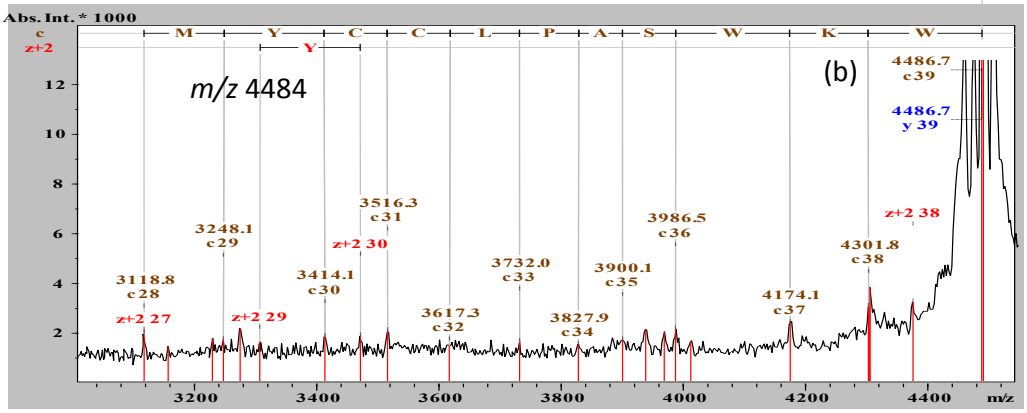


Figure 5. MALDI LIFT-TOF/TOF fragmentation spectra for LC purified, reduced/alkylated (a) m/*z* 4597 (m/z 4943) and (b) 4484 (m/z 4833) peaks showing corresponding b and y ion fragments and their identifications gallin precursors.

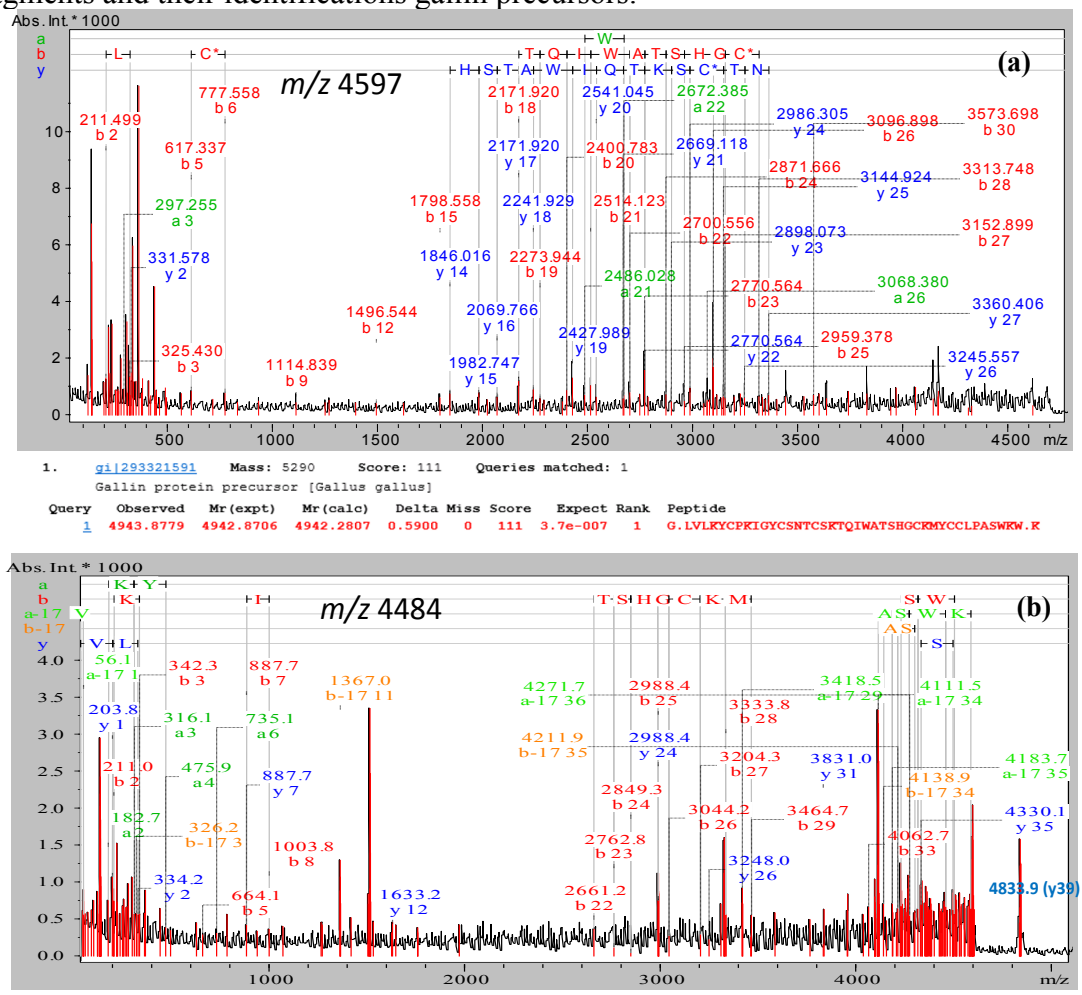
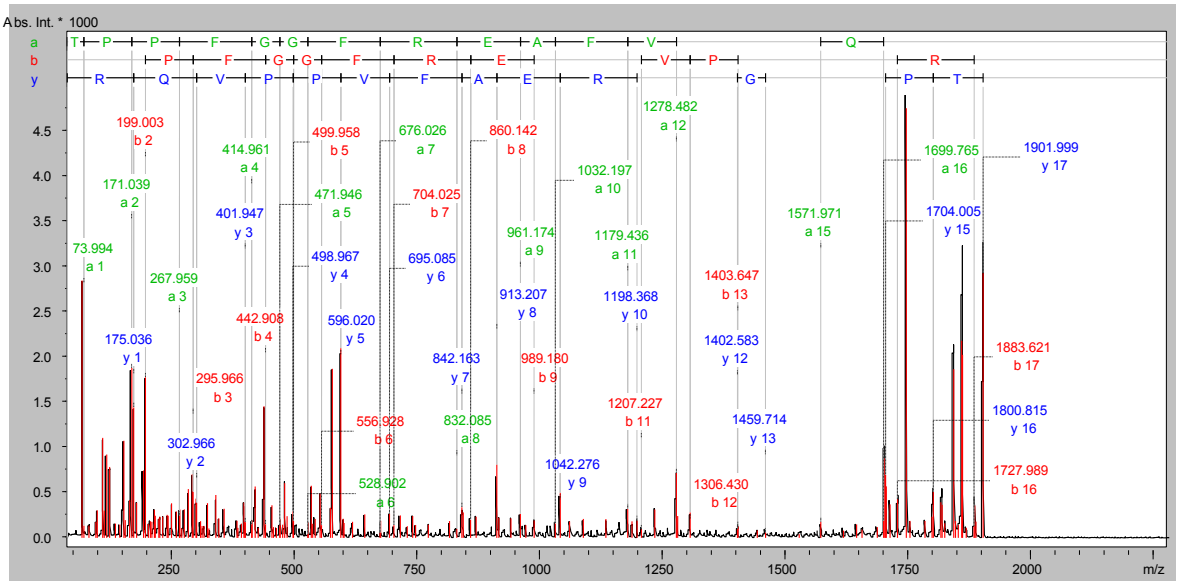


Figure 6. MALDI LIFT-TOF/TOF fragmentation for peak observed at m/z 1902 in direct MALDI-TOF-MS shown in Figure 2. MASCOT MS/MS ion search identified it as a part of clusterin

Figure 6

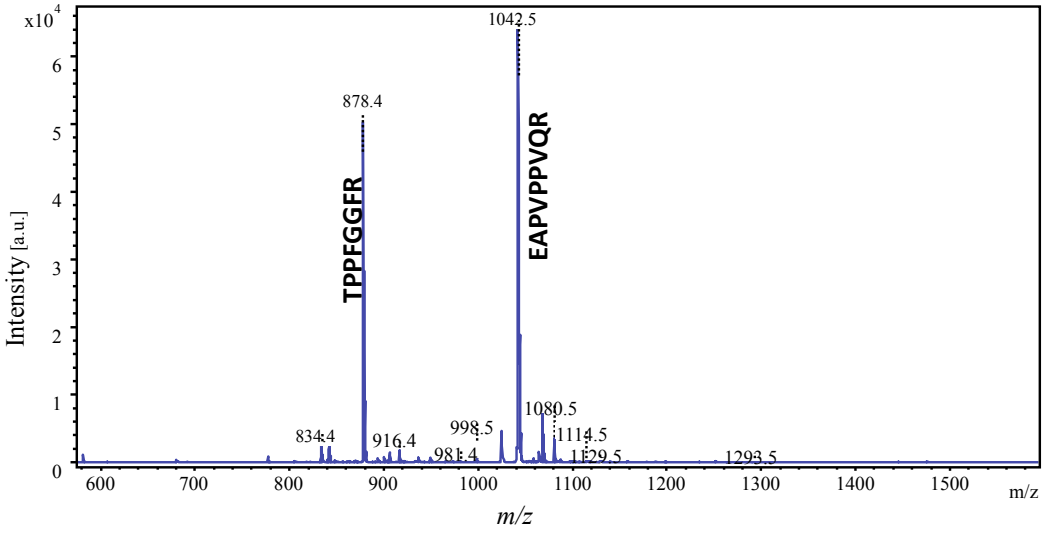


1. gi|4325105 Mass: 51943 Score: 78 Queries matched: 1
clusterin [Gallus gallus]

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Peptide
1	1902.0020	1900.9947	1900.9948	-0.05	0	83	5.4e-005	1	R.TPPFGGFREAFVPPVQR.V

Figure 7. MALDI peptide mass finger print of purified m/z 1902 in direct MALDI-TOF-MS showing tryptic fragments m/z 878 and 1042.

Figure 7

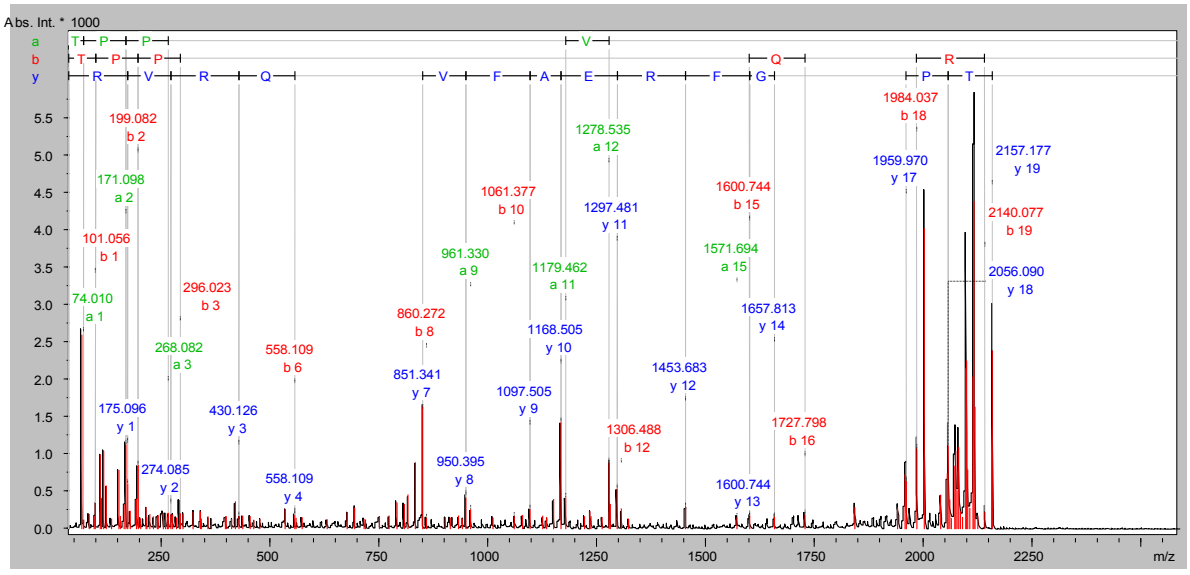


1. gi|4325105 Mass: 51943 Score: 74 Queries matched: 2
clusterin [Gallus gallus]

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Peptide
11	878.4271	877.4198	877.4447	-28.29	0	22	0.92	1	R.TPPFGGFR.E
30	1042.5425	1041.5353	1041.5607	-24.45	0	52	0.00082	1	R.EAFVPPVQR.V

Figure 8. MALDI LIFT-TOF/TOF fragmentation of peak at *m/z* 2157 observed in direct MALDI-TOF-MS in Figure 1 and MASCOT MS/MS ion search showing the corresponding sequence 'TPPFGGFREAFVPPVQRVR,' identified as the clusterin fragment.

Figure 8



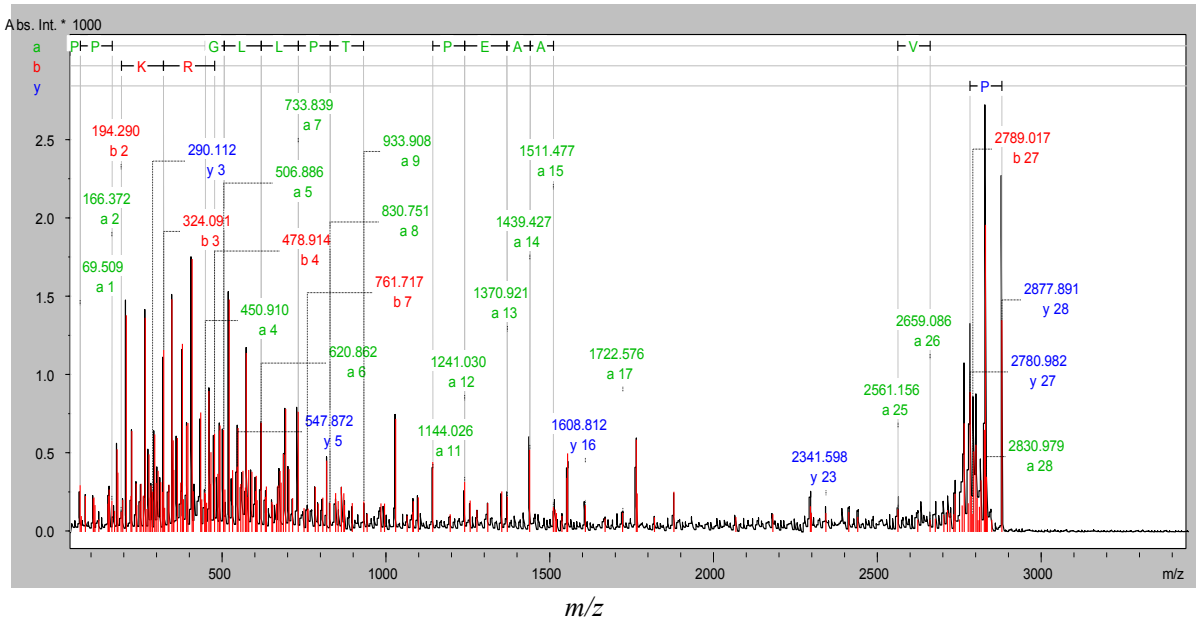
m/z

1. gi|4325105 Mass: 51316 Score: 84 Queries matched: 1
clusterin [Gallus gallus]

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Peptide
1	2157.1721	2156.1648	2156.1644	0.22	0	92	1e-005	1	R.TPPFGGFREAFVPPVQRVR.L

Figure 9. MALDI LIFT-TOF/TOF fragmentation of m/z 2878 peak (Figure 2) and MASCOT MS/MS ion search identification of as phosphatase and actin regulator protein with corresponding fragment sequence PPKRGLLPTNPPEAALPSKPPGDRTVTA.

Figure 9



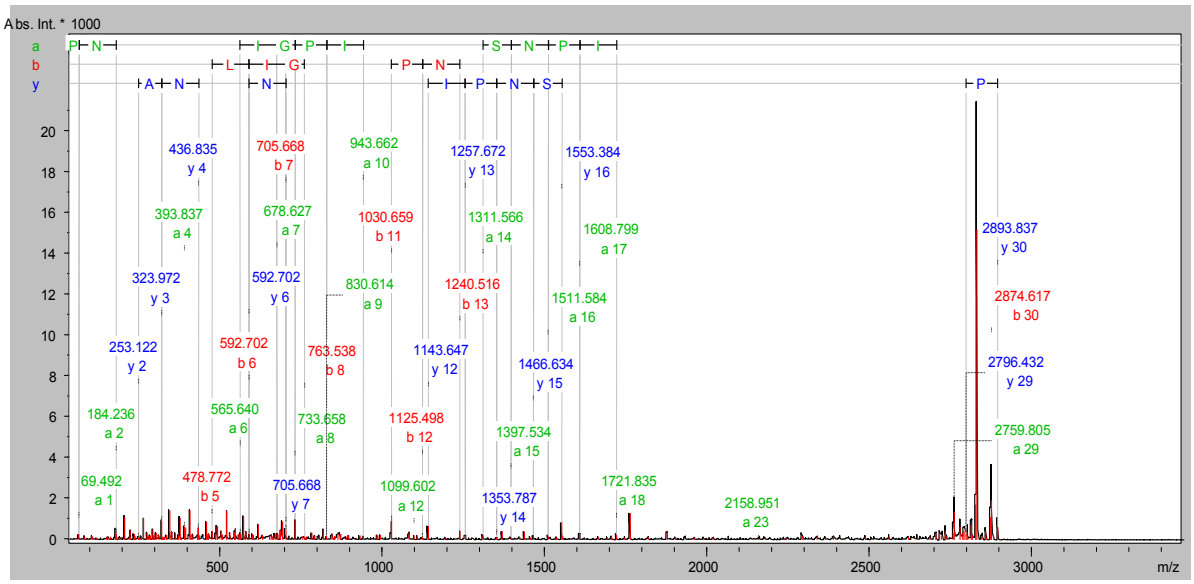
m/z

1. gi|384955725 **Mass:** 80093 **Score:** 52 **Queries matched:** 1
RecName: Full=Phosphatase and actin regulator 4

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Peptide
1	2877.8440	2876.8367	2876.5872	86.7	0	59	0.0036	1	L.PPKRGLLPTNPPEAALPSKPPGDRTVTA.S

Figure 10. MALDI LIFT-TOF/TOF fragmentation of peak at *m/z* 2894 in direct MALDI-TOF-MS (Figure 2) and MASCOT MS/MS ion search showing its identification as a part of sporozoite surface protein 2-like corresponding to the sequence "PNPIGLIGPIGPNVSNPIGLLGPNGPNAFS".

Figure 10



m/z

1. gi|513239416 Mass: 14276 Score: 58 Queries matched: 1
PREDICTED: sporozoite surface protein 2-like, partial [Gallus gallus]

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Peptide
1	2893.8479	2892.8406	2892.5498	101	0	62	0.015	1	V.PNPIGLIGPIGPNVSNPIGLLGPNGPNAFS.P

Figure 11. STRAP annotation of GdHCl extracted, LC/MS/MS identified proteins

Figure 11

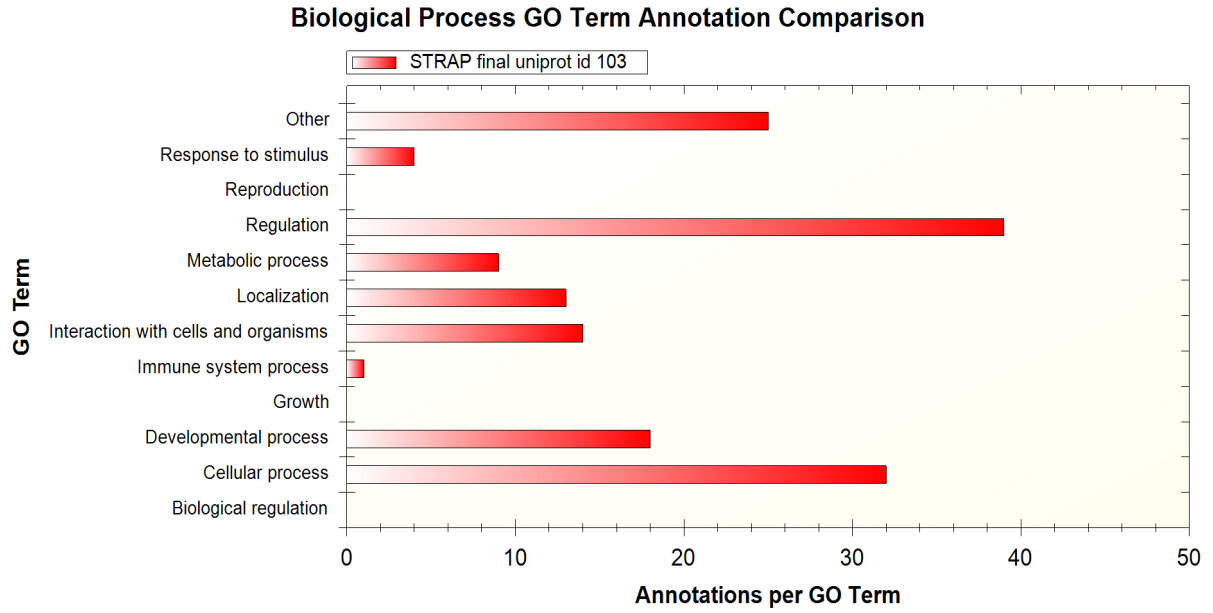
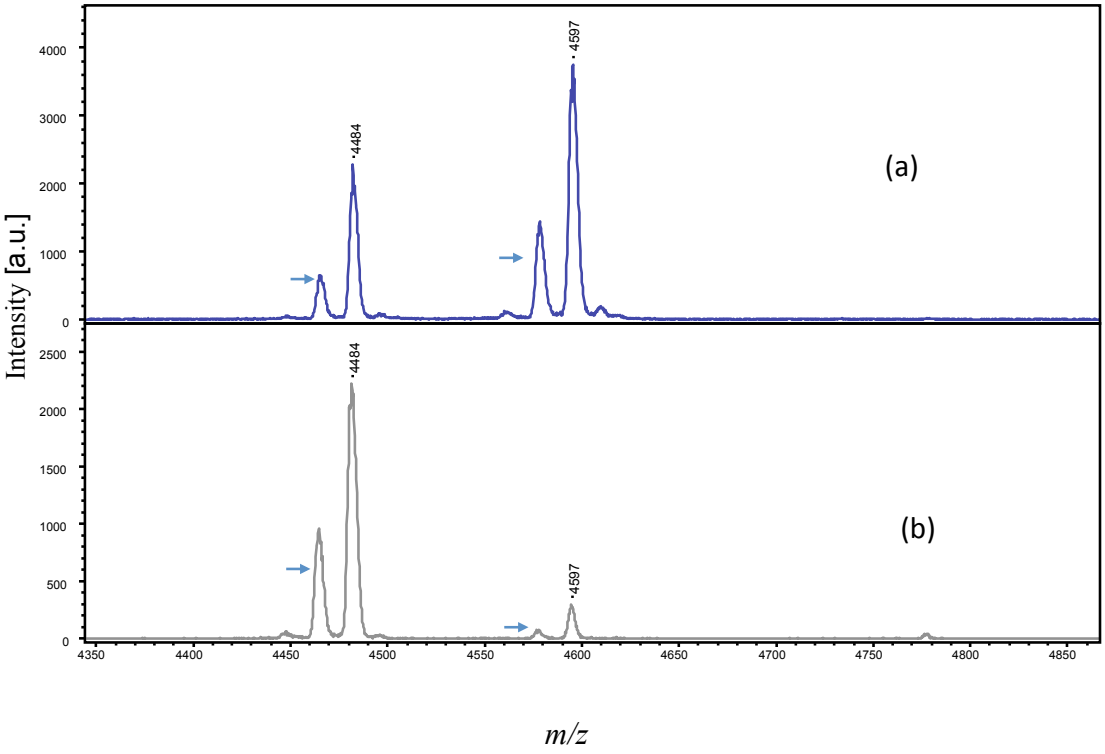


Figure 12. Profiles of m/z 4484 and 4597 peptides expressed in white and brown ESM; the minor peaks (arrow) are corresponding gallin isoforms with loss of H_2O

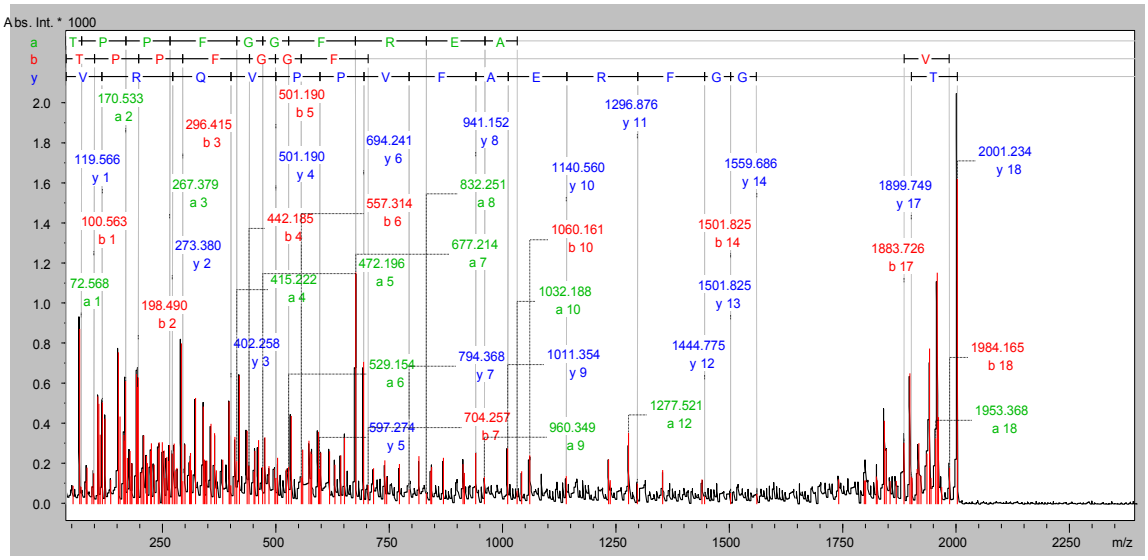
Figure 12



Supplementary Figure and Table legends

Figure S1. MALDI LIFT-TOF/TOF fragmentation for peak observed at m/z 2001 in direct MALDI-TOF-MS.

Figure S1

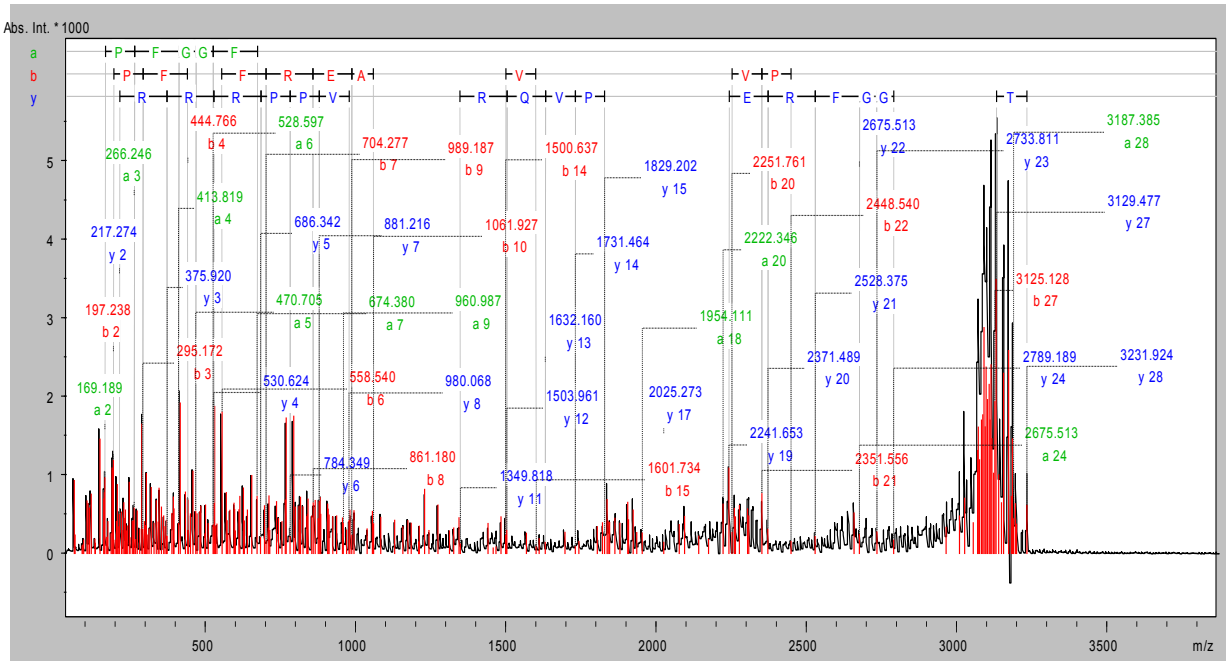


m/z

1. gi|4325105 **Mass:** 51316 **Score:** 56 **Queries matched:** 1
clusterin [Gallus gallus]

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Peptide
1	2001.2130	2000.2057	2000.0632	71.2	0	63	0.008	1	R.TPPFGGFREAFVPPVQRV.R

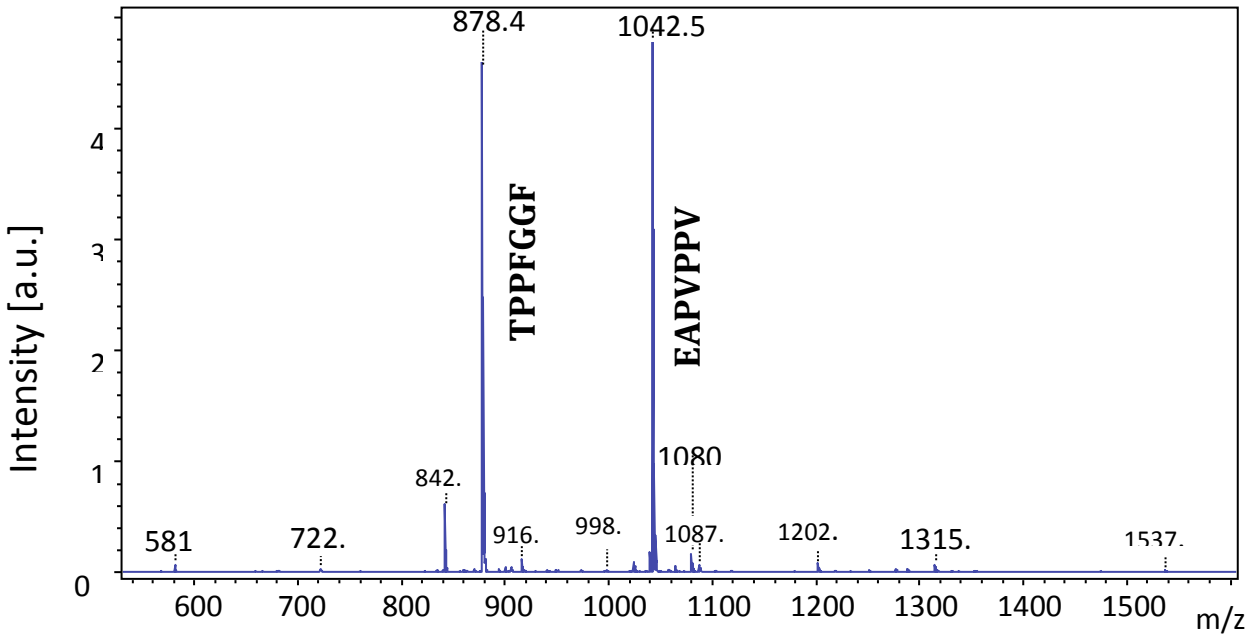
Figure S2. MALDI LIFT-TOF/TOF fragmentation for peak observed at m/z 3231 in direct MALDI-TOF-MS shown in Figure 2. MASCOT MS/MS ion search showed identification as a part of clusterin with a sequence tag, TPPFGGFREAFVPPVQRVRLVPPRRRLS



1. gi|4325105 Mass: 51316 Score: 51 Queries matched: 1
clusterin [Gallus gallus]

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Peptide
1	3232.0840	3231.0767	3230.8418	72.7	0	61	0.0027	1	R.TPPFGGFREAFVPPVQRVRLVPPRRRLS.R

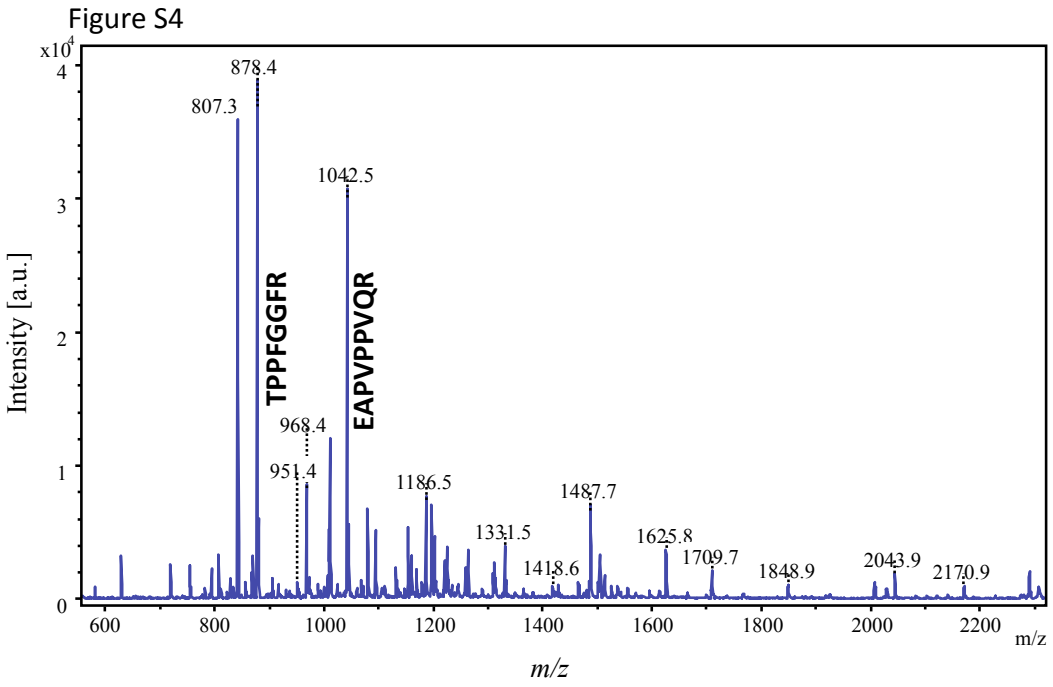
Figure S3. MALDI peptide mass finger print (PMF) of purified *m/z* 2001 in direct MALDI-TOF-MS



1. gi|4325105 Mass: 51943 Score: 49 Queries matched: 2
clusterin [Gallus gallus]

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Peptide
8	878.4251	877.4179	877.4447	-30.53	0	22	0.98	1	R.TPPFGGFR.E
21	1042.5372	1041.5299	1041.5607	-29.57	0	26	0.29	1	R.EAFVPPVQR.V

Figure S4. MALDI peptide mass finger print (PMF) of purified *m/z* 3231 shown by direct MALDI-TOF-MS



1. gi|4325105 Mass: 51943 Score: 34 Queries matched: 2
clusterin [Gallus gallus]

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Peptide
11	878.4245	877.4173	877.4447	-31.21	0	24	0.3	1	R.TPPFGGFR.E
25	1042.5388	1041.5315	1041.5607	-28.03	0	34	0.023	1	R.EAFVPPVQR.V

Supplement Table 1 List of proteins/peptides identified from GdHCl extract of eggshell membrane (ESM).

Row	Accession	Protein	MW [kDa]	Scores	#Peptides
1	gi|71274079	Gallin protein precursor [Gallus gallus]	77.8	2609.0 (M:2609.0)	54
2	gi|83754919	Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin At 2.15 Angstrom Resolution	75.8	2593.4 (M:2593.4)	54
3	gi|34811330	Chain A, Crystal Structure Of S-ovalbumin At 1.9 Angstrom Resolution	42.9	1698.2 (M:1698.2)	34
4	gi|440923753	Chain C, Crystal Structure Of Uncleaved Ovalbumin At 1.95 Angstroms Resolution	42.8	1637.2 (M:1637.2)	32
5	gi|510032768	ovalbumin-related protein X [Gallus gallus gallus]	45.4	732.1 (M:732.1)	17
6	gi|229157	lysozyme	14.3	700.1 (M:700.1)	12
7	gi|345100466	Chain A, Hen Egg White Lysozyme With A Isoaspartate Residue	14.3	697.4 (M:697.4)	14
8	gi|385145541	ovalbumin-related Y [Gallus gallus]	43.8	595.3 (M:595.3)	16
9	gi|513193913	PREDICTED: titin isoform X2 [Gallus gallus]	3652	523.8 (M:523.8)	34
10	gi|513188927	PREDICTED: mucin-6 [Gallus gallus]	291.1	457.4 (M:457.4)	15
11	gi|4325105	clusterin [Gallus gallus]	51.3	443.8 (M:443.8)	11
12	gi|129295	RecName: Full=Ovalbumin-related protein X; AltName: Full=Gene X protein	26.3	405.1 (M:405.1)	9
13	gi|162952006	ovomucoid precursor [Gallus gallus]	22.4	349.6 (M:349.6)	8
14	gi|223464	ovomucoid	20.2	339.5 (M:339.5)	8
15	gi|513191195	PREDICTED: beta-microseminoprotein-like	12.1	286.3 (M:286.3)	6

		[Gallus gallus]			
16	gi|352173	protein,riboflavin binding	25	286.0 (M:286.0)	6
17	gi|63052	unnamed protein product [Gallus gallus]	17.5	272.1 (M:272.1)	5
18	gi|671865	ovomacroglobulin, ovostatin [Gallus gallus]	164	264.0 (M:264.0)	8
19	gi|7441632	ovocleidin - chicken	15.3	240.7 (M:240.7)	5
20	gi|385145531	ovoglobulinG2 type AB [Gallus gallus]	47.4	239.1 (M:239.1)	9
21	gi|22218070	ovoglycoprotein precursor [Gallus gallus]	22.3	238.9 (M:238.9)	5
22	gi|342165190	RecName: Full=Ovocleidin-116; Short=OC-116; Flags: Precursor	76.8	221.1 (M:221.1)	8
23	gi|513167276	PREDICTED: obscurin [Gallus gallus]	1158.7	209.6 (M:209.6)	13
24	gi|212485	ovoinhibitor [Gallus gallus]	51.9	148.3 (M:148.3)	4
25	gi|513178501	PREDICTED: dystonin isoform X4 [Gallus gallus]	920.1	144.0 (M:144.0)	10
26	gi|513206786	PREDICTED: ovoinhibitor [Gallus gallus]	57	137.7 (M:137.7)	4
27	gi|513193378	PREDICTED: dynein heavy chain 7, axonemal isoform X1 [Gallus gallus]	458.9	133.7 (M:133.7)	9
28	gi|223059	ovalbumin N term fragment	4.2	130.7 (M:130.7)	2
29	gi|4204093	egg white lysozyme [Gallus gallus]	4.9	124.8 (M:124.8)	4
30	gi|363734560	PREDICTED: mucin-5B isoform X1 [Gallus gallus]	233.4	122.0 (M:122.0)	6
31	gi|61102692	Xin [Gallus gallus]	216.1	116.5 (M:116.5)	8
32	gi|365813307	Chain A, Crystal Structure Of Monoz-Biotin-Avidin Complex	13.6	113.3 (M:113.3)	3
33	gi|576329	Chain A, Crystal Structure Of Vitelline Membrane Outer Layer	18	112.0 (M:112.0)	4

		Protein I (Vmo-I): A Folding Motif With Homologous Greek Key Structures Related By An Internal Three-Fold Symmetry			
34	gi|513180391	PREDICTED: LOW QUALITY PROTEIN: transcription initiation factor TFIID subunit 1 [Gallus gallus]	216.6	109.6 (M:109.6)	7
35	gi|513195515	PREDICTED: nebulin [Gallus gallus]	752.6	107.4 (M:107.4)	8
36	gi|513206710	PREDICTED: histone-lysine N-methyltransferase, H3 lysine-36 and H4 lysine-20 specific isoform X6 [Gallus gallus]	290.2	107.0 (M:107.0)	6
37	gi|513213183	PREDICTED: golgin subfamily A member 1 isoform X7 [Gallus gallus]	90.2	106.7 (M:106.7)	6
38	gi|1334744	spectrin alpha chain [Gallus gallus]	281.8	105.3 (M:105.3)	7
39	gi|513229885	PREDICTED: sperm flagellar protein 2 [Gallus gallus]	270.1	103.2 (M:103.2)	7
40	gi|102221132	apolipoprotein B [Gallus gallus]	523	102.6 (M:102.6)	6
41	gi|513217982	PREDICTED: protein kinase C-binding protein 1 isoform X22 [Gallus gallus]	132	101.3 (M:101.3)	6
42	gi|513157185	PREDICTED: golgin subfamily B member 1 isoform X3 [Gallus gallus]	362.7	100.6 (M:100.6)	6
43	gi|513190030	PREDICTED: ninein isoform X15 [Gallus gallus]	233.4	99.6 (M:99.6)	7
44	gi|6433844	aczonin [Gallus gallus]	560.4	99.2 (M:99.2)	6
45	gi|513162168	PREDICTED: uro-adherence factor A isoform X1 [Gallus gallus]	245.5	97.2 (M:97.2)	6

46	gi|363727445	PREDICTED: protein piccolo, partial [Gallus gallus]	401.4	96.1 (M:96.1)	6
47	gi|363738135	PREDICTED: LOW QUALITY PROTEIN: chromodomain-helicase-DNA-binding protein 9 [Gallus gallus]	322.2	96.0 (M:96.0)	6
48	gi|63370	unnamed protein product [Gallus gallus]	422.6	95.2 (M:95.2)	7
49	gi|513217433	PREDICTED: centrosome-associated protein CEP250 isoform X14 [Gallus gallus]	287.7	92.4 (M:92.4)	7
50	gi|513187528	PREDICTED: spectrin beta chain, non-erythrocytic 5 [Gallus gallus]	453	92.3 (M:92.3)	6
51	gi|157168357	centromere protein F [Gallus gallus]	339.7	90.4 (M:90.4)	6
52	gi|371928996	keratin 75 [Gallus gallus]	54.3	90.0 (M:90.0)	3
53	gi|116669	RecName: Full=Acetyl-CoA carboxylase; Short=ACC; Includes: RecName: Full=Biotin carboxylase	262.6	89.9 (M:89.9)	6
54	gi|513218156	PREDICTED: death-inducer obliterator 1 isoform X4 [Gallus gallus]	223.5	89.8 (M:89.8)	5
55	gi|293321591	Gallin protein precursor [Gallus gallus]	4.9	88.4 (M:88.4)	2
56	gi|513201109	PREDICTED: vacuolar protein sorting-associated protein 13C isoform X2 [Gallus gallus]	416.6	88.2 (M:88.2)	5
57	gi|513240592	PREDICTED: LOW QUALITY PROTEIN: histone-lysine N-methyltransferase MLL2, partial [Gallus gallus]	575.9	87.7 (M:87.7)	6
58	gi|356991167	E3 ubiquitin-protein ligase HERC2 [Gallus gallus]	528.7	86.8 (M:86.8)	7
59	gi|513214081	PREDICTED: E3 ubiquitin-protein ligase	602.8	86.0 (M:86.0)	7

		RNF213 isoform X6 [Gallus gallus]			
60	gi|513182967	PREDICTED: rho GTPase-activating protein 24 isoform X5 [Gallus gallus]	83.8	85.9 (M:85.9)	5
61	gi|29837126	SMC1 protein cohesin subunit [Gallus gallus]	142.9	85.4 (M:85.4)	5
62	gi|513210403	PREDICTED: LOW QUALITY PROTEIN: acetyl-CoA carboxylase 2 [Gallus gallus]	266.2	85.0 (M:85.0)	6
63	gi|513189629	PREDICTED: nesprin-2 isoform X1 [Gallus gallus]	803.8	84.9 (M:84.9)	6
64	gi|392018	filamin [Gallus gallus]	275.7	84.8 (M:84.8)	5
65	gi|363744378	PREDICTED: DENN domain-containing protein 4C isoform X5 [Gallus gallus]	211.5	84.3 (M:84.3)	4
66	gi|513182982	PREDICTED: rho GTPase-activating protein 24 isoform X10 [Gallus gallus]	73.2	83.9 (M:83.9)	5
67	gi|15341204	cgABP260 [Gallus gallus]	280.3	82.9 (M:82.9)	5
68	gi|513193268	PREDICTED: A-kinase anchor protein 9 isoform X20 [Gallus gallus]	506.8	82.8 (M:82.8)	6
69	gi|513227073	PREDICTED: RNA exonuclease 1 homolog isoform X3 [Gallus gallus]	130.6	82.6 (M:82.6)	6
70	gi|513181431	PREDICTED: uncharacterized protein KIAA1210 isoform X5 [Gallus gallus]	103.6	82.3 (M:82.3)	4
71	gi|513210496	PREDICTED: tetratricopeptide repeat protein 28 [Gallus gallus]	265.1	82.0 (M:82.0)	5
72	gi|513182471	PREDICTED: extracellular matrix protein FRAS1 isoform X2 [Gallus gallus]	439.3	81.6 (M:81.6)	4
73	gi|513176503	PREDICTED: nesprin-1 isoform X6 [Gallus	1010.5	80.6 (M:80.6)	7

		gallus]			
74	gi|513185495	PREDICTED: Alstrom syndrome protein 1 isoform X1 [Gallus gallus]	292.6	79.5 (M:79.5)	5
75	gi|513196869	PREDICTED: nucleoprotein TPR isoform X6 [Gallus gallus]	276.8	78.7 (M:78.7)	6
76	gi|513176284	PREDICTED: utrophin isoform X2 [Gallus gallus]	351.1	78.3 (M:78.3)	5
77	gi|513221651	PREDICTED: microtubule-actin cross-linking factor 1-like, partial [Gallus gallus]	363	78.0 (M:78.0)	6
78	gi|1020104	melanotransferrin/EOS47 [Gallus gallus]	80.9	77.9 (M:77.9)	4
79	gi|7248371	myosin heavy chain [Gallus gallus]	223.3	76.0 (M:76.0)	5
80	gi|118090437	PREDICTED: protocadherin Fat 1 isoform X6 [Gallus gallus]	507.9	76.0 (M:76.0)	5
81	gi|513195972	PREDICTED: LOW QUALITY PROTEIN: abnormal spindle-like microcephaly-associated protein homolog [Gallus gallus]	398.3	75.8 (M:75.8)	6
82	gi|299469458	nuclear mitotic apparatus protein [Gallus gallus]	241.4	75.8 (M:75.8)	5
83	gi|513178510	PREDICTED: dystonin isoform X7 [Gallus gallus]	308.2	75.7 (M:75.7)	5
84	gi|513202440	PREDICTED: microtubule-associated protein 1A [Gallus gallus]	307.1	75.5 (M:75.5)	5
85	gi|91208266	RecName: Full=Cytospin-A; AltName: Full=SPECC1-like protein; AltName: Full=Sperm antigen with calponin homology and coiled-coil domains 1-	124.8	75.2 (M:75.2)	5

		like			
86	gi|158186693	A-kinase anchor protein 9 [Gallus gallus]	455.2	75.1 (M:75.1)	5
87	gi|363731544	PREDICTED: dynein heavy chain 8, axonemal [Gallus gallus]	534.2	74.6 (M:74.6)	5
88	gi|363737124	PREDICTED: dedicator of cytokinesis protein 10 [Gallus gallus]	249.7	74.4 (M:74.4)	3
89	gi|513210175	PREDICTED: kinetochore-associated protein 1 isoform X3 [Gallus gallus]	251	73.9 (M:73.9)	4
90	gi|513179159	PREDICTED: intersectin-2 isoform X6 [Gallus gallus]	172.2	73.7 (M:73.7)	4
91	gi|513158331	PREDICTED: centrosomal protein of 290 kDa isoform X6 [Gallus gallus]	288.8	73.4 (M:73.4)	5
92	gi|62954540	Ovocalyxin-36 precursor [Gallus gallus]	48.8	73.2 (M:73.2)	1
93	gi|513204692	PREDICTED: LOW QUALITY PROTEIN: dynein heavy chain 1, axonemal [Gallus gallus]	489.6	73.2 (M:73.2)	5
94	gi|513183661	PREDICTED: ankyrin-2 [Gallus gallus]	447.9	72.8 (M:72.8)	5
95	gi|478430999	melanoma inhibitory activity protein 3 precursor [Gallus gallus]	221.6	72.6 (M:72.6)	5
96	gi|513218117	PREDICTED: laminin subunit alpha-5 isoform X9 [Gallus gallus]	408.8	72.3 (M:72.3)	5
97	gi|513160180	PREDICTED: ELKS/Rab6-interacting/CAST family member 1 isoform X7 [Gallus gallus]	117.2	72.2 (M:72.2)	5
98	gi|206597434	collagen alpha-2(I) chain precursor [Gallus gallus]	128.8	71.9 (M:71.9)	5
99	gi|50746309	PREDICTED: rho GTPase-activating protein 10 isoform 2 [Gallus gallus]	88.9	71.8 (M:71.8)	5
100	gi|50745053	PREDICTED: structural	127.7	70.9 (M:70.9)	5

		maintenance of chromosomes protein 6 isoform X2 [Gallus gallus]			
101	gi|363736045	PREDICTED: telomere-associated protein RIF1 [Gallus gallus]	254.2	70.8 (M:70.8)	4
102	gi|363737706	PREDICTED: LOW QUALITY PROTEIN: unconventional myosin-Vc [Gallus gallus]	202.8	70.2 (M:70.2)	4
103	gi|116248042	beta-defensin 11 [Gallus gallus]	11.6	70.1 (M:70.1)	1
104	gi|513213292	PREDICTED: myosin-3 [Gallus gallus]	219	70.1 (M:70.1)	4
105	gi|343469213	MPDZ protein [Gallus gallus]	214.1	70.0 (M:70.0)	5
106	gi|363739068	PREDICTED: probable phospholipid-transporting ATPase VB isoform X8 [Gallus gallus]	165.6	69.5 (M:69.5)	4
107	gi|513180457	PREDICTED: testis-expressed sequence 11 protein isoform X8 [Gallus gallus]	101.4	69.4 (M:69.4)	4
108	gi|513168024	PREDICTED: sickle tail protein homolog isoform X16 [Gallus gallus]	156.7	69.2 (M:69.2)	4
109	gi|513194213	PREDICTED: bile salt export pump isoform X2 [Gallus gallus]	148.5	68.2 (M:68.2)	4
110	gi|513175708	PREDICTED: LOW QUALITY PROTEIN: ryanodine receptor 2 [Gallus gallus]	564.6	67.7 (M:67.7)	5
111	gi|513190198	PREDICTED: protein AHNAK2 [Gallus gallus]	389.1	67.3 (M:67.3)	5
112	gi|363734923	PREDICTED: cytoplasmic dynein 1 heavy chain 1 [Gallus gallus]	525.5	67.2 (M:67.2)	4
113	gi|2145309	TBP0 [Gallus gallus]	33.1	67.0 (M:67.0)	4
114	gi|60544838	gonad expressed transcript [Gallus gallus]	177.1	66.9 (M:66.9)	5
115	gi|298542005	unnamed protein product	121.7	66.8 (M:66.8)	5

		[Gallus gallus]			
116	gi|363728442	PREDICTED: coiled-coil domain-containing protein KIAA1407 homolog isoform X2 [Gallus gallus]	106	66.4 (M:66.4)	4
117	gi|513164437	PREDICTED: ADP-ribosylhydrolase like 1 isoform X4 [Gallus gallus]	208.5	66.4 (M:66.4)	3
118	gi|21623677	SPACR [Gallus gallus]	102.6	66.1 (M:66.1)	4
119	gi|118093388	PREDICTED: E3 ubiquitin-protein ligase HECW2 [Gallus gallus]	176	65.9 (M:65.9)	3
120	gi|513192568	PREDICTED: WD repeat-containing protein 96 [Gallus gallus]	204	65.8 (M:65.8)	4
121	gi|513188813	PREDICTED: protein unc-79 homolog isoform X29 [Gallus gallus]	262.5	65.7 (M:65.7)	4
122	gi|513197226	PREDICTED: bromodomain testis-specific protein isoform X6 [Gallus gallus]	102.7	65.6 (M:65.6)	5
123	gi|162417991	protocadherin Fat 3 precursor [Gallus gallus]	501.6	65.3 (M:65.3)	4
124	gi|513225858	PREDICTED: formin-like 1 [Gallus gallus]	131.3	65.2 (M:65.2)	5
125	gi|513208391	PREDICTED: polycystin-1 isoform X3 [Gallus gallus]	479.2	64.9 (M:64.9)	3
126	gi|513229901	PREDICTED: ATP-dependent RNA helicase DHX29, partial [Gallus gallus]	148	64.7 (M:64.7)	4
127	gi|513175724	PREDICTED: LOW QUALITY PROTEIN: lysosomal-trafficking regulator [Gallus gallus]	426.9	64.6 (M:64.6)	5
128	gi|513184797	PREDICTED: biorientation of chromosomes in cell division 1-like isoform X3 [Gallus gallus]	325.3	64.6 (M:64.6)	4
129	gi|513204547	PREDICTED: stabilin-1 [Gallus gallus]	268.7	64.5 (M:64.5)	4

130	gi|513166523	PREDICTED: LOW QUALITY PROTEIN: teneurin-4 isoform X6 [Gallus gallus]	311.5	64.4 (M:64.4)	4
131	gi|253735708	glutathione peroxidase 3 precursor [Gallus gallus]	24.6	63.9 (M:63.9)	1
132	gi|513170156	PREDICTED: transcriptional repressor NF-X1 isoform X16 [Gallus gallus]	119.4	63.8 (M:63.8)	3
133	gi|513160161	PREDICTED: serine/threonine-protein kinase WNK1 isoform X5 [Gallus gallus]	293.8	63.4 (M:63.4)	4
134	gi|363744372	PREDICTED: LOW QUALITY PROTEIN: mitogen-activated protein kinase kinase kinase 1 [Gallus gallus]	170.3	63.2 (M:63.2)	4
135	gi|513211039	PREDICTED: DNA polymerase epsilon catalytic subunit A isoform X1 [Gallus gallus]	260.7	63.0 (M:63.0)	4
136	gi|513187516	PREDICTED: mitogen-activated protein kinase-binding protein 1 isoform X1 [Gallus gallus]	189.4	62.9 (M:62.9)	4
137	gi|438007	alpha-2-macroglobulin receptor [Gallus gallus]	506.8	62.9 (M:62.9)	4
138	gi|363732080	PREDICTED: BEN domain-containing protein 3 isoformX2 [Gallus gallus]	87.6	62.7 (M:62.7)	3
139	gi|513162558	PREDICTED: glutamate receptor ionotropic, kainate 1 isoform X2 [Gallus gallus]	102.1	62.4 (M:62.4)	4
140	gi|60098865	hypothetical protein RCJMB04_13m2 [Gallus gallus]	158.3	62.0 (M:62.0)	3
141	gi|363740639	PREDICTED: myosin heavy chain, skeletal muscle, adult isoform X1 [Gallus gallus]	223.1	62.0 (M:62.0)	3
142	gi|513181168	PREDICTED:	163.2	62.0 (M:62.0)	4

		uncharacterized protein KIAA2022 isoform X7 [Gallus gallus]			
143	gi|211121	agrin [Gallus gallus]	211.3	61.9 (M:61.9)	5
144	gi|3184528	T-Box protein 3 [Gallus gallus]	46.4	61.6 (M:61.6)	4
145	gi|513162834	PREDICTED: E3 ubiquitin-protein ligase TTC3 [Gallus gallus]	226.8	61.2 (M:61.2)	4
146	gi|363737435	PREDICTED: 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase eta-1 isoform X4 [Gallus gallus]	188.8	61.1 (M:61.1)	5
147	gi|513166963	PREDICTED: C2 domain-containing protein 3 isoform X6 [Gallus gallus]	256.9	61.0 (M:61.0)	4
148	gi|118085134	PREDICTED: cytoplasmic dynein 2 heavy chain 1 isoform X2 [Gallus gallus]	491.7	61.0 (M:61.0)	3
149	gi|513165698	PREDICTED: protein furry homolog isoform X2 [Gallus gallus]	343.8	60.9 (M:60.9)	5
150	gi|513211222	PREDICTED: HORMA domain-containing protein 2 isoform X7 [Gallus gallus]	38.6	60.9 (M:60.9)	3
151	gi|226823291	hydrocephalus inducing homolog [Gallus gallus]	564.4	60.6 (M:60.6)	4
152	gi|513178325	PREDICTED: LOW QUALITY PROTEIN: filamin-A-interacting protein 1 [Gallus gallus]	137.9	60.1 (M:60.1)	4
153	gi|513228901	PREDICTED: Nipped-B homolog-like isoform X7 [Gallus gallus]	291.1	60.1 (M:60.1)	4
154	gi|513176211	PREDICTED: mitogen-activated protein kinase kinase kinase 4 isoform X3 [Gallus gallus]	178.5	59.9 (M:59.9)	4
155	gi|344925838	FYVE and coiled-coil domain-containing protein 1 [Gallus gallus]	176.8	59.9 (M:59.9)	3

156	gi|513162041	PREDICTED: pleckstrin homology-like domain, family B, member 2 isoform X3 [Gallus gallus]	147.7	59.8 (M:59.8)	4
157	gi|10241574	teneurin-2 [Gallus gallus]	310.6	59.8 (M:59.8)	4
158	gi|389616152	TBC1 domain family member 1 [Gallus gallus]	134.2	59.8 (M:59.8)	3
159	gi|513188188	PREDICTED: uncharacterized protein LOC423333 isoform X7 [Gallus gallus]	193.9	59.7 (M:59.7)	4
160	gi|513202856	PREDICTED: pleckstrin homology domain-containing family G member 4B isoform X1 [Gallus gallus]	125.1	59.6 (M:59.6)	4
161	gi|513192909	PREDICTED: ATP-binding cassette sub-family A member 12 [Gallus gallus]	428.2	59.6 (M:59.6)	5
162	gi|2463529	DNA topoisomeraseII_beta [Gallus gallus]	183.1	59.5 (M:59.5)	4
163	gi|513183646	PREDICTED: protocadherin Fat 4 [Gallus gallus]	543.7	59.4 (M:59.4)	4
164	gi|513170392	PREDICTED: lysine-specific histone demethylase 1B isoform X1 [Gallus gallus]	65.9	59.1 (M:59.1)	4
165	gi|513210289	PREDICTED: LOW QUALITY PROTEIN: probable E3 ubiquitin-protein ligase HECTD4 [Gallus gallus]	486.2	59.1 (M:59.1)	3
166	gi|513229854	PREDICTED: microtubule-associated protein 1B isoform X2 [Gallus gallus]	288.8	58.7 (M:58.7)	4
167	gi|513172748	PREDICTED: oxygen-regulated protein 1 [Gallus gallus]	390.1	58.6 (M:58.6)	4
168	gi|513222086	PREDICTED: suppressor of tumorigenicity 14 protein homolog isoform	94.1	58.4 (M:58.4)	4

		X16 [Gallus gallus]			
169	gi|513209631	PREDICTED: dynein heavy chain 3, axonemal isoform X11 [Gallus gallus]	432.9	58.1 (M:58.1)	4
170	gi|513169783	PREDICTED: LOW QUALITY PROTEIN: golgin subfamily A member 4 [Gallus gallus]	264.1	57.5 (M:57.5)	3
171	gi|14278285	Chain A, Crystal Structure Of Avian Atic, A Bifunctional Transformylase And Cyclohydrolase Enzyme In Purine Biosynthesis At 1.75 Ang. Resolution	64.2	57.3 (M:57.3)	4
172	gi|50733622	PREDICTED: CD83 antigen [Gallus gallus]	23.6	57.2 (M:57.2)	3
173	gi|513178813	PREDICTED: myelin transcription factor 1-like isoform X30 [Gallus gallus]	111	57.0 (M:57.0)	4
174	gi|513199927	PREDICTED: leucine-rich repeat-containing protein 31 isoform X2 [Gallus gallus]	64.1	56.8 (M:56.8)	3
175	gi|50593343	axin protein 1 transcript variant 1 [Gallus gallus]	94.8	56.5 (M:56.5)	2
176	gi|513175430	PREDICTED: baculoviral IAP repeat-containing protein 6 [Gallus gallus]	506.2	56.4 (M:56.4)	3
177	gi|313661353	rho-associated protein kinase 1 [Gallus gallus]	158.6	56.4 (M:56.4)	4
178	gi|363734028	PREDICTED: LOW QUALITY PROTEIN: 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-2 [Gallus gallus]	138.5	56.4 (M:56.4)	4
179	gi|462740	RecName: Full=Neuronal cell adhesion molecule; Short=Nr-CAM; AltName: Full=Neuronal surface protein Bravo;	141.8	56.2 (M:56.2)	3

		Short=gBravo; AltName: Full=NgCAM-related cell adhesion molecule; Short=Ng-CAM-related; Flags: Precursor			
180	gi|513182151	PREDICTED: SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5 isoform X1 [Gallus gallus]	116.6	56.0 (M:56.0)	4
181	gi|513187539	PREDICTED: cytosolic phospholipase A2 epsilon isoform X2 [Gallus gallus]	91.7	55.9 (M:55.9)	3
182	gi|513158072	PREDICTED: LOW QUALITY PROTEIN: gamma-tubulin complex component 6 [Gallus gallus]	203.3	55.8 (M:55.8)	4
183	gi|227016	apolipoprotein AI	28.8	55.7 (M:55.7)	3
184	gi|513229146	PREDICTED: chondroitin sulfate proteoglycan 4-like isoform X2 [Gallus gallus]	275.9	55.6 (M:55.6)	3
185	gi|513163146	PREDICTED: peripheral plasma membrane protein CASK isoform X11 [Gallus gallus]	94.5	55.3 (M:55.3)	3
186	gi|510936992	chromodomain-helicase-DNA-binding protein 2 [Gallus gallus]	212.7	54.9 (M:54.9)	4
187	gi|513199109	PREDICTED: kinesin family member 1A isoform X5 [Gallus gallus]	192.4	54.4 (M:54.4)	4
188	gi|117380068	cortactin-binding protein 2 [Gallus gallus]	177.9	54.4 (M:54.4)	3
189	gi|513188106	PREDICTED: bromodomain adjacent to zinc finger domain protein 1A isoform X7 [Gallus gallus]	168.3	54.3 (M:54.3)	3
190	gi|513174643	PREDICTED: ninein-like protein isoform X10	62.8	54.1 (M:54.1)	4

		[Gallus gallus]			
191	gi|513193788	PREDICTED: neurobeachin-like 1 isoform X12 [Gallus gallus]	306.2	54.0 (M:54.0)	3
192	gi|53130528	hypothetical protein RCJMB04_8i12 [Gallus gallus]	109.8	53.9 (M:53.9)	3
193	gi|513172897	PREDICTED: LOW QUALITY PROTEIN: regulating synaptic membrane exocytosis protein 2 [Gallus gallus]	181.8	53.8 (M:53.8)	3
194	gi|513158188	PREDICTED: DNA helicase B isoform X3 [Gallus gallus]	116.1	53.7 (M:53.7)	2
195	gi|76468580	aldehyde oxidase 2 [Gallus gallus]	147.7	53.6 (M:53.6)	4
196	gi|118095631	PREDICTED: probable E3 ubiquitin-protein ligase HERC1 isoform X8 [Gallus gallus]	532.6	53.0 (M:53.0)	4
197	gi|513200951	PREDICTED: S phase cyclin A-associated protein in the endoplasmic reticulum isoform X6 [Gallus gallus]	154.5	52.8 (M:52.8)	3
198	gi|513166677	PREDICTED: atherin-like [Gallus gallus]	33.5	52.7 (M:52.7)	2
199	gi|513194247	PREDICTED: low-density lipoprotein receptor-related protein 2 isoform X9 [Gallus gallus]	521.8	52.5 (M:52.5)	4
200	gi|14017756	chick atrial myosin heavy chain [Gallus gallus]	221.7	52.3 (M:52.3)	3
201	gi|513181916	PREDICTED: probable ATP-dependent RNA helicase DDX60 isoform X1 [Gallus gallus]	210.8	51.9 (M:51.9)	4
202	gi|513209317	PREDICTED: tetratricopeptide repeat protein 18 isoform X1 [Gallus gallus]	138.2	51.8 (M:51.8)	3
203	gi|513158974	PREDICTED:	207	51.6 (M:51.6)	3

		transcription factor 20 isoform X7 [Gallus gallus]			
204	gi|513185632	PREDICTED: attractin, partial [Gallus gallus]	144.3	51.5 (M:51.5)	3
205	gi|513232435	PREDICTED: proteasome-associated protein ECM29 homolog isoform X3 [Gallus gallus]	203.9	51.4 (M:51.4)	4
206	gi|513203915	PREDICTED: polycystic kidney disease protein 1-like 2 [Gallus gallus]	273.3	51.4 (M:51.4)	4
207	gi|53133498	hypothetical protein RCJMB04_17e23 [Gallus gallus]	49.4	51.4 (M:51.4)	3
208	gi|513165204	PREDICTED: von Willebrand factor A domain-containing protein 8-like [Gallus gallus]	213.4	51.3 (M:51.3)	3
209	gi|513163290	PREDICTED: maestro heat-like repeat-containing protein family member 2B-like isoform X9 [Gallus gallus]	141.2	51.2 (M:51.2)	3
210	gi|513212577	PREDICTED: protein PRRC2B isoform X15 [Gallus gallus]	245.8	51.1 (M:51.1)	4
211	gi|483968268	mRNA turnover protein 4 homolog [Gallus gallus]	28	50.8 (M:50.8)	3
212	gi|513211075	PREDICTED: scavenger receptor class F member 2 isoform X2 [Gallus gallus]	102.3	50.7 (M:50.7)	3
213	gi|363731756	PREDICTED: usherin [Gallus gallus]	573.9	50.7 (M:50.7)	3
214	gi|513171368	PREDICTED: dynein heavy chain 5, axonemal-like [Gallus gallus]	533.7	50.7 (M:50.7)	4
215	gi|513178065	PREDICTED: midasin isoform X4 [Gallus gallus]	632.3	50.4 (M:50.4)	3
216	gi|513209920	PREDICTED: probable ATP-dependent RNA	116.8	50.4 (M:50.4)	3

		helicase DHX37 isoform X3 [Gallus gallus]			
217	gi|513221021	PREDICTED: lysine-specific histone demethylase 1A, partial [Gallus gallus]	86.3	50.3 (M:50.3)	3
218	gi|513194426	PREDICTED: tetratricopeptide repeat protein 21B isoform X3 [Gallus gallus]	157.3	50.2 (M:50.2)	3
219	gi|53133818	hypothetical protein RCJMB04_20k2 [Gallus gallus]	86.2	50.2 (M:50.2)	3
220	gi|211622	alpha-3 collagen type VI [Gallus gallus]	339.4	50.0 (M:50.0)	3
221	gi|513200349	PREDICTED: mediator complex subunit 12-like isoform X3 [Gallus gallus]	237.8	49.6 (M:49.6)	3
222	gi|293651608	cytoplasmic linker associated protein 2 [Gallus gallus]	164.8	49.5 (M:49.5)	3
223	gi|5733818	gephyrin [Gallus gallus]	79.7	49.5 (M:49.5)	3
224	gi|2330003	glutamine rich protein, partial [Gallus gallus]	112.2	49.5 (M:49.5)	4
225	gi|513211995	PREDICTED: FK506-binding protein 15 isoform X8 [Gallus gallus]	138.3	49.4 (M:49.4)	4
226	gi|513191098	PREDICTED: golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1 isoform X5 [Gallus gallus]	200.2	49.4 (M:49.4)	3
227	gi|363728726	PREDICTED: protein dopey-2 [Gallus gallus]	257.7	49.3 (M:49.3)	2
228	gi|118102546	PREDICTED: inositol 1,4,5-trisphosphate receptor type 3 isoform X2 [Gallus gallus]	304.6	49.3 (M:49.3)	4
229	gi|50582493	vitellogenin [Gallus gallus]	162.5	49.3 (M:49.3)	3
230	gi|513175768	PREDICTED: AT-rich interactive domain-containing protein 4B	146.3	49.2 (M:49.2)	3

		isoform X2 [Gallus gallus]			
231	gi|513224576	PREDICTED: pleckstrin homology domain-containing family A member 6 isoform X27 [Gallus gallus]	114.2	49.2 (M:49.2)	3
232	gi|20140635	RecName: Full=Transferrin receptor protein 1; Short=TR; Short=TfR; Short=TfR1; Short=Trfr	85.6	49.0 (M:49.0)	4
233	gi|363738939	PREDICTED: SH3 and PX domain-containing protein 2B isoform X3 [Gallus gallus]	96.4	48.8 (M:48.8)	3
234	gi|513171922	PREDICTED: growth regulation by estrogen in breast cancer-like isoform X13 [Gallus gallus]	194.6	48.7 (M:48.7)	3
235	gi|146219852	breast cancer 2, early onset [Gallus gallus]	377.5	48.7 (M:48.7)	3
236	gi|53136870	hypothetical protein RCJMB04_35e7 [Gallus gallus]	94.1	48.5 (M:48.5)	3
237	gi|513223426	PREDICTED: nucleoporin 210kDa-like isoform X7 [Gallus gallus]	188.4	48.5 (M:48.5)	2
238	gi|513179755	PREDICTED: tudor domain-containing protein 6 [Gallus gallus]	172.7	48.4 (M:48.4)	4
239	gi|513190759	PREDICTED: activating signal cointegrator 1 complex subunit 1 isoform X7 [Gallus gallus]	40.8	48.1 (M:48.1)	3
240	gi|513184258	PREDICTED: NF-X1-type zinc finger protein NFXL1 isoform X3 [Gallus gallus]	84.4	47.9 (M:47.9)	4
241	gi|363735853	PREDICTED: alkyldihydroxyacetonephosphate synthase, peroxisomal [Gallus	70.7	47.8 (M:47.8)	3

		gallus]			
242	gi|513232874	PREDICTED: Dmx-like 1 isoform X4 [Gallus gallus]	272.6	47.8 (M:47.8)	3
243	gi|186703014	PNPLA7 [Gallus gallus]	147.6	47.7 (M:47.7)	3
244	gi|513239041	PREDICTED: maestro heat-like repeat-containing protein family member 2B-like isoform X5 [Gallus gallus]	128.8	47.6 (M:47.6)	3
245	gi|60099181	hypothetical protein RCJMB04_32g20 [Gallus gallus]	138	47.5 (M:47.5)	3
246	gi|513178375	PREDICTED: regulating synaptic membrane exocytosis protein 1 isoform X12 [Gallus gallus]	174	47.4 (M:47.4)	3
247	gi|363733842	PREDICTED: LOW QUALITY PROTEIN: regulator of G-protein signaling 12 [Gallus gallus]	166.4	47.4 (M:47.4)	4
248	gi|313747559	A-kinase anchor protein 8-like [Gallus gallus]	80.4	47.1 (M:47.1)	4
249	gi|513229372	PREDICTED: A disintegrin and metalloproteinase with thrombospondin motifs 6 isoform X10 [Gallus gallus]	109.6	47.1 (M:47.1)	3
250	gi|513200909	PREDICTED: chondroitin sulfate proteoglycan 4 isoform X5 [Gallus gallus]	266.8	47.0 (M:47.0)	3
251	gi|513229093	PREDICTED: integrin alpha-2 [Gallus gallus]	129.6	47.0 (M:47.0)	3
252	gi|513164384	PREDICTED: LOW QUALITY PROTEIN: E3 SUMO-protein ligase RanBP2 [Gallus gallus]	336.3	46.9 (M:46.9)	3
253	gi|241982727	protein ELYS [Gallus gallus]	252.5	46.8 (M:46.8)	3
254	gi|513221255	PREDICTED: splicing factor, proline- and glutamine-rich isoform	68.7	46.7 (M:46.7)	3

		X6 [Gallus gallus]			
255	gi|513176328	PREDICTED: androglobin [Gallus gallus]	182.1	46.6 (M:46.6)	3
256	gi|313851036	cytoskeleton-associated protein 5 [Gallus gallus]	225.2	46.6 (M:46.6)	3
257	gi|513191260	PREDICTED: LOW QUALITY PROTEIN: WD repeat- and FYVE domain-containing protein 4 [Gallus gallus]	357.7	46.5 (M:46.5)	3
258	gi|60098943	hypothetical protein RCJMB04_16d21 [Gallus gallus]	88.6	46.4 (M:46.4)	3
259	gi|50742516	PREDICTED: TGF-beta-activated kinase 1 and MAP3K7-binding protein 2 isoform X2 [Gallus gallus]	76.7	46.4 (M:46.4)	3
260	gi|349732129	rho GTPase-activating protein 29 [Gallus gallus]	151.7	46.2 (M:46.2)	3
261	gi|513200221	PREDICTED: leucine-, glutamate- and lysine-rich protein 1 isoform X21 [Gallus gallus]	77.7	46.2 (M:46.2)	3
262	gi|534285973	Chain A, Crystal Structure Of Chicken Galectin 2	14.9	46.2 (M:46.2)	3
263	gi|53129447	hypothetical protein RCJMB04_5l15 [Gallus gallus]	86.5	46.0 (M:46.0)	3
264	gi|513225769	PREDICTED: protein TANC2 isoform X19 [Gallus gallus]	205.4	46.0 (M:46.0)	3
265	gi|118082738	PREDICTED: nucleolar protein 12 [Gallus gallus]	24.9	45.9 (M:45.9)	2
266	gi|513226968	PREDICTED: tRNA-dihydrouridine(47) synthase [NAD(P)(+)]-like [Gallus gallus]	89.5	45.9 (M:45.9)	2
267	gi|513163173	PREDICTED: probable ubiquitin carboxyl-terminal hydrolase FAF-X isoform X3 [Gallus gallus]	289.5	45.8 (M:45.8)	2
268	gi|363727703	PREDICTED: apoptotic	142.2	45.8 (M:45.8)	2

		protease-activating factor 1 isoformX4 [Gallus gallus]			
269	gi|513192043	PREDICTED: von Willebrand factor A domain-containing protein 2 isoform X4 [Gallus gallus]	76.8	45.7 (M:45.7)	3
270	gi|363733636	PREDICTED: LOW QUALITY PROTEIN: DNA-directed RNA polymerase I subunit RPA1 [Gallus gallus]	192.5	45.5 (M:45.5)	3
271	gi|513200631	PREDICTED: LOW QUALITY PROTEIN: unconventional myosin-IXa [Gallus gallus]	301.4	45.5 (M:45.5)	3
272	gi|513171872	PREDICTED: LOW QUALITY PROTEIN: erythrocyte membrane protein band 4.1-like 3 [Gallus gallus]	158.4	45.5 (M:45.5)	2
273	gi|513199614	PREDICTED: DIS3 mitotic control homolog (S. cerevisiae)-like 2 isoform X2 [Gallus gallus]	126.4	45.3 (M:45.3)	3
274	gi|513163748	PREDICTED: FERM and PDZ domain-containing protein 4 isoform X3 [Gallus gallus]	194.3	45.3 (M:45.3)	3
275	gi|1096715	DNA methyltransferase	172.8	45.2 (M:45.2)	3
276	gi|513192529	PREDICTED: LOW QUALITY PROTEIN: kinesin-like protein KIF20B [Gallus gallus]	207	45.1 (M:45.1)	2

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III. Nutritional effects of egg shell membrane supplements on chicken performance and immunity

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ABSTRACT

Eggshell membranes (**ESM**) contain a variety of proteins and peptides which help in the development of embryo and provide protection to it. Many of the peptides and proteins associated with ESM have antimicrobial, immune-modulatory, and adjuvant properties. We hypothesized that the membrane byproducts from egg, provided as post hatch nutritional supplements to chickens, may improve their performance and immunity. To explore its effect, we fed 3 groups of broiler chicks with feed containing 0, 0.2% and 0.4% ESM from day 1 post hatch through 14 days and regular feed thereafter. The birds were individually weighed at the onset of the experiment and at weekly intervals until the termination at third week when they were bled and euthanized. The relative weights of liver, spleen, bursa, and heart, hematology profiles, clinical chemistry variables including serum IgM, IgG and corticosterone concentrations measured. The chickens in the ESM treated groups showed a statistically significant increase in BW with no impact on relative organ weights. Compared with controls, the WBC and lymphocyte percentage increased in chickens fed 0.4% ESM whereas the monocyte percentage decreased at both levels of ESM. Except for the serum protein which increased in ESM fed birds no other metabolic clinical chemistry variables showed any significant change. Both IgM and IgG(Y) levels were elevated and corticosterone levels reduced in chickens fed ESM supplemented diets. Our results suggest that ESM supplements during the early phases of growth may improve immunity and stress variables, and enhance their growth performance without any detrimental effect on other physiological parameters.

Key words*:* egg shell membrane, chicken, growth, immunity, stress

INTRODUCTION

Eggshells are byproducts of the poultry industry, which consist largely of calcareous outer shells underlined by proteinaceous membranes and the proteins that adsorb to these membranes from egg white (Hincke, et al., 2012; Mann, 2007; Mine and Kovacs-Nolan, 2006). The shell membranes (**ESM**) are fibrous structural proteins made up of collagens and keratins that are generally resistant to conventional gastric proteases. However there are also numerous other proteins and peptides with antimicrobial, antioxidant, and immune-modulatory properties such as lysozyme, ovotransferrin, ovalbumin, globulins, ovomucins, and defensins present in these membranes (Miksík, et al., 2007). Many of these proteins are functionally similar to some milk proteins which confer post-natal protection to newborns, help maturation of gut, and shape their microbiome (Lawrence and Pane, 2007; Rose and Hincke, 2009). Antimicrobial peptides not only provide protection against a wide range of microbes including bacteria and fungi but also can function as adjuvants enhancing immunity against foreign antigens (Brown and Hancock, 2006). In view of the need for alternatives to antibiotics in meat animal production (Seal, et al., 2013; Thacker, 2013), exploring the potential of egg byproducts to improve immunity and disease resistance in poultry is logical. We hypothesized that the factors present in the ESM may help modulate immunity and performance of chickens if provided as post hatch nutrient supplements which is the objective of this study.

MATERIALS AND METHODS

Preparation of eggshell and ESM

Unfertilized fresh eggs removed of albumen and yolk were washed by mild scrubbing inside of the shell under running water and peeled to obtain membranes. The membranes were washed by stirring with excess water for 2-3 hours, lyophilized, and finally ground to powdery flakes using

a commercial blender. To determine the membrane yield, the individual eggs and their membranes were processed separately. For preliminary trials, ESM and whole shells with or without membranes were ground separately, and used as supplements to evaluate their effects on chicken performance using BW and relative organ weights as the variables. The above preparations were mixed with the grower diet formulated per NRC specification (NRC, 1994) using a feed mixer. The amount of eggshell and ESM were set to the concentrations of 5% whole shell, 4.8% shell without membrane, and 0.2% ESM based on the observation that a large egg yields approximately 5-6 g shell and 0.2-0.25 g of ESM. Based on those initial trials, subsequent studies were done using only the ESM preparations at 0.2 and 0.4% levels, respectively

ESM and feed analysis

The nitrogen (**N**), calorie, and selective mineral content of ESM supplemented feed were analyzed in the Central Analytical Laboratory of the University of Arkansas using randomly sampled ESM powder and feed. Dumas N analyzer, bomb calorimeter, and inductively coupled plasma spectroscopy (**ICP**) were used for respective analyses.

Chicken treatments

Studies were approved by the University of Arkansas, Institutional Animal Care and Use Committee. In all trials day-old male broiler chicks from local hatchery (Cobb) were used. In the preliminary trial, the birds were divided into 3 groups consisting of 16 birds each, placed in 2 replicate battery cages, and provided *ad libitum* access to feed and water. The chickens were provided specified diets from day 1 through 14 and regular diets thereafter till the termination of experiments. The follow up and final trial reported here was done using ESM at 2 concentrations, 0.2% and 0.4%, respectively. Birds were monitored daily for mortality and

welfare. The BW of the chickens were measured at the beginning and at weekly intervals thereafter. All birds were necropsied at three weeks of age. *Blood collection and organ weight*

On day 21, the chickens were weighed and 6 from each of the replicate cages were bled by cardiac puncture; the blood was collected in Vaccutainer tubes containing EDTA for hematology and clot accelerator for serum clinical chemistry analyses. The chickens were killed by cervical dislocation and the weights of liver, heart, spleen, and bursa recorded, and calculated as percentage of BW. The blood with clot accelerator were kept at room temperature for 2 hours, centrifuged at 2,500g to separate serum, and stored in aliquots at -20 °C until the assays were done.

Hematology

Hematology measurements were done with EDTA anti-coagulated blood within 2 h of bleeding using a Cell-Dyn 3500 blood analysis system (Abbott Diagnostics, Abbott Park, IL), standardized for avian blood. The white blood cell (**WBC**), heterophil (**H**), lymphocyte (**L**), monocyte (**M**), eosinophil (**E**), basophil (**B**), red blood cell (**RBC**), and thrombocyte counts, hemoglobin, hematocrit, mean corpuscular volume (**MCV**), microhematocrit (**MCH**), red blood cell distribution width (**RDW**) values were measured, and the heterophil to lymphocyte ratios (**H/L**) calculated.

Serum chemistry, corticosterone, IgG, and IgM determination

The serum was used to determine clinical chemistry variables using a clinical chemistry analyzer (Ciba Corning Diagnostics Corp; Medfield MA). The parameters included protein, glucose, cholesterol, calcium, phosphorous, magnesium, triglycerides, blood urea nitrogen (**BUN**),

alanine aminotransferase (**ALT**), aspartyl aminotransferase (**AST**), gamma glutamyl transferase (**GGT**), creatinine, creatine kinase, and alkaline phosphatase. Serum corticosterone levels were measured using a Detect X enzyme immunoassay kit™ purchased from Arbor Assays (Ann Arbor, MI). Serum samples from 12 birds in each group were diluted 1:20 using the assay buffer provided in the kit, and the immunoassay done per instructions in the kit. The concentrations of corticosterone in serum samples were calculated from a standard curve obtained using the supplied standard. The results were expressed as nanograms of corticosterone per ml. Similarly, the IgM and IgG concentrations of the sera were determined in triplicates using reagents obtained from Bethyl Laboratory (Montgomery, TX), following the suggested instructions. Eight well strips (BD Falcon) were coated with either goat anti chicken IgM or IgG antibodies and the assays performed per respective instructions using sera diluted to 1:20,000 for IgM and 1:5000 for IgG as determined in preliminary assays. The goat anti chicken IgM- or IgG-horse radish peroxidase (**HRP**) were used as secondary antibodies respectively. The HRP enzyme activity was measured using tetramethylbenzidine (**TMB**) substrate as the end point. The concentrations of antibodies in the sera were calculated from their respective standard curves obtained using a reference calibrator serum supplied in the kit. The results were reported as mg/ml serum.

Statistical analyses

The relative organ weights were calculated as percentage of whole BW. All results were evaluated using Duncan's multiple range test using SAS software (SAS, 2009) and a *P*-value of $\leq$0.05 considered significant.

RESULTS

The preliminary trial with shell with or without membranes caused a substantial reduction in the BW while ESM alone supported growth (not shown). Similarly, the relative weights of both heart and liver increased significantly in groups receiving feed with shell containing preparations indicating their toxic effects. The chickens receiving feed supplemented with ESM only did not show any change in relative organ weights (not shown). The results of the final trial are shown later.

The analysis of ESM showed the N content ~86 % of total mass. When ESM added at 0.2% or 0.4% levels to feed, showed negligible differences compared with the total protein, caloric, or elemental content of regular diet (Table 1).

Mortality, health, and BW

Chickens fed control or ESM supplemented diets showed no mortality during the trial. The birds in overall appeared healthy and alert with no signs of sickness or lethargy. The BW showed increased differences in birds fed 0.4% ESM supplemented diet starting from first week of growth (Fig 1). At final week both treatments showed statistically higher BW relative to the controls. The BW and relative organ weight changes are shown in Table 2. There were no changes in relative heart, liver, spleen, and bursa weights of birds fed ESM supplemented diet compared with controls.

Blood Differential count

There was a significant increase in WBC, lymphocyte, RBC, and HCT values, and a decrease in monocyte counts of chickens fed 0.4% ESM compared with controls. In chickens receiving 0.2% ESM there was no significant change in blood cell counts except for monocytes which decreased as compared with control birds (Table 3).

Serum clinical chemistry, corticosterone, IgM, and IgG assays

Except for the total protein content of serum there were no changes in any of the clinical chemistry parameters including AST, ALT, GGT, BUN, uric acid, Ca, or P (Table 4). IgM and IgG content showed significant increases in the sera of chickens fed both 0.2% and 0.4% levels of ESM (Fig 2). The corticosterone levels on the contrary, showed significant reduction in the sera of birds fed 0.4% ESM supplemented diet and numerically lower at 0.2% level (Fig 3).

DISCUSSION

Salvaged egg byproducts from defective eggs have been shown to improve livestock performance based on their nutritive values (Al-Harthi, et al., 2011; Schmidt, et al., 2007). But the use of eggshell membranes as feed supplements has been little explored. Considering the large numbers of immunomodulatory proteins and peptides that are present in shell membrane (Cordeiro and Hincke, 2011; Miksík, et al., 2007; Mine, 2007), we hypothesized that ESM may have beneficial effects on the physiology of chickens. Inclusion of ESM in the diet not only caused a moderate to significant weight gain but also elevated both serum IgM and IgG levels indicative of modulation of humoral immunity. There was no change in relative weights suggestive of any negative or inflammatory effect of ESM. The changes in some blood cell parameters such as WBC and lymphocyte counts that were increased with 0.4% ESM fed birds along with their antibody (IgM and IgG) response, may suggest a stimulation of their adaptive

immune response. Although the monocyte counts decreased at both treatment levels, the heterophil counts and H/L ratio showed only numerical decrease at 0.4% levels of ESM. Stress is a major factor that decreases monocyte counts. Our results showed that the blood corticosterone levels were reduced in chickens fed ESM diets suggesting a lower levels of stress in these birds although the mechanism for its decrease is not understood. Stress and inflammation can also cause a loss of BW and present other signs of sickness such as lethargy that was not observed in ESM fed birds. Low levels of stress can also imply better feeding behavior (Bunnett, 2005) that would contribute to increase in BW. The sickness was also not evident from clinical chemistry variables such as the AST, ALT, and GGT values which are linked to hepatic dysfunction and poultry myopathy (MacRae, et al., 2006). Similarly, there was no elevation in the levels of blood urea nitrogen (BUN), uric acid, and creatinine that would indicate kidney dysfunction or creatine kinase which is a sign of muscle dysfunction. The increases in blood lymphocyte, IgG, and IgM levels indicate a modulation of immunity as compared with control birds. IgM is a natural antibody produced by B1 lymphocytes that fights infection, prevents inflammation, reacts with a variety of foreign antigens including pathogen associated molecules, activates complement, foreruns, and stimulates IgG response (Boes, 2000; Ehrenstein and Notley, 2010; Grönwall and Silverman, 2014). Similarly, an increase in the levels of IgG in ESM fed chickens also suggests a modulation of adaptive immune response. These antibodies play vital roles for protection against a variety of microbial pathogens (Jeurissen, et al., 2000; Sharma, 1997). Whether the antibody response to ESM is transient or it establishes a lasting resistance to certain infection needs to be verified.

The shell membrane is a highly crosslinked matrix that contains many proteins and peptides such as defensins which can potentially behave as adjuvants (Brown and Hancock, 2006; Zhang, et

al., 2010). It can also bind and carry foreign antigens to provide vaccine-like effects. Lactotransferrin, same as ovotransferrin in ESM, was shown to help maturation of dendritic cells of intestine and improve gut immunity (Spadaro, et al., 2008; Spadaro, et al., 2014). Chickens fed genetically engineered rice, expressing lactoferrin and lysozyme, showed improvement of gastrointestinal function with antibiotic-like effects (Humphrey, et al., 2002). Lysozyme was also shown to have similar effects in pig (Oliver and Wells, 2013).

One of the major imperatives of meat-animal production is to improve immunity (disease resistance) without sacrificing growth while abstaining from the use of antibiotics. The ESM supplement appears to have beneficial effect in chickens while it reduces stress and modulates immunity without sacrificing the growth potential of the birds.

ACKNOWLEDGEMENTS

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Table 1. Protein, calorie and selective elemental content of regular and ESM supplemented

Variables	Control Feed	Control Feed +ESM 0.2%	Control Feed +ESM 0.4%
Protein (%)	22.1	22.2	22.2
Calories/kg	4252	4221	4212
Calcium (ppm)	11531	11524	11037
Magnesium (ppm)	1552	1602	1619
Sodium (ppm)	1511	1469	1339
Phosphate (ppm)	8074	7852	7668
Sulfur (ppm)	2284	2347	2357
Zinc (ppm)	134	115	116

Table 2. BW and the relative organ weights of chicken fed diets with and without ESM (n=16)

Parameters	**Control**	**+ESM 0.2%**	**+ESM0.4%**
Body weight (grams)	905.38±18.35[b]	967.50±12.97[a]	958.00±16.89[a]
Relative heart weight (%)	0.50±0.01[a]	0.54±0.02[a]	0.54±0.01[a]
Relative liver weight (%)	2.21±0.07[a]	2.46±0.20[a]	2.25±0.05[a]
Relative spleen weight (%)	0.08±0.01[a]	0.10±0.01[a]	0.11±0.01[a]
Relative bursa weight (%)	0.16±0.01[a]	0.17±0.01[a]	0.20±0.01[a]

Values with different superscripts in a row are significantly different ($P \leq 0.05$).

Fig 1. Effect of ESM supplement on weekly BW (n=16). *denotes statistically significant differences ($p < 0.05$) compared with the control fed chickens. The BW of chickens fed both levels of ESM supplemented diets showed statistically significant increases at 3-wk of age indicated by **.

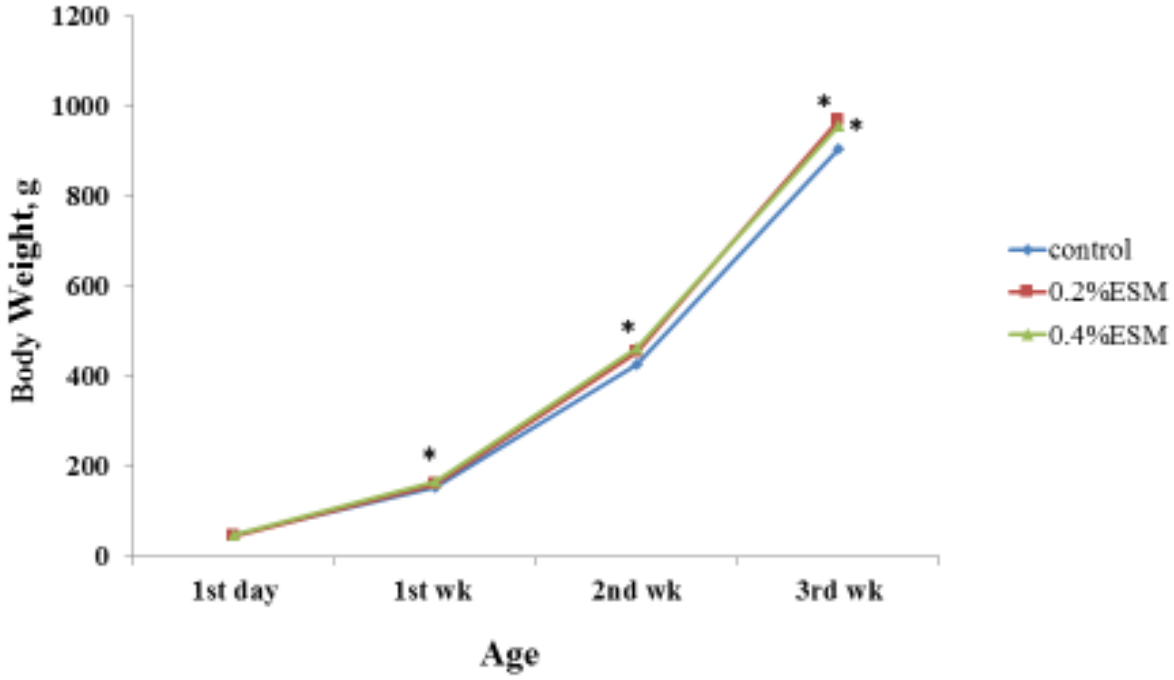


Table 3. Hematology profiles of chickens fed with and without ESM supplemented feed (n=12)

Variables	Control	+ESM 0.2%	+ESM 0.4%
White blood cell (WBC) ($x10^3/\mu L$)	28.61±2.87[b]	35.45±2.25[b,a]	37.5±2.30[a]
Heterophil (%)	14.83±1.30[a]	11.96±1.22[a,b]	11.2±1.19[a,b]
Lymphocyte (%)	74.81±2.02[b]	80.0±1.77[b,a]	81.6±1.55[a]
Monocyte (%)	8.02±0.84[a]	5.53±0.70[b]	4.95±0.37[b]
Eosinophil (%)	0.02±0.01[a]	0.02±0.01[a]	0.02±0.00[a]
Basophil (%)	2.34±0.12[a]	2.50±0.14 [a]	2.17±0.18[a]
Red blood cell (RBC) ($\times 10^6/\mu L$)	2.08±0.02[b]	2.09±0.03[b]	2.2±0.03[a]
Thrombocyte (k/μL)	13.36±0.65[a]	11.81±0.70[a,b]	9.93±0.39[b]
Heterophil/Lymphocyte (H/L)	0.21±0.03[a]	0.16±0.02[a]	0.14±0.02[a,b]
Hemoglobin (g/dL)	7.00±0.10[a]	6.92±0.09[a]	7.06±0.08[a]
Hematocrit (%)	52.78±0.67[b]	52.65±0.77[b]	55.67±0.68[a]
Mean corpuscular volume (MCV) (fL)	254.06±1.66[a]	252.33±0.82[a]	257.40±1.68[a]
Red cell distribution width (RDW) (%)	11.76±0.16[a]	11.79±0.11[a]	11.91±0.14[a]

Values with different superscripts in a row are significantly different ($P \leq 0.05$)

Table 4. Clinical chemistry variables of serum from 3 wk-old chickens fed with or without ESM (n=12)

Parameters	Control	+ESM 0.2%	+ESM 0.4%
Albumin (g/dL)	0.98±0.03[b]	1.03±0.08[b]	1.05±0.02[b,a]
Alkaline phosphate (U/L)	757.83±139.78[a]	958.75±186.52[a]	797.58±168.70[a]
Alanine transferase (U/L)	2.61±0.51[a]	2.08±0.57[a]	2.65±0.37[a]
Aspartate aminotransferase(U/L)	189.60±4.64[a]	194.02±6.16[a]	193.63±5.46[a]
Blood urea nitrogen (mg/μL)	1.12±0.09[a]	1.12±0.11[a]	1.19±0.43 [a]
Calcium (mg/dL)	8.80±0.16[a,b]	9.11±0.21[a]	8.47±0.13[b]
Cholesterol (mg/dL)	107.50±3.27[a]	110.50±5.89[a]	109.17±3.46[a]
Creatinine kinase (U/L)	177.08±22.97[a]	150.75±25.56[a]	267.25±61.65[a]
Creatinine (mg/dL)	0.31± 0.02[a]	0.27±0.02[a]	0.28±0.02[a]
Gamma-glutamyl transferase (U/L)	14.41±5.13[a]	14.25±0.70[a]	14.9±0.57[a]
Glucose (mg/dL)	230.41±5.13[a]	240.08±5.90[a]	238.17±0.06[a]
Phosphorous (mg/dL)	4.33±0.08[a]	4.46±0.23[a]	4.45±0.06[a]
Low density lipoprotein (mg/dL)	115.50±13.03[a]	119.75±10.34[a]	133.33±10.13[a]
Total protein (g/dL)	2.23±0.04[c]	2.85± 0.09[b]	3.18±0.04[a]

Triglycerides (mg/dL)	54.17±4.75[a]	62.58± 6.58[a]	62.75±6.91[a]
Uric Acid (mg/dL)	5.87±0.23[a]	6.23±0.29[a]	5.97±0.32[a]
Magnesium (mEq/L)	1.60±0.05[a]	1.60±0.05[a]	1.67±0.03[a]
Iron (µg/dL)	84.40±3.97[a]	86.55±7.12[a]	82.50±3.70[a]

Values with different superscripts in a row are significantly different ($P \leq 0.05$)

Fig 2. Comparison of serum IgM and IgG levels of chickens fed control, 0.2%, and 0.4% ESM supplemented diets. The IgM and IgG levels were measured as mg/ml ± SEM (n=12 each). Values with different superscripts are significantly different ($P \leq 0.05$).

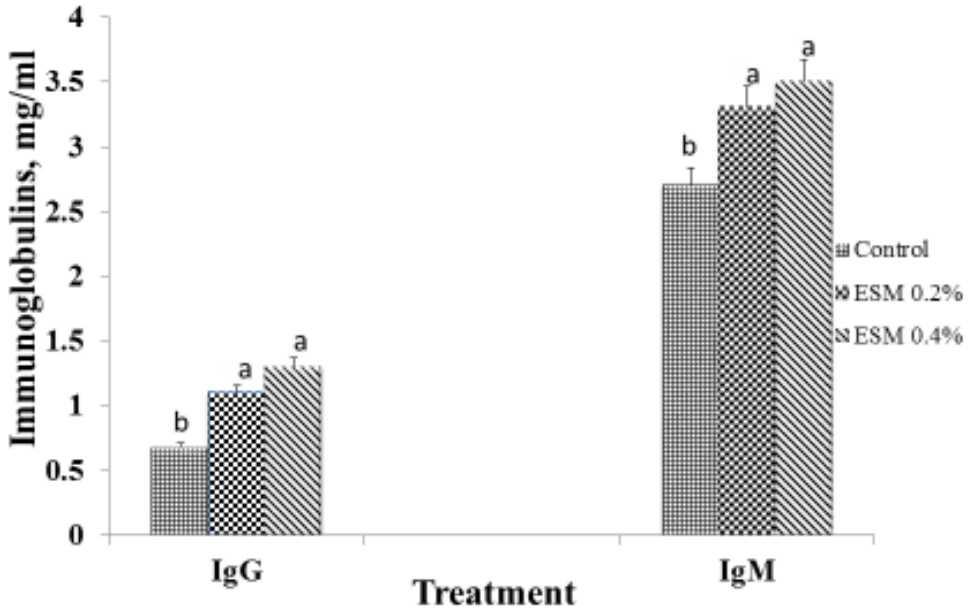
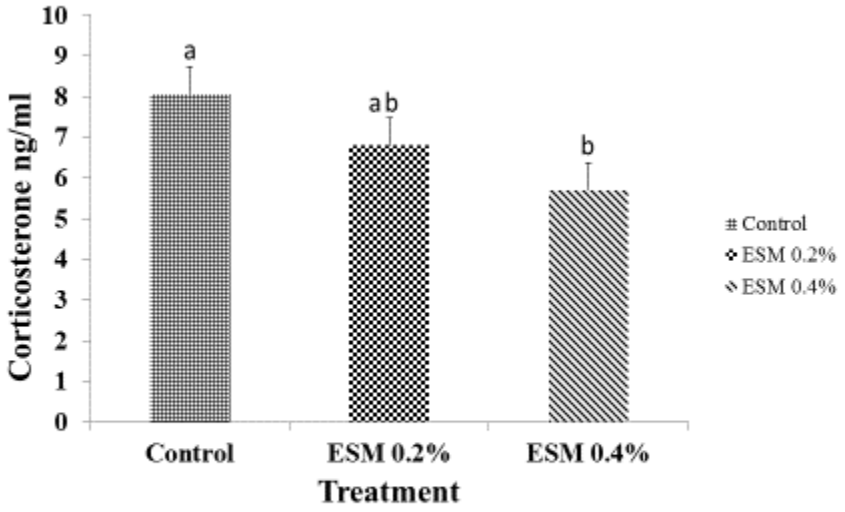


Fig 3. Serum corticosterone levels of chickens fed diets supplemented with or without ESM. The concentration of corticosterone was measured as ng/ml and shown as mean ± SEM. Values with different superscripts are significantly different ($P \leq 0.05$).


Corticosterone ng/ml
10
9
8
7
6
5
4
3
2
1
0
a
a b
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Control
ESM 0.2%
ESM 0.4%
Treatment
Control
ESM 0.2%
ESM 0.4%

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IV. Protein profiles of hatchery derived egg shell membrane

Abstract

Eggshell membranes protect growing embryo are interlaced around the albumen and form a meshwork, which can trap the invading bacteria and prevent it from further penetrating in the egg. The microstructures present on egg membranes are formed of these fibrous proteins, which are knotted together to form a net to obstruct the passage of microorganisms. We hypothesize that eggshell membranes from hatched eggs will be richer in fetal proteins and their characterization possibly might shed light into their biological relevance in providing physical and chemical defense to the growing embryo. We want to explore the eggshell membranes in the context of waste material left after the eggs are hatched. We extracted the proteins and peptides by two methods and analyzed them with mass spectrometry techniques. The proteins and peptides from hatched eggshell membranes (HESM) were extracted with methanol and also with a chaotropic agent. Both the extracts were subjected to in solution digestion, the protein and peptide profiles were determined by LC-MS/MS. The results from hatched egg membranes showed the presence of not only the presence of proteins (ovalbumin, ovocledin, lysozyme) which is found in unfertilized egg membranes but also many new proteins such as zona pleucida, filamin, lumican which can be major players in the growth, and development of the embryo.

Introduction

Egg shells from hatchery waste have been considered to be useful for biological and biomedical applications (Abeyrathne, et al., 2013; Kovacs-Nolan, et al., 2005) . The empty egg shells largely consist of the outer calcareous matrix and underlying membranes that are not only proteinaceous but laced with many proteins of embryonic origins as well as a variety of microbial and hatchery contaminants (Das, et al., 2002; Mine, et al., 2003). The embryonic proteins and peptides may

be useful to bring about specific physiological modulation; however, their potential has not been tested. Previously, we found that shell membranes prepared from unfertilized eggs when fed to chickens post hatch for 2 weeks affected their growth performance, and immunity (Makkar, et al., 2015b) these membranes were abound with numerous antimicrobial and cell associated peptides (Makkar, et al., 2015a). Following those studies we observed that hatchery egg shell membrane (HESM) fed to post hatch chickens not only improved growth performance but also protected the chickens against lipopolysaccharide (LPS) induced anorexia/cachexia (unpublished). Considering that the differences between 2 membrane preparations can be considerable such as the unfertilized egg shell membrane may have only certain types of proteins and peptides inherently acquired from reproductive tract, whereas the HESM may have been differentially enriched with proteins of embryonic, blood, feather, and microbial origins, we were interested to determine their protein profile. The results of these studies are described in the current report.

Chemicals and reagents. All reagents and devices such as C18 Nu tips (Glysci.com), Spectra/Por membranes (Spectrumlabs.com), BCA protein assay kit, Pierce C18 spin columns, MS grade trypsin (Fisher Scientific.com), peptide calibration standard II (*m/z* 500-16000, Bruker Daltonics, Bremen, Germany), and 2-iodoacetamide (IAA) (MP Biomedicals, OH) were purchased from their respective vendors. All other reagents and supplies including 1, 4-dithiothreitol (DTT), 2, 5-dihydroxybenzoic acid (DHB), were purchased from Sigma Aldrich (St. Louis, MO).

Material and methods

Empty eggshell with membranes were obtained from a local hatchery and allowed to dry at room temperature under the hood. The membranes were separated manually and ground to a powdered form using an IKA mill (find specification). The membrane powders were extracted with 4 M guanidine hydrochloride (GdHCl) containing 20 mM EDTA, and 50 mM Na-acetate, pH 5.8 and 70% methanol containing 0.1% acetic acid stirred in 20 volumes of respective solutions overnight at 4^{o} C. The extracts were centrifuged at 21,000 g for 15 min, and the supernatant dialyzed against excess 50 mM ammonium carbonate solution with 3 changes using 1,000 Da Spectra/Por membranes. The protein concentrations of both the extracts were measured using BCA protein assay kit. The extracts were concentrated by vacuum evaporation by means of a speed vac or lypholization and approximately 50 μg of both were reduced, alkylated and trypsin digested and desalted with C18 columns and subjected to LC/MS/MS. Each of these extractions was done in 2 trials and the studies were repeated twice.

LC-MS/MS analysis of methanol and guanidine extracted proteins. The protein concentrations of GdHCL was adjusted to 5mg/ml. Approximately 50 μg of GdHCl extracted proteins was subjected to reduction and alkylation, and digested with trypsin at the protein: trypsin ratios of 50:1 for 24 h at 37^{o}C . The tryptic digest was desalted with Pierce C18 spin columns and chromatographed on a capillary C_{18} column (150 x 0.1 mm, 3.5 μm particle size, 300 Å pore size, Zorbax SB) attached to an Agilent 1200 series HPLC, interfaced with a Bruker Amazon-SL quadrupole ion trap mass spectrometer, and captive spray source. Tryptic peptides were separated at a solvent flow rate of 1.6 μL/min with 0 to 40 % gradients of 0.1% FA (solvent A) and ACN in 0.1% FA solvent B (solvent B). Each time the samples were run three times as technical repeats and the results from 2 replicate studies were processed using Skyline software

(https://skyline.gs.washington.edu/labkey/project/home/begin.view) for peptide dominance and scores automatically without introducing any manual bias. The same strategy was applied to the methanol extract of HESM.

LC-MS/MS analyzed proteins. Peaks were picked in the LC-MS/MS (MS^n) chromatogram using Bruker default settings. Bruker Proteinscape bioinformatics suite coupled with MASCOT 2.1 was used to search NCBI Gallus protein database for identification. The parent ion mass tolerance and fragment ion mass tolerance were both set at 0.6 Da. A MASCOT decoy database search was performed with all the datasets. A score threshold of 45 or more was used as a high probability match for protein identifications. The proteins with only <1% false discovery rate (FDR) and at least 1unique peptide were reported. Functional annotation for these proteins was performed using Gene Ontology tool powered by PANTHER (http://geneontology.org)

Results

The guanidine HCl extract of HESM showed the presence of more than 100 proteins (Table 1) and 47 proteins (Table 2) in the methanol. Along with ovoalbumin, ovolcledin which are present in ESM, the membranes from fertilized eggs also contains tubulin, annexin, collagen, titin, desmin in abundance. Several chaperon proteins such as heat shock 10, 60 and 70 are also found. Proteins associated with antimicrobial properties such as lysozyme, gallinacin, keratin, cystatin are also reported. Some of the proteins are unique in methanol extract, which are not found in gunadine HCl extract such as gallinacin 9, thymosin beta 4, septins. The functional annotation by PANTHER shows that most of the proteins are involved in metabolic and cellular processes in terms of their biological relevance. In terms they are mainly involved of their molecular function Structural, binding and catalytic activity. We also found 50 proteins of bacterial origin,

which gives us a clue that these membranes acts as a trap for bacteria and prevent their egression inside the egg and acts as both physical and chemical barrier for the growing embryo.

Discussion

Cordieri et.al also reported the presence of fibronectin, vitellogenin, apolipoproteins in fertilized ESM (Cordeiro and Hincke, 2015) . The fertilized membranes are more rich in cytoskeleton proteins such as stratifin, fibronectin, annexin. Fibronectin plays very important role in embryogenesis and it is involved in the cell migration, attachment and differentiation during the process of growth of an embryo. (Nicosia, et al., 1993) (Risau and Lemmon, 1988).

Defensins are an important category of antimicrobial peptides that contains highly conserved cysteine residues. They are important arsenals of our innate immune system cells and are multifunctional in nature. The chemotactic properties of defensins helps in the recruitment of the immune cells to the site of infection, eliminate the pathogens and modulate the immune system. (Guaní-Guerra, et al., 2010; Hazlett and Wu, 2011; Jäger, et al., 2012). Gallin, a member of defensin family found in egg white and now also reported in membranes have potent antimicrobial activity against E.coli (Gong, et al., 2010).

Actin, mimecan, apolipoprotein, annexin, lumican are involved in developmental process. Mimecan is unique to fertilized membranes and is extracellular matrix glycoprotein, involved in the formation of tissues (Funderburgh, et al., 1997). Lumican another extracellular protein is clinically significant in term of providing transparency to the cornea by aligning with collagen fibrils (Kao, et al., 2006; Wang, et al., 2014). Lum knock out mice are reported to have abnormality in skin, heart tissues and cornea (Chakravarti, 2002)

Thioredoxin, lactate dehydrogenase, serpin are major players in catalytic activity. Thioredoxins are antioxidants and help to prevent oxidative stress (Lu and Holmgren, 2014; Nordberg and

Arnér, 2001). Serpins are protease inhibitors and also important constituents of blood clotting and inflammation processes (Devlin and Bottomley, 2005; Rau, et al., 2007). Proteins are actual functional molecules and indicators of pathological condition or pharmacological process and can be good candidates to target for drug designing. Understanding the constituent proteins and peptides may shed light into their functions associated with the development and protection of the embryo and improve egg quality.

The biological interpretation of the proteomic dataset in itself is a big challenge. Therefore analysis of each single protein by referring to the literature is a limiting factor, in finding the insights of the functional aspect of these proteins. (Fredrickson, et al., 2013) (Thompson, et al., 2012). But still with the annotation software's we can broadly classify these proteins into categories based on their roles in growth, development and several other metabolic functions. Functional analysis of HESM proteins showed that majority of them is involved in cellular process, regulation and also development process.

Table 1: Proteins identified in methanol extract by LC-MS/MS

ID	Description	scores	mw	#Peptides
ENSGALP00000000275	Ovalbumin [Source:UniProtKB/Swiss-Prot;Acc:P01012]	629.9	42.9	11
ENSGALP00000000325	Ovomucoid [Source:UniProtKB/Swiss-Prot;Acc:P01005]	338.1	22.6	7
ENSGALP00000000726	Ovocleidin-116 [Source:UniProtKB/Swiss-Prot;Acc:F1NSM7]	225	76.8	2
ENSGALP00000000876	orosomucoid 1 (ovoglycoprotein) precursor [Source:RefSeq peptide;Acc:NP_989872]	167	22.3	3
ENSGALP00000001532	peptidyl-prolyl cis-trans isomerase FKBP1A [Source:RefSeq peptide;Acc:NP_989661]	163.5	8.9	3
ENSGALP00000002523	Lysozyme C [Source:UniProtKB/Swiss-Prot;Acc:P00698]	129.2	16.2	2
ENSGALP00000005544	keratin 8, type II [Source:HGNC Symbol;Acc:HGNC:6446]	119.1	42.1	2
ENSGALP00000006093	Gallus gallus SH3 domain binding glutamic acid-rich protein like (SH3BGRL), mRNA. [Source:RefSeq mRNA;Acc:NM_001012574]	109.6	12.9	2
ENSGALP00000006097	Ubiquitin-fold modifier 1 [Source:UniProtKB/Swiss-Prot;Acc:Q5ZMK7]	102.1	9	1
ENSGALP00000008163	Gallinacin-9 [Source:UniProtKB/Swiss-Prot;Acc:Q6QLR1]	85.7	7.3	2
ENSGALP00000009976	Ovocalyxin-36 precursor [Source:RefSeq peptide;Acc:NP_001026032]	83.7	58.3	1
ENSGALP00000010763	Gallus gallus diazepam binding inhibitor (GABA receptor modulator, acyl-CoA binding protein) (DBI), mRNA. [Source: RefSeq mRNA;Acc:NM_204576]	69.8	9.6	2
ENSGALP00000012729	Gallinacin-10 [Source: UniProtKB/Swiss-Prot;Acc:Q6QLQ9]	62.6	7.1	2
ENSGALP00000013908	keratin, type I cytoskeletal 19 [Source: RefSeq peptide;Acc:NP_990340]	62.3	46	3
ENSGALP00000014919	Usher syndrome 1C [Source: HGNC Symbol;Acc:HGNC:12597]	51.9	100.1	1
ENSGALP00000016177	Uncharacterized protein [Source:	50.9	21	1

	UniProtKB/TrEMBL;Acc:E1C8H4]			
ENSGALP00000016632	thymosin, beta 4 [Source:RefSeq peptide;Acc:NP_001001315]	47.8	5	2
ENSGALP00000017755	collagen, type XVI, alpha 1 [Source: HGNC Symbol;Acc:HGNC:2193]	42	156.6	1
ENSGALP00000018601	signal peptidase complex subunit 1 homolog [Source: RefSeq peptide;Acc:NP_001165115]	37.9	27.2	1
ENSGALP00000019412	zinc finger BED domain-containing protein 4 [Source: RefSeq peptide;Acc:NP_001186470]	37.8	132.4	2
ENSGALP00000019758	alpha-D-globin (HBAD), mRNA. [Source: RefSeq mRNA;Acc:NM_001004375]	37.3	15.7	1
ENSGALP00000019988	utrophin [Source: HGNC Symbol;Acc:HGNC:12635]	36.5	398.6	2
ENSGALP00000020194	fatty acid-binding protein, heart [Source: RefSeq peptide;Acc:NP_001026060]	34.6	14.8	1
ENSGALP00000024777	nociceptin precursor [Source: RefSeq peptide;Acc:NP_001171980]	34.4	21.4	1
ENSGALP00000025120	polycystic kidney and hepatic disease 1 (autosomal recessive) [Source: HGNC Symbol;Acc:HGNC:9016]	32.7	440	1
ENSGALP00000025439	serine peptidase inhibitor, Kazal type 2 (acrosin-trypsin inhibitor) [Source: HGNC Symbol;Acc:HGNC:11245]	26.7	6	1
ENSGALP00000026777	probable arginyl-tRNA synthetase, mitochondrial [Source: RefSeq peptide;Acc:NP_001264948]	25.2	65.3	1
ENSGALP00000026846	transforming, acidic coiled-coil containing protein 1 [Source: HGNC Symbol;Acc:HGNC:11522]	24.5	86.4	2
ENSGALP00000026863	elaC ribonuclease Z 2 [Source :HGNC Symbol;Acc:HGNC:14198]	24.2	94.2	1
ENSGALP00000027483	Fibroblast growth factor 2 [Source: UniProtKB/Swiss-Prot;Acc:P48800]	22.8	16.2	1
ENSGALP00000027541	nuclear receptor coactivator 2 [Source: HGNC Symbol;Acc:HGNC:7669]	21.5	44.7	1
ENSGALP00000030659	WD repeat-containing protein 36 [Source:RefSeq peptide;Acc:NP_001038099]	21.2	98.2	1
ENSGALP00000031518	large tumor suppressor kinase 1 [Source:HGNC Symbol;Acc:HGNC:6514]	21.2	127.7	1
ENSGALP00000031725	septin 3 [Source:HGNC	20.5	40	1

	Symbol;Acc:HGNC:10750]			
ENSGALP00000035930	High mobility group protein B1 [Source:UniProtKB/Swiss-Prot;Acc:Q9YH06]	20	24.9	1
ENSGALP00000036403	tRNA (adenine-N(1)-)-methyltransferase non-catalytic subunit TRM6 [Source:RefSeq peptide;Acc:NP_001026212]	19.8	54.2	1
ENSGALP00000038283	fibrinogen silencer binding protein [Source:HGNC Symbol;Acc:HGNC:43653]	19	36.4	1
ENSGALP00000038735	SH3 domain binding glutamate-rich protein like 3 [Source:HGNC Symbol;Acc:HGNC:15568]	18.8	10.5	1
ENSGALP00000038904	UPF3 regulator of nonsense transcripts homolog B (yeast) [Source:HGNC Symbol;Acc:HGNC:20439]	18.4	56.9	1
ENSGALP00000038912	mutated in colorectal cancers [Source:HGNC Symbol;Acc:HGNC:6935]	18.3	112.6	1
ENSGALP00000039913	Polyubiquitin-B Ubiquitin [Source:UniProtKB/Swiss-Prot;Acc:P0CG62]	15.5	109.6	1
ENSGALP00000040476	neuregulin 2 [Source:HGNC Symbol;Acc:HGNC:7998]	15	65.5	1

Table 2: Proteins identified in guanidine HCL extract of eggshell membranes by LC-MS/MS

Protein id	Description	Score	Mw	# Peptides
ENSGALP00000010405	ovotransferrin precursor [Source:RefSeq peptide;Acc:NP_990635]	2328.2	77.8	45
ENSGALP00000036403	Ovalbumin [Source:UniProtKB/Swiss-Prot;Acc:P01012]	1751.1	42.9	28
ENSGALP00000039176	Actin, cytoplasmic type 5 [Source:UniProtKB/Swiss-Prot;Acc:P53478]	1470.9	41.8	29
ENSGALP00000005654	fibronectin precursor [Source: RefSeq peptide;Acc:NP_001185641]	1383	273.1	33
ENSGALP00000019372	protein-glutamine gamma-glutamyltransferase 4 [Source:RefSeq peptide;Acc:NP_001006368]	1307.4	78.9	28
ENSGALP00000016648		1267.7	23.9	17
ENSGALP00000015988	Actin, alpha cardiac muscle 1 [Source: UniProtKB/Swiss-Prot;Acc:P68034]	1267.2	42	24
ENSGALP00000006093	keratin, type I cytoskeletal 19 [Source: RefSeq peptide;Acc:NP_990340]	1110.2	46	28
ENSGALP00000005836	keratin, type I cytoskeletal 14-like [Source: RefSeq peptide;Acc:NP_001264913]	904.6	50	23
ENSGALP00000016632	keratin 8, type II [Source: HGNC Symbol;Acc:HGNC:6446]	870.4	42.1	17
ENSGALP00000006090	keratin, type I cytoskeletal 14 [Source:RefSeq peptide;Acc:NP_001001311]	845.1	51	20
ENSGALP00000015687	collagen alpha-2(I) chain precursor [Source:RefSeq peptide;Acc:NP_001073182]	805.3	128.8	19
ENSGALP00000038912	Gallus gallus alpha-D-globin (HBAD), mRNA. [Source:RefSeq mRNA;Acc:NM_001004375]	776.9	15.7	13
ENSGALP00000005971	Annexin A2 [Source:UniProtKB/Swiss-Prot;Acc:P17785]	740.3	38.6	17
ENSGALP00000016177	Lysozyme C [Source:UniProtKB/Swiss-Prot;Acc:P00698]	712.1	16.2	11
ENSGALP00000035593	Hemoglobin subunit beta [Source:UniProtKB/Swiss-	712.1	16.5	12

	Prot;Acc:P02112]			
ENSGALP00000016651	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:H9KZP6]	701.3	28	16
ENSGALP00000019031	serum albumin precursor [Source:RefSeq peptide;Acc:NP_990592]	673.2	64	17
ENSGALP00000038904	Gallus gallus hemoglobin, alpha 1 (HBAA), mRNA. [Source:RefSeq mRNA;Acc:NM_001004376]	668.8	15.4	13
ENSGALP00000018373	decorin precursor [Source:RefSeq peptide;Acc:NP_001025918]	654.7	39.6	15
ENSGALP00000014107	vimentin [Source:RefSeq peptide;Acc:NP_001041541]	641.7	53.2	14
ENSGALP00000035339	Gallus gallus ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (ATP5B), nuclear gene encoding mitochondrial protein, mRNA. [Source:RefSeq mRNA;Acc:NM_001031391]	538.6	52.9	10
ENSGALP00000035591	epsilon globin [Source:RefSeq peptide;Acc:NP_001026660]	522.6	16.4	11
ENSGALP00000006098	keratin, type I cytoskeletal 15 [Source: RefSeq peptide;Acc:NP_001001312]	486.5	47.9	15
ENSGALP00000043135	titin [Source:HGNC Symbol;Acc:HGNC:12403]	456.8	3397.7	27
ENSGALP00000041526	Gallus gallus histone cluster 1, H4-VI, germinal H4 (similar to human histone cluster 1, class H4 genes) (HIST1H46), mRNA. [Source: RefSeq mRNA;Acc:NM_001037845]	449	11.4	6
ENSGALP00000022528	zona pellucida sperm-binding protein 1 precursor [Source: RefSeq peptide;Acc:NP_990014]	443.2	99.7	10
ENSGALP00000003737	alpha-enolase [Source: RefSeq peptide;Acc:NP_990451]	397.4	47.3	8
ENSGALP00000003695	keratin 6A [Source: RefSeq peptide;Acc:NP_001001313]	397	57	9
ENSGALP00000038799	Tropomyosin alpha-1 chain [Source: UniProtKB/Swiss-Prot;Acc:P04268]	392.9	32.9	12
ENSGALP00000010210	myeloid protein 1 precursor [Source: RefSeq peptide;Acc:NP_990809]	388.4	35.2	7
ENSGALP00000042171	transgelin 2 [Source: HGNC Symbol;Acc:HGNC:11554]	382	29	8
ENSGALP00000023278	Glyceraldehyde-3-phosphate dehydrogenase [Source:	366.1	34.9	8

	UniProtKB/Swiss-Prot;Acc:P00356]			
ENSGALP00000032184	annexin A1 [Source: RefSeq peptide;Acc:NP_996789]	334	38.5	9
ENSGALP00000002368	Zona pellucida sperm-binding protein 3 Processed zona pellucida sperm-binding protein 3 [Source: UniProtKB/Swiss-Prot;Acc:P79762]	331.6	46.7	3
ENSGALP00000025606	Elongation factor 1-alpha 1 [Source:UniProtKB/Swiss-Prot;Acc:Q90835]	328.2	50.1	10
ENSGALP00000037266	Gallus gallus histone cluster 1, H1.01 (similar to human histone cluster 1, class H1 genes) (HIST1H101), mRNA. [Source: RefSeq mRNA;Acc:NM_001040642]	326.7	22.5	7
ENSGALP00000041690	peptidyl-prolyl cis-trans isomerase A [Source: RefSeq peptide;Acc:NP_001159798]	323.2	15.9	6
ENSGALP00000011510	Apolipoprotein A-I Proapolipoprotein A-I [Source: UniProtKB/Swiss-Prot;Acc:P08250]	320.7	30.7	8
ENSGALP00000042590	keratin 18, type I [Source:HGNC Symbol;Acc:HGNC:6430]	318.3	19	5
ENSGALP00000018370	Lumican [Source: UniProtKB/Swiss-Prot;Acc:P51890]	317	38.6	8
ENSGALP00000009563	Annexin [Source: UniProtKB/TrEMBL;Acc:E1C8K3]	313.8	36.7	8
ENSGALP00000017755	Ovocleidin-116 [Source: UniProtKB/Swiss-Prot;Acc:F1NSM7]	312.2	76.8	6
ENSGALP00000019120	heat shock 70 kDa protein [Source: RefSeq peptide;Acc:NP_001006686]	300.9	69.9	11
ENSGALP00000013964	tubulin beta-3 chain [Source: RefSeq peptide;Acc:NP_001074329]	272.9	49.8	7
ENSGALP00000002197	gelsolin precursor [Source: RefSeq peptide;Acc:NP_990265]	272.4	85.8	10
ENSGALP00000005544	Ovomucoid [Source: UniProtKB/Swiss-Prot;Acc:P01005]	269.7	22.6	5
ENSGALP00000018265	Serpin H1 [Source: UniProtKB/Swiss-Prot;Acc:P13731]	264.9	45.7	1
ENSGALP00000043256	Fructose-bisphosphate aldolase C [Source: UniProtKB/Swiss-Prot;Acc:P53449]	257.2	39.3	7
ENSGALP00000028845	keratin 4, type II [Source:HGNC Symbol;Acc:HGNC:6441]	256.4	58.7	8
ENSGALP00000010510	heat shock cognate 71 kDa protein [Source: RefSeq	255.4	70.8	2

	peptide;Acc:NP_990334]			
ENSGALP00000023396	Triosephosphate isomerase [Source: UniProtKB/Swiss-Prot;Acc:P00940]	231.3	26.6	4
ENSGALP00000023085	Gallus gallus actinin, alpha 4 (ACTN4), mRNA. [Source :RefSeq mRNA;Acc:NM_205126]	217.2	71.6	5
ENSGALP00000018742	creatine kinase B-type [Source: RefSeq peptide;Acc:NP_990641]	216.4	40.2	8
ENSGALP00000005607	proline/arginine-rich end leucine-rich repeat protein [Source: HGNC Symbol;Acc:HGNC:9357]	210.9	42.9	6
ENSGALP00000018424	desmin [Source: HGNC Symbol;Acc:HGNC:2770]	204.5	48.8	6
ENSGALP00000034108	Pyruvate kinase PKM [Source: UniProtKB/Swiss-Prot;Acc:P00548]	200.1	57.8	6
ENSGALP00000021743	Histone H2B [Source: UniProtKB/TrEMBL;Acc:F1NF30]	193	13.5	1
ENSGALP00000014097	destrin [Source: RefSeq peptide;Acc:NP_990859]	189.9	18.4	7
ENSGALP00000023926	Uncharacterized protein [Source: UniProtKB/TrEMBL;Acc:E1C6R9]	182.5	19.9	4
ENSGALP00000020275	Beta-galactoside-binding lectin [Source: UniProtKB/Swiss-Prot;Acc:P07583]	176.9		
ENSGALP00000017578	anterior gradient 2 [Source: HGNC Symbol;Acc:HGNC:328]	173.9	19.8	4
ENSGALP00000039133	14-3-3 protein epsilon [Source: UniProtKB/Swiss-Prot;Acc:Q5ZMT0]	173.4	26.6	3
ENSGALP00000025593	collagen alpha-1(XII) chain precursor [Source: RefSeq peptide;Acc:NP_990352]	171.6	339.6	1
ENSGALP00000010853		161.2	452.4	3
ENSGALP00000042357	H2A histone family, member X [Source: HGNC Symbol;Acc:HGNC:4739]	159.5	15	3
ENSGALP00000042528	cold-inducible RNA-binding protein [Source:RefSeq peptide;Acc:NP_001026518]	159.5	18..6	2
ENSGALP00000008163	orsomucoid 1 (ovoglycoprotein) precursor [Source:RefSeq peptide;Acc:NP_989872]	158.4	22.3	3
ENSGALP00000018498	heat shock protein 90kDa alpha (cytosolic), class A member 1 [Source: HGNC Symbol;Acc:HGNC:5253]	157.7	83.2	3
ENSGALP00000041	tropomyosin beta chain [Source:	157.6	28.7	7

937	RefSeq peptide;Acc:NP_990777]			
ENSGALP00000001474	78 kDa glucose-regulated protein [Source: UniProtKB/Swiss-Prot;Acc:Q90593]	155.8	72	5
ENSGALP00000025280	Thioredoxin [Source: UniProtKB/Swiss-Prot;Acc:P08629]	155.4	11.7	4
ENSGALP00000006240	collagen alpha-3(VI) chain precursor [Source:RefSeq peptide;Acc:NP_990865]	155.2	339.4	5
ENSGALP00000019399	Transgelin [Source: UniProtKB/Swiss-Prot;Acc:P19966]	147.2	22.3	5
ENSGALP00000033366	Ribonuclease homolog [Source:UniProtKB/Swiss-Prot;Acc:P30374]	143.2	15.9	1
ENSGALP00000020967	ovalbumin-related protein Y [Source: RefSeq peptide;Acc:NP_001026172]	141.6	43.8	5
ENSGALP00000028277	Histone H2A.Z [Source: UniProtKB/Swiss-Prot;Acc:Q5ZMD6]	139.7	13.6	3
ENSGALP00000040188	S100 calcium binding protein A12 [Source: HGNC Symbol;Acc:HGNC:10489]	138.4	18.5	4
ENSGALP00000036122	14-3-3 protein theta [Source: UniProtKB/Swiss-Prot;Acc:Q5ZMD1]	138.3	27.8	1
ENSGALP00000002888	vitellogenin-2 precursor [Source: RefSeq peptide;Acc:NP_001026447]	136.8	205	6
ENSGALP00000019033	Alpha-fetoprotein [Source: UniProtKB/TrEMBL;Acc:E1BV96]	133.9	71.1	6
ENSGALP00000040672	stratifin [Source: HGNC Symbol;Acc:HGNC:10773]	133.4	27.7	4
ENSGALP00000010852	Uncharacterized protein [Source: UniProtKB/TrEMBL;Acc:F1NZY2]	133.1	233.4	2
ENSGALP00000026846	Gallinacin-10 [Source: UniProtKB/Swiss-Prot;Acc:Q6QLQ9]	127.7	7.1	2
ENSGALP00000043172	Cystatin [Source: UniProtKB/Swiss-Prot;Acc:P01038]	123.6	16.3	2
ENSGALP00000041913	Myosin regulatory light chain 2, smooth muscle major isoform [Source:UniProtKB/Swiss-Prot;Acc:P02612]	117.8	19.8	4
ENSGALP00000000876	fatty acid-binding protein, heart [Source:RefSeq peptide;Acc:NP_001026060]	116.4	14.8	4
ENSGALP00000038626	L-lactate dehydrogenase A chain [Source:RefSeq peptide;Acc:NP_990615]	116.2	36.5	4
ENSGALP00000011	60S acidic ribosomal protein P0	114.8	34.6	2

717	[Source:RefSeq peptide;Acc:NP_990318]			
ENSGALP00000011961	phosphatidylethanolamine-binding protein 1 [Source: RefSeq peptide;Acc:NP_001185571]	113.9	20.9	4
ENSGALP00000000062	Uncharacterized protein [Source: UniProtKB/TrEMBL;Acc:H9KYP2]	113.8	67.8	5
ENSGALP00000043361	Uncharacterized protein [Source: UniProtKB/TrEMBL;Acc:R4GMA5]	113.2	26.7	2
ENSGALP00000043060	Mimecan [Source: UniProtKB/Swiss-Prot;Acc:Q9W6H0]	110.1	33.2	3
ENSGALP00000013267	rab GDP dissociation inhibitor beta [Source: RefSeq peptide;Acc:NP_990335]	106.7	50.7	3
ENSGALP00000034078	Nucleoside diphosphate kinase [Source: UniProtKB/Swiss-Prot;Acc:O57535]	104	17.3	4
ENSGALP00000019365	annexin A5 [Source: RefSeq peptide;Acc:NP_001026709]	103.3	36.2	5
ENSGALP00000026126	60S ribosomal protein L8 [Source: RefSeq peptide;Acc:NP_001264657]	102.2	28	4
ENSGALP00000005520	aldehyde dehydrogenase 9 family, member A1 [Source: HGNC Symbol;Acc:HGNC:412]	101.7	57.1	3
ENSGALP00000000316	major vault protein [Source: RefSeq peptide;Acc:NP_001006336]	96.5	93.7	6
ENSGALP00000036963	ribosomal protein L15 [Source: HGNC Symbol;Acc:HGNC:10306]	95.8	24.1	2
ENSGALP00000020094	Histone H5 [Source: UniProtKB/Swiss-Prot;Acc:P02259]	95.4	20.7	3
ENSGALP00000014317	isocitrate dehydrogenase 1 (NADP+), soluble [Source: HGNC Symbol;Acc:HGNC:5382]	93.3	46.9	2
ENSGALP00000008498	transketolase [Source:HGNC Symbol;Acc:HGNC:11834]	91.9	68.4	3
ENSGALP00000039326	60S ribosomal protein L19 [Source: RefSeq peptide;Acc:NP_001026100]	85.7	23.2	2
ENSGALP00000014912	Protein S100-A11 [Source: UniProtKB/Swiss-Prot;Acc:P24479]	85	11.4	1
ENSGALP00000013574	Protein disulfide-isomerase A3 [Source: UniProtKB/Swiss-Prot;Acc:Q8JG64]	84.9	56.1	4
ENSGALP00000016361	ribosomal protein S3A [Source:RefSeq peptide;Acc:NP_001075886]	84.7	29.8	4
ENSGALP00000040606	CD99 antigen precursor [Source: RefSeq peptide;Acc:NP_001185580]	79.3	18.2	2

ENSGALP00000006938	annexin A6 [Source: RefSeq peptide;Acc:NP_990061]	77.5	75.2	2
ENSGALP00000040966	Uncharacterized protein [Source: UniProtKB/TrEMBL;Acc:R4GG07]	74.1	13.2	1
ENSGALP00000007490	moesin [Source: HGNC Symbol;Acc:HGNC:7373]	73.8	68.5	3
ENSGALP00000038677	Gallus gallus phosphoglycerate mutase 1 (brain) (PGAM1), mRNA. [Source: RefSeq mRNA;Acc:NM_001031556]	73.4	23.7	3
ENSGALP00000029440	Vitelline membrane outer layer protein 1 [Source: UniProtKB/Swiss-Prot;Acc:P41366]	69.1	20.2	1
ENSGALP00000024468	40S ribosomal protein S15 [Source: UniProtKB/Swiss-Prot;Acc:P62846]	68	16.9	2
ENSGALP00000032611	vitellogenin-1 precursor [Source: RefSeq peptide;Acc:NP_001004408]	67.6	210.6	3
ENSGALP00000003455	peptidyl-prolyl cis-trans isomerase B precursor [Source: RefSeq peptide;Acc:NP_990792]	66.6	16.2	2
ENSGALP00000029968	60S ribosomal protein L3 [Source: RefSeq peptide;Acc:NP_001006241]	65.8	26.1	3
ENSGALP00000025929	hydroxyacyl-coenzyme A dehydrogenase, mitochondrial [Source: RefSeq peptide;Acc:NP_001264826]	64.9	34.4	2
ENSGALP00000001013	60S ribosomal protein L22 [Source:RefSeq peptide;Acc:NP_989472]	64.7	14.6	2
ENSGALP00000007680	60S ribosomal protein L6 [Source: RefSeq peptide;Acc:NP_989483]	64	33.9	2
ENSGALP00000021618	L-lactate dehydrogenase B chain [Source: UniProtKB/Swiss-Prot;Acc:P00337]	61.6	36.3	3
ENSGALP00000038462	protein TENP [Source: RefSeq peptide;Acc:NP_990357]	61.3	47.4	2
ENSGALP00000011689	protein disulfide-isomerase precursor [Source: RefSeq peptide;Acc:NP_001185639]	61.1	55.8	4
ENSGALP00000003431	nucleophosmin [Source: RefSeq peptide;Acc:NP_990598]	60.5	30.3	2
ENSGALP00000041639	ribosomal protein S26 [Source: HGNC Symbol;Acc:HGNC:10414]	59	18	2
ENSGALP00000008131	vinculin [Source: HGNC Symbol;Acc:HGNC:12665]	58.9	114.3	1
ENSGALP00000024078	WD repeat-containing protein 1 [Source: RefSeq peptide;Acc:NP_001004402]	58.5	66.5	1

ENSGALP00000023089	ribosomal protein, large, P2 [Source: HGNC Symbol;Acc:HGNC:10377]	57.8	14.2	1
ENSGALP00000007476	protein SET [Source: RefSeq peptide;Acc:NP_001025862]	57.7	32.1	1
ENSGALP00000012462	ribosomal protein S25 [Source: HGNC Symbol;Acc:HGNC:10413]	57.5	13.7	1
ENSGALP00000000509	heterogeneous nuclear ribonucleoprotein M [Source: RefSeq peptide;Acc:NP_001026103]	53	76	4
ENSGALP00000014298	60S ribosomal protein L12 [Source: RefSeq peptide;Acc:NP_001264608]	52.2	17.7	1
ENSGALP00000027030	60S ribosomal protein L31 [Source: RefSeq peptide;Acc:NP_001264684]	52.2	14.7	2
ENSGALP00000027665	acetyl-CoA acetyltransferase, mitochondrial [Source: RefSeq peptide;Acc:NP_001264708]	51.9	44.1	2
ENSGALP00000039447	heat shock protein beta-1 [Source: RefSeq peptide;Acc:NP_990621]	51.9	21.8	1
ENSGALP00000025745	Uncharacterized protein [Source: UniProtKB/TrEMBL;Acc:F1P304]	50.8	24.5	3
ENSGALP00000039530	heterogeneous nuclear ribonucleoprotein H [Source: RefSeq peptide;Acc:NP_989827]	50.4	56.5	2
ENSGALP00000038435	protein-glutamine gamma-glutamyltransferase 2 [Source: RefSeq peptide;Acc:NP_990779]	49.5	77.7	1
ENSGALP00000002333	Uncharacterized protein [Source: UniProtKB/TrEMBL;Acc:F1NPG6]	47.6	93.8	2
ENSGALP00000006284	heterogeneous nuclear ribonucleoprotein H3 [Source: RefSeq peptide;Acc:NP_001012610]	46.3	36.6	2
ENSGALP00000021314	caldesmon [Source: RefSeq peptide;Acc:NP_989489]	44.7	86.4	2
ENSGALP00000026392	ribosomal protein S7 [Source:HGNC Symbol;Acc:HGNC:10440]	43	22.3	2
ENSGALP00000026123	ATP-dependent RNA helicase DDX3X [Source: RefSeq peptide;Acc:NP_001025971]	42.8	72.6	1
ENSGALP00000027012	lysozyme g precursor [Source: RefSeq peptide;Acc:NP_001001470]	42.2	23.3	3
ENSGALP00000019979	Uncharacterized protein [Source: UniProtKB/TrEMBL;Acc:F1NI80]	41.2	41.1	1
ENSGALP00000010800	actin-related protein 2/3 complex subunit 4 [Source :RefSeq peptide;Acc:NP_001244213]	41.1	19.7	8
ENSGALP00000025	Carbonic anhydrase 2 [Source:	41.1	29	1

525	UniProtKB/Swiss-Prot;Acc:P07630]			
ENSGALP00000039575	guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 [Source: RefSeq peptide;Acc:NP_001012853]	40.8	37.3	2
ENSGALP00000028211	glycerol-3-phosphate dehydrogenase 1 (soluble) [Source: HGNC Symbol;Acc:HGNC:4455]	40.2	38.6	1
ENSGALP00000014746	Gallus gallus heat shock 10kDa protein 1 (chaperonin 10) (HSPE1), nuclear gene encoding mitochondrial protein, mRNA. [Source: RefSeq mRNA;Acc:NM_205067]	39.6	12.1	2
ENSGALP00000035996	superoxide dismutase [Source:RefSeq peptide;Acc:NP_990395]	39.3	15.7	1
ENSGALP00000012481	60S ribosomal protein L4 [Source: RefSeq peptide;Acc:NP_001007480]	38.6	46.7	2
ENSGALP00000006308	ribosomal protein L23a [Source:HGNC Symbol;Acc:HGNC:10317]	38.3	17.6	2
ENSGALP00000010414	heterogeneous nuclear ribonucleoprotein G [Source:RefSeq peptide;Acc:NP_001073196]	38	41.5	2
ENSGALP00000001914	chloride intracellular channel 4 [Source:HGNC Symbol;Acc:HGNC:13518]	31.5	27.7	1
ENSGALP00000041423	glutathione peroxidase 1 [Source:RefSeq peptide;Acc:NP_001264782]	31.4	17.9	1
ENSGALP00000008802	40S ribosomal protein S2 [Source:RefSeq peptide;Acc:NP_001264093]	31	30.7	1
ENSGALP00000013122	60 kDa heat shock protein, mitochondrial [Source:UniProtKB/Swiss-Prot;Acc:Q5ZL72]	30.8	60.9	1
ENSGALP00000009511	60S ribosomal protein L5 [Source:UniProtKB/Swiss-Prot;Acc:P22451]	30.7	33.9	2
ENSGALP00000006222	calreticulin 3 [Source:HGNC Symbol;Acc:HGNC:20407]	29.8	48	1
ENSGALP00000041109	capping protein (actin filament), gelsolin-like [Source:HGNC Symbol;Acc:HGNC:1474]	29.5	72.4	1
ENSGALP00000033411	ribosomal protein S21 [Source:HGNC Symbol;Acc:HGNC:10409]	28.8	9.1	1
ENSGALP00000029993	rho GDP-dissociation inhibitor 2 [Source:RefSeq	28.1	23.2	1

	peptide;Acc:NP_001264293]			
ENSGALP00000035366	isocitrate dehydrogenase [Source:RefSeq peptide;Acc:NP_001026770]	26.7	50.4	1
ENSGALP00000035959	carbonyl reductase [Source:RefSeq peptide;Acc:NP_001025966]	24.4	30.3	1
ENSGALP00000041772	Protein syndesmos [Source:UniProtKB/Swiss-Prot;Acc:Q9IAY5]	23.5	33.8	1
ENSGALP00000003373	40S ribosomal protein S17 [Source:UniProtKB/Swiss-Prot;Acc:P08636]	20	9.5	1
ENSGALP00000037222	Heterogeneous nuclear ribonucleoprotein D-like [Source: UniProtKB/Swiss-Prot;Acc:Q5ZI72]	17.3	33.4	1

Table 3: Proteins identified in bacterial database

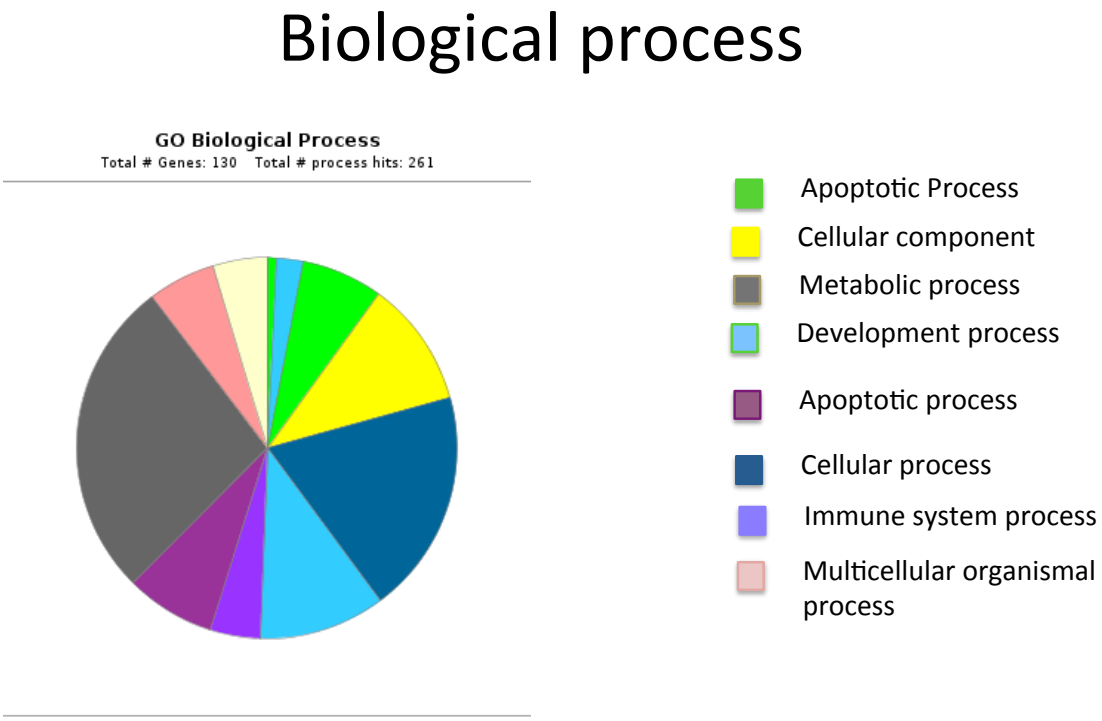
	Accession	Protein	MW [kDa]	Scores	#Peptides
1	gi|294828133	histidine kinase/response regulator hybrid protein [Leptospira interrogans serovar Lai str. 56601]	116.9	77.8	4
2	gi|517357534	serine/threonine protein kinase [Streptomyces sp. HmicA12]	67.9	65.8	2
3	gi|655245428	protein kinase [Nocardioides sp. J54]	72.9	63.7	2
4	gi|124514345	Precorrin-4 C11-methyltransferase [Leptospirillum rubarum]	29.5	56.8	2
5	gi|206742631	soluble lytic murein transglycosylase [Thermodesulfovibrio yellowstonii DSM 11347]	73.8	55.6	2
6	gi|292642245	SWIM zinc finger domain protein [Enterococcus faecium PC4.1]	42.4	54.1	2
7	gi|695172258	aminotransferase [Sphingomonas taxi]	33.6	49.5	1
8	gi|345633807	LuxR family transcriptional regulator [Streptomyces zinciresistens K42]	24.0	48.9	1
9	gi|19705092	hypothetical protein FN1787 [Fusobacterium nucleatum subsp. nucleatum ATCC 25586]	73.8	44.1	1
10	gi|124515012	putative hydrolase, haloacid dehalogenase-like family [Leptospirillum rubarum]	24.5	43.7	1
11	gi|422886479	cold shock-like protein [Alcaligenes sp. HPC1271]	7.5	42.3	1
12	gi|618782811	ribonucleotide-diphosphate reductase subunit alpha [Pseudomonas aeruginosa M10]	107.0	38.8	1
13	gi|311693188	ATPase components of ABC transporters with duplicated ATPase domains [Marinobacter adhaerens HP15]	24.5	71.7	2
14	gi|297550774	serine/threonine protein kinase	73.8	65	2

		with TPR repeats [Ktedonobacter racemifer DSM 44963]			
15	gi|405587154	transcription termination factor Rho [Bergeyella zoohelcum CCUG 30536]	65.9	63.3	2
16	gi|618771208	F0F1 ATP synthase subunit alpha [Pseudomonas aeruginosa M10]	55.4	61	1
17	gi|588290902	prolyl-tRNA synthetase [Thalassolituus oleivorans R6-15]	63.9	55.7	2
18	gi|114739665	isocitrate dehydrogenase, NADP-dependent [Hyphomonas neptunium ATCC 15444]	45.6	52.6	1
19	gi|755437351	der GTPase activator family protein [Yersinia kristensenii]	21.2	49.5	1
20	gi|394456251	hypothetical protein O71_08395 [Pontibacter sp. BAB1700]	45.3	45.8	1
21	gi|452006359	mutant NtrC-like activator [Pseudomonas stutzeri NF13]	51.1	45	1
22	gi|618789844	oxidoreductase [Pseudomonas aeruginosa M10]	26.2	44.4	1
23	gi|328474119	GTP-binding protein LepA [Vibrio parahaemolyticus 10329]	65.9	43.1	1
24	gi|651910070	hypothetical protein [Butyrivibrio sp. AC2005]	52.1	40.4	1
25	gi|311694265	glutathione synthase/ribosomal protein S6 modification enzyme [Marinobacter adhaerens HP15]	58.3	43.8	1
26	gi|546198376	MULTISPECIES: ribosomal protein L25, Ctc-form [Bacteria]	20.7	43.8	1
27	gi|726045696	acetyltransferase [Candidatus Scalindua brodae]	17.6	43.4	1
28	gi|452009578	hypothetical protein B381_02321 [Pseudomonas stutzeri NF13]	32.3	32.1	1
29	gi|258592528	putative Histidine kinase [Candidatus Methylomirabilis oxyfera]	86.4	85.2	3
30	gi|516628378	MULTISPECIES: F0F1 ATP synthase subunit alpha [Bacteria][Archaea]	55.6	84.4	4
31	gi|292642035	hypothetical protein CUO_2557 [Enterococcus faecium PC4.1]	97.6	80.2	3
32	gi|695170760	2-keto-4-pentenoate hydratase [Sphingomonas taxi]	35.8	78.4	3
33	gi|380733894	serine/threonine protein kinase [Corallococcus coralloides DSM	126.9	77	3

		2259]			
34	gi|292637908	NADH:ubiquinone oxidoreductase, Na(+)-translocating, A subunit [Bacteroides xylanisolvens SD CC 2a]	55.7	70.5	2
35	gi|114737610	putative helicase [Hyphomonas neptunium ATCC 15444]	42.9	69.2	3
36	gi|288328957	tetratricopeptide repeat protein [Prevotella sp. oral taxon 317 str. F0108]	67.0	62.7	2
37	gi|618792792	selenocysteine synthase [Pseudomonas aeruginosa M10]	49.7	62	2
38	gi|300402166	DNA mismatch repair domain protein [Escherichia coli MS 84-1]	67.9	62	2
39	gi|394454349	tex-like protein [Pontibacter sp. BAB1700]	83.4	54.4	1
40	gi|695170101	phosphoadenosine phosphosulfate reductase [Sphingomonas taxi]	27.7	51.1	1
41	gi|618777703	ATP-binding protein [Pseudomonas aeruginosa M10]	38.4	49.7	1
42	gi|291518125	Uncharacterized protein conserved in bacteria [Butyrivibrio fibrisolvens 16/4]	9.1	46.6	1
43	gi|726045751	hypothetical protein SCABRO_01635 [Candidatus Scalindua brodae]	23.3	46.2	1
44	gi|292643035	ribonuclease HIII [Enterococcus faecium PC4.1]	33.9	45.2	1
45	gi|618771210	F0F1 ATP synthase subunit beta [Pseudomonas aeruginosa M10]	49.5	45	1
46	gi|691636805	hypothetical protein IA69_10970 [Massilia sp. JS1662]	99.2	44.7	1
47	gi|726045041	hypothetical protein SCABRO_02256 [Candidatus Scalindua brodae]	15.4	44.6	1
48	gi|635597237	uncharacterized protein conserved in bacteria [Comamonadaceae bacterium B1]	45.0	43.7	1
49	gi|114740197	putative fimbrial assembly protein [Hyphomonas neptunium ATCC 15444]	26.7	42.5	1
50	gi|667096584	lytic transglycosylase	20.7	41	1

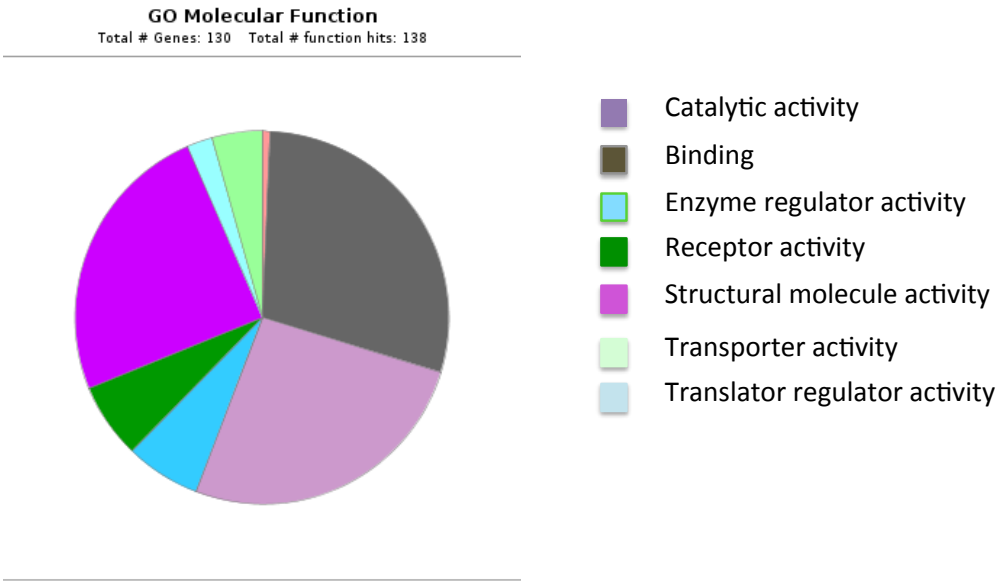
		[Xanthomonas vasicola pv. vasculorum NCPPB 895]			

Figure 1: Functional annotation of proteins by **P**rotein **A**nalysis **th**rough **E**volutionary **R**elationships (PANTHER) a) biological process b) Molecular Functions



b)

Molecular function



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V. Nutritional supplement of hatchery eggshell membrane improves poultry performance and provides resistance against lipopolysaccharide induced effects

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Abstract

Eggshells are significant part of hatchery waste which consist of calcium carbonate crust, membranes, and proteins and peptides of embryonic origins along with other entrapped contaminants such as microbes. We hypothesized that using this product as a nutritional additive in poultry diet may confer better immunity to the chickens in the paradigm of mammalian milk that enhances immunity. Therefore, we investigated the effect of hatchery eggshell membranes (HESM) as a short term feed supplement on growth performance and immunity of chickens under bacterial lipopolysaccharide (LPS) challenged condition. Three studies were conducted to find the effect of HESM supplement on post hatch chickens. In the first study, the chickens were fed either a control diet or diets containing 0.5% whey protein or HESM as supplement and evaluated at 5 weeks of age using growth, hematology, clinical chemistry, plasma immunoglobulins, and corticosterone as variables. The second and third studies were done to compare the effects of LPS on control and HESM fed birds at 5 weeks of age where the HESM was also treated with ethanol to inactivate bacterial factors, and the effects of LPS evaluated at 4 and 24 h of treatment. HESM supplement caused a numerical but nonsignificant weight gain in 2 experiments and consistently decreased the blood corticosterone levels. LPS caused a significant loss in body weight at 24 h following its administration but the HESM supplemented birds showed significantly less body weight loss compared with the control fed birds. The WBC, heterophil/lymphocyte ratio, and the levels of IgG were low in chickens fed HESM supplement diet compared with the control fed group. LPS challenge increased the expression of pro-inflammatory cytokine gene IL-6 but the HESM fed birds showed its effect curtailed also, favored the up-regulation of some anti-inflammatory genes compared with control fed chickens.

Post hatch supplementation of HESM appears to modulate immunity, and increase their resistance to endotoxin.

Key words: Hatchery eggshell membrane, chicken, lipopolysaccharide, gene expression, corticosterone

Introduction

Eggshells which constitute a significant part of hatchery waste consist of calcareous crust, shell membranes, proteins and peptides of embryonic origins, and entrapped contaminants including microbes [1, 2]. Proteomic analysis of the eggshell membranes (ESM) have shown the presence of over 200 proteins and peptides belonging to structural, antimicrobial, and cell-regulatory genre [3-5] with the hatchery eggshell membrane (HESM) enriched with many blood derived proteins (Makkar et al., in preparation). We hypothesized that HESM as a feed supplement may be beneficial to post hatch poultry in the paradigm of mammalian milk, which contain many similar proteins and peptides such as lactoferrin, lysozyme, albumin, and other factors that help gastrointestinal development and help development of immunity in neonates [6, 7]. However, the functional stability of these proteins to harsh processes such as, drying, decontamination, and passage through the gastrointestinal tract is not known. Reports in the literature have shown the biological effects of different enzymes, antibodies, recombinant cytokines, and other bioactive protein additives in animal feed [8-13]. Previously, we showed that nutritional supplement of eggshell membrane (ESM) from fresh unfertilized eggs given to the chickens during first 2 weeks post hatch, improved growth, increased serum immunoglobulins, and reduced several stress variables such as plasma corticosterone, heterophils, and heterophil/lymphocyte ratios[14]. The growth supportive effects of fetal proteins have also been demonstrated in other experimental models [15, 16], The muco-adhesive membrane particles may also act as carriers of microbial antigens along with other adjuvant-like proteins and peptides [17, 18] that help to develop resistance or tolerance to pathogens. Hence, the objective of this research was to explore the effect of HESM supplements on the performance of post hatch chickens stressed with endotoxin during the grow-out period.

Materials and Method

Preparation of HESM and its sterilization

Empty eggshells collected from a hatchery were dried at room temperature and the membranes separated from the shells and pulverized to powders and flakes with an IKA mill (Cole Parmer). The protein nitrogen content of the membrane powder before and after mixing with feed were estimated by Duma's nitrogen analyzer using duplicate samples [14]. Three feeding experiments were conducted: study 1 utilized intact HESM while the studies 2 and 3 utilized HESM flakes sterilized with ethanol to reduce bacterial and endotoxin contaminants. In studies with ethanol sterilization, the HESM flakes were treated with 3 volumes (w/v) of reagent grade ethanol, enough to wet the flakes, and then air dried in a chemical hood without decantation. The effect of this treatment was evaluated using bacterial colony count assays [19] and the production of nitrite by HTC macrophages due to endotoxin [20]. Briefly, duplicate samples of untreated and ethanol treated HESM powders were extracted with sterile saline at the concentrations of 100 mg/ mL at room temperature for 2 h and centrifuged at 21,000 g. Respective supernatants were serially diluted and 100 µl of each sample was plated on agar plates in triplicate, and incubated for 24 h at 37^{o}C to evaluate for bacterial growth. The same extracts were also evaluated for endotoxin activities using nitrite production by the HTC chicken macrophages following 24 h of stimulation and compared with Salmonella LPS (1µg/ml) used as a positive control [20].

Experimental Schedule

The animal study protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Arkansas. Newly hatched Cobb 500 male chicks were raised on floor pens at a density of 8 square feet /bird with 23:1 light: dark schedule and provided feed

formulated per National Research Council [21] specification and *ad libitum* water. The HESM was added at 0.5% level to broiler starter diet based on previous experiments. In Study 1 the effects of crude HESM and a comparable level of whey protein powder were tested on the growth performance and general physiological parameters of 5-wk-old chickens as described later. In Studies 2 and 3, the HESM powder was ethanol sterilized and used as the feed supplement where the effects of *Salmonella typhimurium* lipopolysaccharide was evaluated following 24 or 4 h of treatment. In all the experiments, the chickens were fed diets containing the supplement for 14 days post hatch then switched to un supplemented diet for the rest of the time until necropsy. The birds were monitored daily for mortality, welfare and evaluated weekly for body weight (BW), and feed consumption. The BW of the birds were measured before LPS injection and prior to necropsy when necessary.

In Study 1, 72 one day-old chicks were divided into 3 groups each with 24 birds in two replicate pens. The three groups received diets as follows: 1) control feed with no supplement, 2) feed containing 0.5% whey protein powder as a secondary control to find whether the effect was due to protein supplement alone, and 3) feed containing 0.5% HESM. Prior to necropsy, 6 birds from each pen (12/group) were bled by cardiac puncture, blood collected using EDTA containing Vacutainer as well as rapid serum tubes (BD Falcon) for hematology and clinical chemistry assays respectively [14].

Studies 2 and 3 were done with ethanol sterilized HESM. Growth performance of the birds along with differnt physiological changes including the effects of *Salmonella typhimurium* LPS (cat # HC4060 Sigma-Aldrich, St. Louis, MO) were determined. In Study 2, the day-old chickens were allocated into 2 groups and given feed with or without 0.5% HESM as described above then switched to regular feed through 5 weeks of age. On day 34, 12 birds in each group

were injected intramuscularly in the thigh with LPS at the concentration of 1 mg/kg BW in saline and the rest received equal volumes of saline. The effect of LPS was monitored visually for 5 h following injection with the BW measured before and after 24 h of injection. Prior to necropsy, 12 chickens/group were bled for hematology and clinical chemistry assays. At necropsy, the weights of selective organs from all the birds were recorded.

In Study 3, the effect of LPS on splenic expression of selective genes associated with different immune function were determined. Chickens from control and HESM groups received either saline or LPS injection as described earlier. Four h after the injection 6 chickens from each group were killed and the spleens placed in liquid nitrogen for RNA extraction, and the rest killed after 24 h to record BW and organ weights.

Necropsy

The liver, heart, spleen, and bursa weights from all birds were used to calculate the percentage relative to BW. In Study 2, a cm length of ileum below the pancreatic loop was excised from each of six control and HESM fed birds and fixed in Carnoy's fluid for ~5 h, transferred to 70% alcohol then processed for histology. Six micron paraffin sections were stained with periodic acid Schiff (PAS) hematoxylin staining and examined for villus health, mucous secretion, and gross abnormality by visual observation. The sections were photographed in BX Olympus microscope.

Hematology

Blood cell counts along with hemoglobin content, mean corpuscular volume (MCV), hematocrit, microhematocrit (MCH), red blood cell distribution width (RDW) values were measured using EDTA anticoagulated blood by the use of Cell-Dyn 3500 blood analysis system (Abbott

Diagnostics, Abbott Park, IL) standardized for avian blood and the heterophil to lymphocyte ratios (H/L) calculated.

Serum assays

The serum metabolic parameters were assayed using a clinical chemistry analyzer (Ciba Corning Diagnostics Corp, Medfield, MA). Corticosterone concentrations were measured by Detect X enzyme immunoassay kit™ (Arbor Assays, Ann Arbor, MI) using predetermined dilutions of sera [14]. The IgM, IgG, and IgA concentrations were similarly, determined using respective assay kits from Bethyl Laboratory (Montgomery, TX) with the serum diluted to 1:1000 with the manufacturer supplied buffer for IgA, 1:50,000 for IgG, and 1:20,000 for IgM, respectively. The concentrations of antibodies in the sera were calculated from their respective standard curves.

Gene expression

The expressions of inflammation regulatory genes such as pro-inflammatory (IL-1β, IL-6, IFN-γ), anti-inflammatory (IL-4, IL-10, IL-12), and immunosuppressive, wound repair supportive factors (TGF-β3 and vascular endothelial growth factor (VEGF) [22-24] were determined using splenic tissue RNA and quantitative RT-PCR. Six frozen spleens from each treatment group were split into 4 quarters and ~ 100 mg of tissues from equivalent region of each spleen were extracted with Tri Reagent (Sigma-Aldrich) to prepare RNA. Complementary DNA (cDNA) was synthesized using 1 μg of RNA and qScript™ cDNA SuperMix (Quanta biosciences) following manufacturer's protocol. Quantitative real-time PCR was performed using SYBR® Green PCR Master mix (Life technologies) in an ABI 7500 Real-Time PCR system (Applied Biosystems, Carlsbad, CA). A 25 μl reaction containing 5 μl cDNA (1 μg of RNA equivalent) and primers specific against chicken IL-1β, IL-4, IL-6, IL-10, IFN-γ, TGF-β3, VEGF and IL-12

(Supplementary Table 1) were subjected to PCR with initial denaturation at 95°C for 10 minutes followed by 40 PCR cycles as follows: 95 °C for 15 s, 58 °C for 1 min. Expression of target genes were analyzed by the $2^{-\Delta\Delta Ct}$ method [25] with 18S RNA used as reference gene.

Statistical analyses

All results were evaluated using Duncan's t test using SAS software [26] and a *P*-value of $\leq$0.05 considered to be significant.The results are shown as mean±SEM.

Results

HESM

The average protein content of HESM was determined to be approximately 88% (w/w) the addition of which did not significantly alter the protein content of feed (Control: 25.1% and HESM: 25.3%, n=2 samples/group). The number of bacterial colonies showed a significant reduction from 30,000 / ml in untreated HESM extract to less than 5 colonies in ethanol treated HESM. Similarly, the ethanol treatment reduced the endotoxin content of HESM judged by a significantly low level of nitrite production by the HTC cells (Figure. 1)

Effect on BW, mortality

In Study 1, there were no significant differences in body weight (BW) or relative organ weights of the birds given HESM supplemented feed compared with either control or whey protein supplemented groups (Supplementary Table 2). In both Studies 2 and 3, the birds fed HESM supplement diet showed a slight but statistically nonsignificant increase in BW and no differences in relative organ weights compared with control group. Cumulative mortality rate in

all 3 experiments combined, showed no differences between control and HESM fed chicks (Table 1).

LPS effect

LPS treated chickens showed symptoms of sickness indicated by lack of activity, eyelid closure, and feed avoidance within 3 h of treatment and decreases in BW by 24 h. The relative liver weights were significantly increased and bursa weights decreased in LPS treated groups although this effect was not seen with heart and spleen. The chickens that received HESM showed comparatively less ($p \leq 0.05$) BW loss relative to control fed birds (Table 1, Figure. 2).

Hematology and serum chemistry

The results from Study 2 and the effect of LPS are shown in Table 2. HESM treatment *per se* had no effect on lymphocyte (L), monocyte (M), heterophil (H), or basophil (B) percentages, and H/L ratios. On LPS treatment, there was an increase in percentages of heterophil, monocyte, and basophils, and H/L ratios, and a reduction in lymphocyte counts in both groups. The relative decrease in heterophil and increase in the lymphocyte counts resulted in a significant decrease of H/L ratios in HESM group compared with controls and challenged with LPS (Table 2). There were few other changes including increased hematocrit in HESM birds and treated with LPS. HESM produced a moderate decrease in serum protein, calcium, and magnesium levels some of which increased upon LPS treatment. LPS caused a decrease in serum iron and increase in triglycerides in both groups (Table 3). The cholesterol and HDL levels were down regulated in serum of control birds as compared to HESM when challenged with LPS. Neither alanine nor the aspartate amino transferases were affected by HESM indicating the lack of liver toxicity.

Serum immunoglobulins and corticosterone

In Study 1 there was no changes in serum IgM levels of chickens fed whey protein or intact HESM but the IgG levels decreased with HESM (Supplementary Table 3). Similar trend was observed in the 2nd study that upon LPS treatment increased the serum IgM while the IgG level remained unchanged in HESM fed birds. Neither treatment had any effect on serum IgA (Table 4). The corticosterone was consistently lower in both studies in HESM fed birds but with LPS treatment, it increased moderately reaching to the same levels as control birds (Table 4).

Gene expression

The splenic gene expression results are shown in Table 5. Chickens fed regular diet and challenged with LPS had a significant increase in IL-6 gene expression compared with HESM fed group (Figure 3). The anti-inflammatory gene IL-10 showed a significant increase in the HESM group when challenged with LPS (Figure 4). The IL-4 gene was downregulated in HESM birds but on LPS treatment its expression was significantly higher compared with control fed chickens (Figure 5). There was no change in the expressions of IFN-g or IL-12. But the TGF-β expression showed a significant decrease by LPS treatment in both control and HESM fed groups whereas the VEGF downregulated in HESM birds regardless of LPS treatment.

Histology

There were no differences in the overall health of intestine between the control and HESM diet fed birds judged by villus morphology, muscularis, and mucus deposition (Supplementary Figure 1).

Discussion

Our results show that feeding HESM is beneficial to chickens particularly in decreasing stress levels and improving resistance to LPS-induced changes. These results are consistent with our previous report where the egg shell membranes (ESM) from fresh unfertilized eggs improved the performance of 3 week old chickens with respect to body weight and downregulated corticosterone and other stress parameters[14]. In the previous study with ESM we observed an increase in the levels of IgG and IgM at 3 weeks but in the present study the IgM levels appeared not to be affected which may be due to later sampling time of 5 weeks when the early response to antigens tend to subside [27-29]. However, the cause of IgG downregulation in HESM fed birds is not understood.

Weight loss is a hallmark of endotoxemia in both mammals and birds which is mediated through several pro inflammatory cytokines such as IL-1, IL-6, and TNF-α [30-33]. These cytokines not only cause hypophagia but also promote protein catabolism [34]. The HESM appears to curb the effect of endotoxin promoting weight loss also modifies the splenic expression of cytokine genes that are associated with inflammation [35-39]. Similarly, there was a persistent downregulation of corticosterone and other stress markers such as heterophil to lymphocyte ratios [40] in HESM fed birds that could account for their better performance. Glucocorticoids can not only be anti-anabolic but also immunosuppressive [41]. Lower stress can improve feeding and decrease susceptibility to pathogens in poultry [42]. However, the mechanism by which the ESM lower the stress parameters is not understood since the effect appears to persist beyond the period of discontinuation of feeding HESM. Hypothetically a decreased serum level of adrenal steroids can be expected upon endocrine exhaustion occurring under conditions such as chronic endotoxemia. However, it is not the case in this study because

the HESM was not only deplete of endotoxin but also, the chickens fed on it showed any sign of sickness judged from their BW, intestinal pathology and blood profiles.

Although the expression of IL-6, was upregulated by LPS treatment in both feed groups, it was significantly low in birds fed HESM. Similarly, there were also the upregulation of IL-4 and IL-10, both of which are considered as anti-inflammatory cytokines implicated in the development of immune tolerance [43, 44]. Anti-inflammatory effect of natural ESM has been reported in experimental models of joint inflammation where the effects were attributed to the proteoglycan content of the preparation [45, 46]. Similar findings were reported by Shi et.al in mice where the effect of hydrolysate of eggshell membrane provided protection against dextran sodium sulfate induced intestinal inflammation [47]. The TGF-β expression was lower in both feed groups injected with LPS while the VEGF showed consistently lower expression in HESM birds. Since these growth factors help tissue repair and angiogenesis, associated with the resolution of inflammation, [23] their downregulation during early phases of inflammation is likely. However, the decrease in VEGF expression in birds fed HESM treatment is not understood. Whether the patterns of expressions of pro and anti-inflammatory cytokines have any relevance in curbing the body weight loss in HESM fed chickens is not known. Evidently, a modified immune response due to HESM confers resistance to endotoxin induced changes. As the susceptibility to infection can increase in immunocompromised individual likewise, it may confer tolerance to disease in immune strengthened birds.

There were no significant differences in IgM or IgA levels of chickens fed either control or HESM diet with or without LPS challenge. By contrast, the IgG levels were reduced in birds fed

HESM that did not substantially change even after LPS treatment. Hypogammaglobinemia with normal IgM and IgA have been noted in human patients with physical trauma such as burn and nephrosis [48]. But the chickens fed HESM had neither physical trauma nor their clinical chemistry showed any indication of dysregulated kidney function such as hypoalbumenemia and hyperlipidemia that can be associated with nephrotic conditions. The HESM induced down regulation of serum corticosterone is consistent with our previous results with ESM [14]. We presume that post hatch exposure to HESM which is laden with different regulatory proteins and peptides and the remnants of bacterial and parasite contaminants possibly, condition the neuro-immune system lowering the disposition of birds to stress and higher tolerance to LPS. In newly hatched birds as in mammalian neonates, the immune and neuroendocrine system is immature and prone to epigenetic conditioning. At this stage not only the maternal but also other biodiverse factors such as diets, and microbes provide signals that can shape immunity and establish tolerance and resistance to pathogens [49-52]. There is increasing evidence showing that the neonatal exposure to stress, diets and microbiome have long term effect on immunity, health, and wellbeing of individuals [53, 54]. Besides, the enteric system houses the second largest density of neurons that could be impacted by bioactive factors thereby can influence immunity; for example, it is now known that the immune functions of lymphoid organs such as spleen can be prone to control through neural output of autonomic system and T cell regulation is subject to cholinergic output [55, 56]. Thus, the bioactive embryonic factors in HESM modulating the immune response of chickens is a possibility. Also, it is now well recognized that maternal factors such as milk along with exposure to microbiome are important factors for establishing disease resistance and post-natal conditioning in mammals [57]. The plethora of proteins and peptides present in the eggshell membrane could simulate those effects in chickens.

From the foregoing discussion it is clear that HESM supplementation of feed is beneficial to post hatch poultry and it curtails the harming effects of LPS. Whether the effects are due to the bioactive proteins and peptides or some other factors is not known. Very little is known as to whether and how food associated bioactive proteins influence immunity because most omnivorous birds and mammals rely on some sort of raw proteins and peptides for their early nutrition which could provide epigenetic conditioning of immune system and build their resistance against common infections. The postnatal immune system being immature but plastic it certainly provides opportunity for nutritional modulation for building better immunity [58] In conclusion, our results show that HESM supplement can be a sustainable feed additive to improve immunity and health physiology of poultry.

Acknowledgment

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Author Contribution

Conceived and designed by NCR, SKM, experiments performed by SKM, NCR, GR, BP, ZYZ, data analyzed by SKM, NCR, manuscript written by SKM, NCR, discussed and approved by NCR and AMD.

Table 1. Body weight (BW) and the relative organ weights (% BW) of 5-week-old chicken fed diets with sterilized HESM and challenged with LPS for 24 h: Studies 2 and 3 combined (n=32-36).

	Saline		LPS	
Parameters	**Control**	**HESM**	**Control**	**HESM**
BW (g)	2238.27±33.38[a]	2327.23±40.77[a]	2021.30±37.02[c]	2135.38±28.55[b]
Heart	0.52±0.01[a]	0.51±0.01[a]	0.52±0.01[a]	0.52±0.01[a]
Liver	2.63±0.04[b]	2.48±0.06[b]	3.41±0.08[a]	3.30±0.09[a]
Spleen	0.12±0.01[a]	0.12±0.00[a]	0.13±0.01[a]	0.13±0.01[a]
Mortality (%)*	9±1.53[a]	14.33±3.48[a]	-	-

Values with different superscripts in a row are significantly different ($P \leq 0.05$). *Results based on 3 experiments

Table 2.Hematology profiles of chickens fed with or without HESM containing feed and treated with LPS: Study 2 (n=12)

	Saline		LPS	
Parameters	**Control**	**HESM**	**Control**	**HESM**
WBC (10^3/μL)	49.76±1.42 [a]	46.01±1.75 [b]	54.09± 3.12 [a]	45.60±3.0[b]
Heterophil (H) (%)	11.44±0.36[c]	12.62±0.46[c]	30.85±2.35[a]	22.94±3.14[b]
Lymphocytes (L) (%)	83.81±0.65[a]	82.31±0.70[a]	61.80±2.22[c]	69.92±3.10[b]
(H/L)	0.14±0.01[c]	0.15±0.01[c]	0.50±0.06[a]	0.32±0.05[b]
Monocytes (M) (%)	2.32±0.18 [b]	2.75±0.28[b]	4.31±0.33[a]	4.01±0.26[a]
Eosinophil (E) (%)	0.02±0.01[a]	0.01±0.00 [a]	0.02±0.00 [a]	0.02±0.00[a]
Basophil (B) (%)	1.99±0.17[b]	2.3±0.18[b]	2.99±0.19[a]	3.03±0.17[a]
Red blood cell (× 10^6/μL)	2.18±0.04[a]	2.18±0.04[a]	2.24±0.03[a]	2.33±0.02[a]
Hemoglobin (g/dL)	6.77±0.09[b]	6.80±0.085[b]	6.92±0.062[b]	7.17±0.073[a]
Hematocrit (%)	59.26 ±1.15[b]	60.27±0.98[b]	60.11±0.68[b]	63.02±0.65[a]
Mean corpuscular volume (MCV)(fL)	271.23±1.73[b]	276.16±1.69[a]	267.32±1.58[b]	271.07±1.16[b]
Thrombocyte (k/μL)	0.03±0.03[a]	0.00±0.00[a]	0.64±0.44[a]	0.003±0.00 [a]

Values with different superscripts in a row are significantly different ($P \leq 0.05$).

Table 3.. Serum clinical chemistry variables of 5 week-old chickens fed with regular diet or the diet supplemented with 0.5% HESM and challenged with LPS: Study 2 (n=12).

	Saline		LPS	
Parameters	**Control**	**HESM**	**Control**	**HESM**
Albumin (g/dL)	1.15± 0.02[a,b]	1.08± 0.02 [b]	1.17± 0.02[a]	1.19± 0.03[a]
Glucose(mg/dL)	213.15±4.18[a]	216.15± 3.64 [a]	199.08± 4.14[b]	212.85± 5.63[a]
Inorganic phosphate (mg/dL)	3.31±0.16[b]	3.28± 0.13[b]	3.28± 0.17[b]	4.01± 0.21[a]
Total protein(g/dL)	3.12± 0.06[a]	2.76± 0.04[b]	3.15 ± 0.05[a]	3.22± 0.01[a]
Alkaline phosphate (U/L)	193.77± 24.24[a,b]	226.31± 28.60[a]	139.77 ± 14.90[b]	192.85±24.93[a,b]
Alanine aminotransferase (U/L)	3.85 ± 0.62[a]	2.49± 0.49[a]	2.75± 0.44[a]	3.85± 0.56[a]
Aspartate Aminotransferase (U/L)	309.45±17.4[a]	348.67±29.45 [a]	324.95±18.49[a]	380.83±24.72[a]
Blood urea nitrogen (mg/μL)	1.61± 0.13[a]	1.05± 0.15 [a]	1.33± 0.08 [a]	1.30± 0.08[a]
Magnesium (mEq/L)	1.90± 0.05[a]	1.58± 0.04 [b]	1.73± 0.05 [a ,b]	1.68± 0.11[b]
Calcium (mg/dL)	10.53± 0.23[a]	7.99± 0.26 [c]	10.28± 0.29[a]	9.40± 0.27 [b]

Cholesterol (mg/dL)	165.15± 6.91[a]	166.00± 4.16[a]	138.54±4.05[b]	161.31 ± 6.20[a]
Creatinine kinase(U/L)	546.8±83.43[a,b]	821.9 ± 166.59[a]	288.9± 36.06[b]	388.7± 46.39[b]
Triglycerides (mg/dL)	55.00± 4.86 [b]	51.07± 3.28[b]	93.00± 5.49[a]	86.31± 6.93[a]
High density lipoprotein (mg/dL)	45.15± 2.81[a]	44.77± 1.28 [a]	35.69± 1.24 [b]	41.23± 1.61[a]
Iron (μg/dL)	99.54± 4.94 [a]	93.85± 4.80 [a]	55.46± 5.18 [b]	57.77± 9.27 [b]

Values with different superscripts in a row are significantly different ($P \leq 0.05$).

Table 4.. Serum IgG, IgM, IgA, and corticosterone levels of chickens fed regular NRC diet or diets supplemented with ethanol sterilized HESM and challenged with LPS for 24 h.: Study 1 (n=12)

	Saline		LPS	
Parameters	**Control**	**HESM**	**Control**	**HESM**
IgM (mg/ml)	2.84±0.30[b.a]	1.93±0.24[b]	3.83±0.40[a]	3.50±0.47[a]
IgG (mg/ml)	4.78±0.68[a]	1.23±0.17[b]	3.53±0.53[a]	0.98±0.17[b]
IgA (mg/ml)	0.56±0.15[a]	0.54±0.22[a]	1.18±0.17[a]	0.81±0.39[a]
Corticosterone	7.74±0.95[a]	5.01±0.53[b]	6.7±0.60[a,b]	6.58± 0.96[a,b]

Values with different superscripts in a row are significantly different ($P \leq 0.05$).

Table 5. Study 3. The expression of splenic genes quantified by RT-PCR in birds fed with and without HESM and injected with LPS or saline (n=6)

	Saline		LPS	
Parameters	**Control**	**HESM**	**Control**	**HESM**
IL-1	1.00± 0.24^{b,a}	0.75±0.11^{b}	1.32±0.25^{b,a}	1.83±0.61^{a}
IL-6	1.00±0.24^{b}	1.08±0.17^{b}	3.52±0.37^{a}	2.42±0.49^{b}
IL-10	1.00± 0.10^{b,c}	0.89±0.19^{c}	2.3±0.34^{b}	4.26±0.95^{a}
IFN-γ	1.00±0.28^{a}	0.99±0.26^{a}	0.68±0.13^{a}	1.15±0.31^{a}
TGF-β	1.00±0.20^{a}	0.97±0.14^{a}	0.28±0.04^{b}	0.30±0.04^{b}
IL-12	1.00±0.13^{a}	1.43±0.32^{a}	1.13±0.26^{a}	1.64±0.19^{a}
VEGF	1.00±0.13^{a}	0.32±0.08^{b}	0.86±0.11^{a}	0.31±0.04^{b}
IL-4	1.00±0.13^{b,c}	0.67±0.12^{c}	1.76±0.32^{b}	2.82±0.68^{a}

Values with different superscripts in a row are significantly different ($P \leq 0.05$).

Figure legends

Figure 1. Nitrite production at 24 h by the HTC chicken macrophage in response to different treatments.

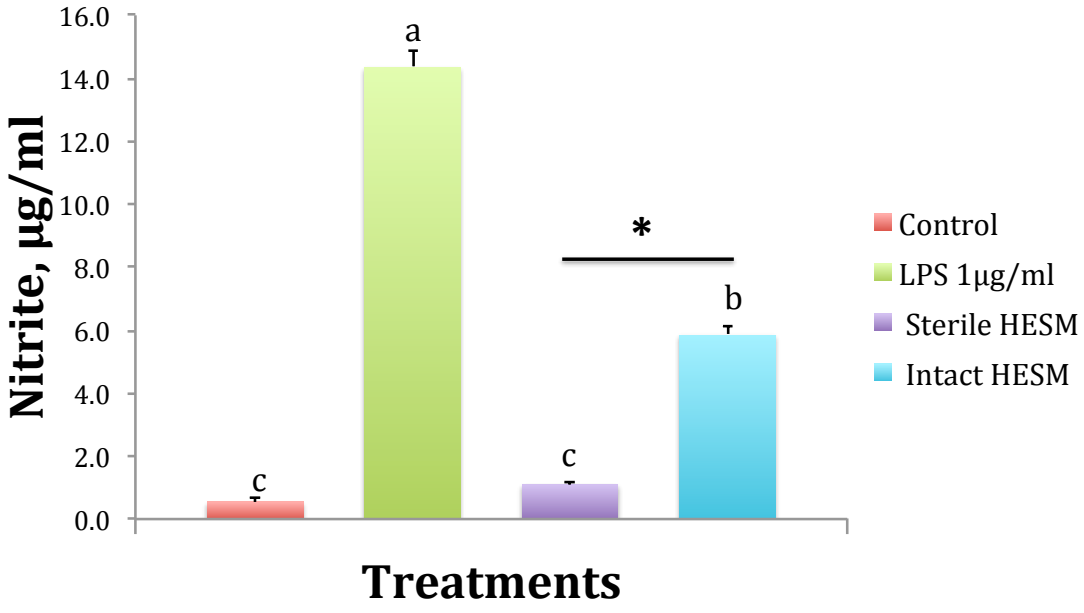
Figure 2. Effect of HESM on body weight of 5 week old birds, challenged with LPS for 24h n=(32-26). Values with different superscripts are significantly different.

Figure 3. Comparison of splenic IL-6 gene expression in chickens fed control and HESM diet and challenged with LPS or Saline for 4 h (n=6). Values with different superscripts are significantly different ($p \leq 0.05$).

Figure 4. Splenic IL-10 gene expression comparison of chickens fed control or HESM supplemented diet and treated with LPS or Saline for 4 h (n=6). Values with different superscripts are significantly different ($p \leq 0.05$).

Figure 5 Comparison of splenic IL-4 gene expression in chickens fed control and HESM diet and challenged with LPS or Saline for 4 h (n=6). Values with different superscripts are significantly different ($p \leq 0.05$).

Figure 1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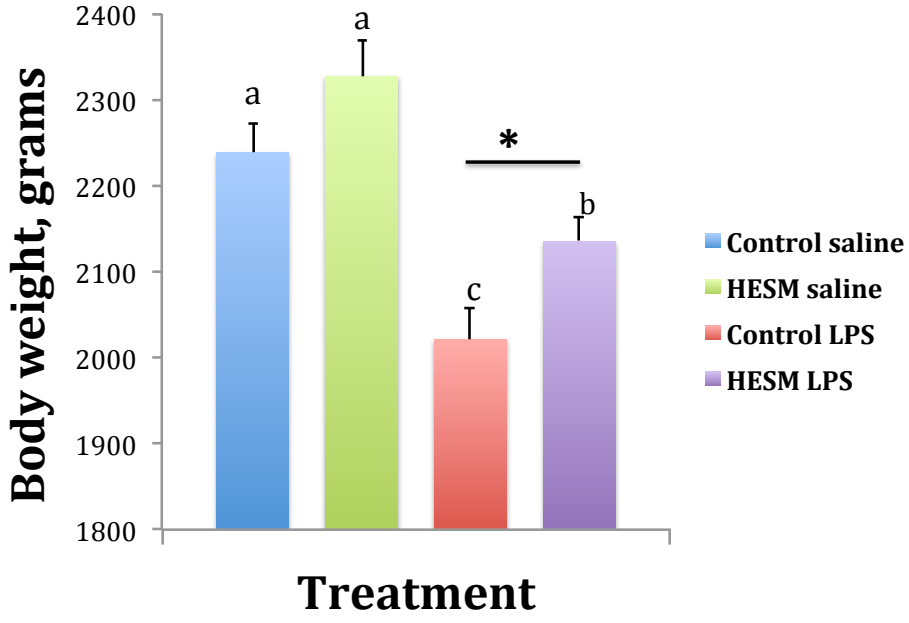
10.0
8.0
6.0
4.0
2.0
0.0
Nitrite, µg/ml
a
c
c
b
*
Control
LPS 1µg/ml
Sterile HESM
Intact HESM
Treatments

Figure 2


2400
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2100
2000
1900
1800
Body weight, grams
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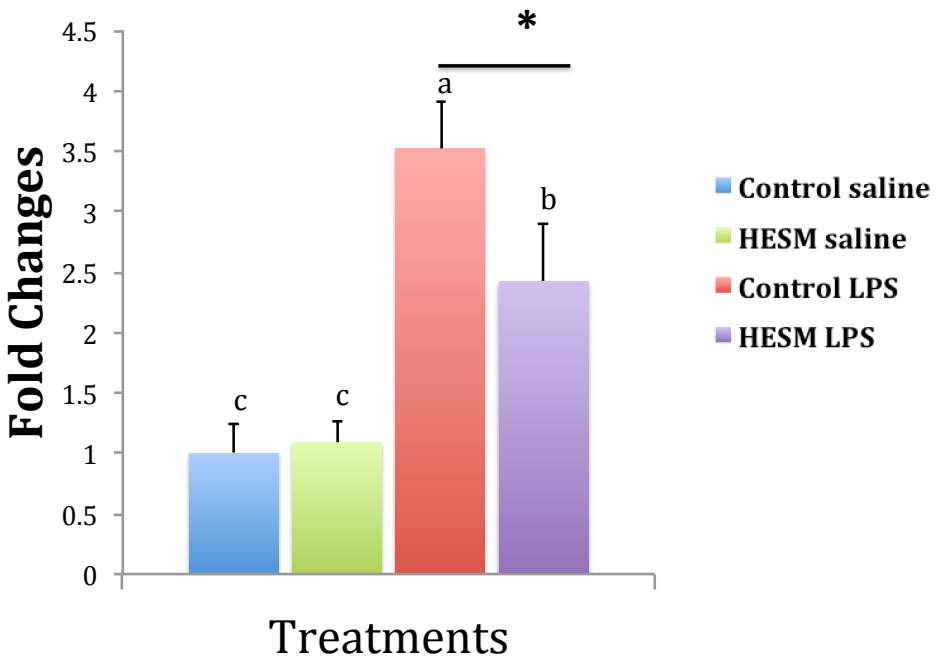
a
c
b
*
Treatment
Control saline
HESM saline
Control LPS
HESM LPS

Figure 3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Fold Changes
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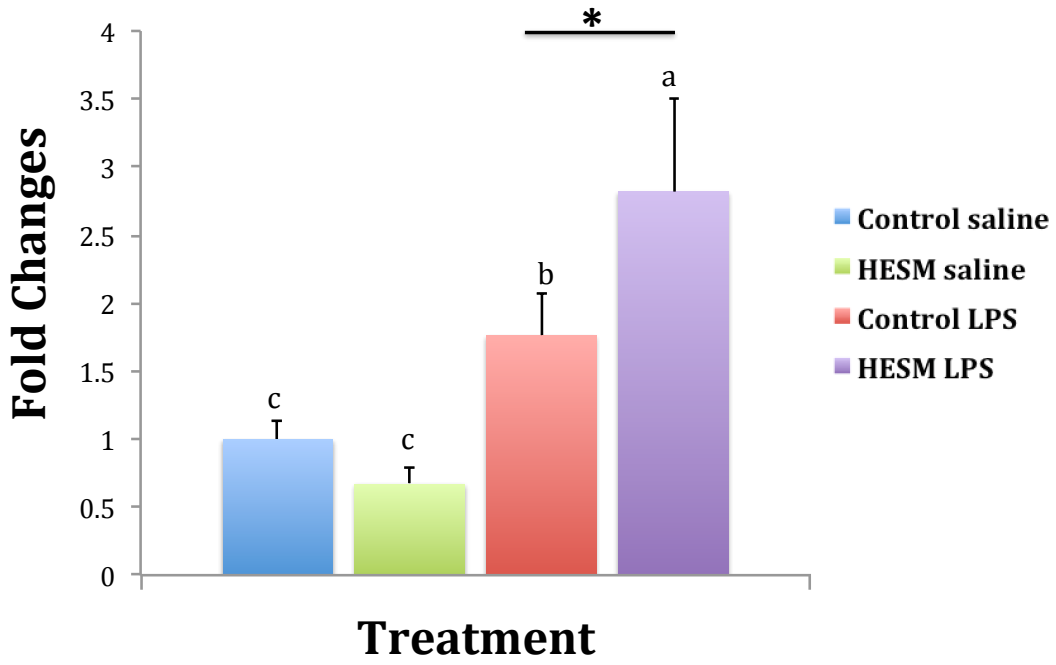
Treatments
Control saline
HESM saline
Control LPS
HESM LPS

Figure 4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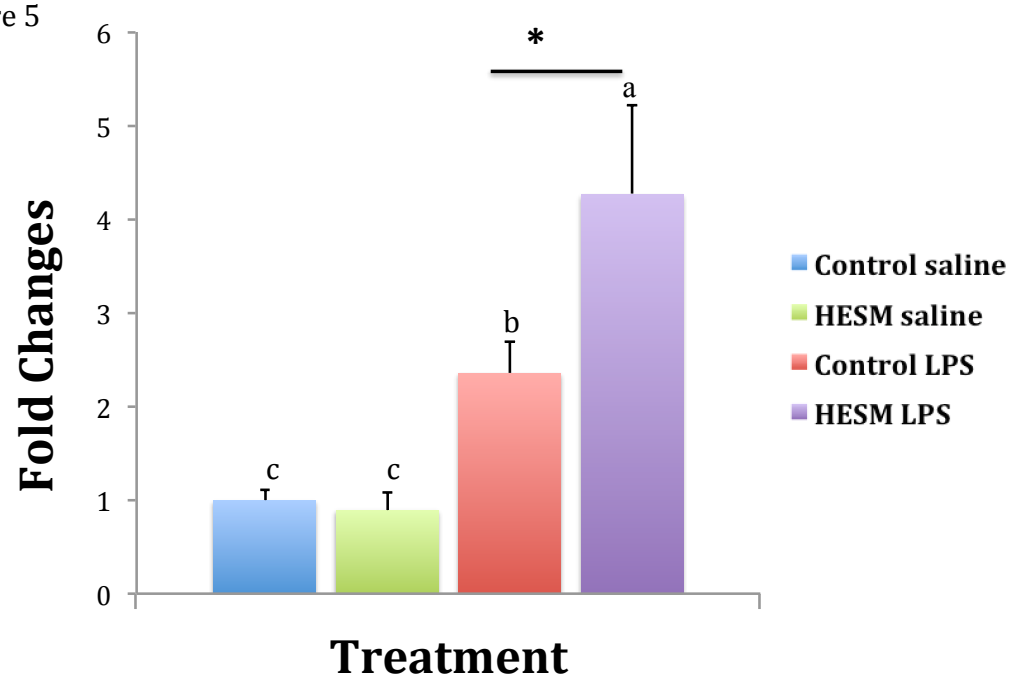
Fold Changes
Treatment
Control saline
HESM saline
Control LPS
HESM LPS

Figure 5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Fold Changes
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Treatment
Control saline
HESM saline
Control LPS
HESM LPS

Supplementary Table 1. PCR primers and accession numbers of candidate genes for chicken cytokine and other proteins

Target genes	Accession number	Primer sequences	Length of product (bp)
IL-1β	NM_204524.1	SF：CGAGGAGCAGGGACTTTGC SR：GAAGGTGACGGGCTCAAAAA	71
IL-6	NM_204628.1	SF：GCTTCGACGAGGAGAAATGC SR:GGTAGGTCTGAAAGGCGAACAG	63
IL-10	NM_001004414.2	SF：CGCTGTCACCGCTTCTTCA SR：CGTCTCCTTGATCTGCTTGATG	63
IFN-γ	NM_205149	SF：AAAGCCGCACATCAAACACA SR：GCCATCAGGAAGGTTGTTTTTC	64
TGF-β3	NM_205454.1	SF：TGCGGCCAGATGAGCAT SR：TGCACATTCCTGCCACTGA	55
18S rRNA	NC_006088.3	SF：TCCCCTCCCGTTACTTGGAT SR：GCGCTCGTCGGCATGTA	60
IL-12	NC-46430425	SF:TGCCCAGTGCCAGAAGGA SR:TCAGTCGGCTGGTGCTCTT	57
VEGF-A	GI 160358852	SF:AAATTCACAGACTCACGTTGCAA SR: ATCTGCAAGTGCGCTCGTTT	61
IL-4	NM_0010079.1	SF: GCTCTCAGTGCCGCTGATG SR: GAAACCTCTCCCTGGATGTCAT	60

Supplementary Table 2. Study 1. Body weight (BW) and the relative organ weights (% BW) of 5 week-old chicken fed diets containing 0.5% whey protein powder or 0.5% HESM (n=20-23).

Parameters	Control	0.5% whey protein	0.5% HESM
BW (g)	2153.95±45.97[a]	2219.65±37.20[a]	2148.00±37.74[a]
Heart	0.56±0.02[a]	0.53±0.02[a]	0.57±0.02[a]
Liver	2.20±0.04[a]	2.18±0.05[a]	2.3±0.06[a]
Spleen	0.11±0.01[a]	0.12±0.01[a]	0.11±0.01[a]
Bursa (%)	0.15±0.01 [a]	0.16±0.02[a]	0.16±0.01[a]

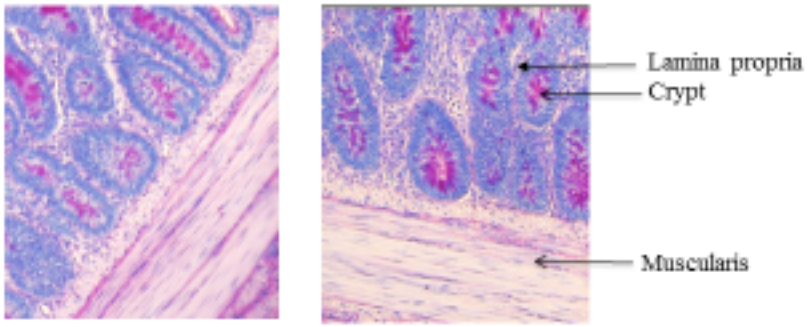
Values with different superscripts in a row are significantly different ($P \leq 0.05$).

Supplementary Table 3. Study 1. Comparison of serum IgG, IgM, and corticosterone levels of chickens fed with regular NRC diet or 0.5% whey protein, or HESM supplemented diet. The results are shown as mean ± SEM. (n=12/ group)

Parameters	**Control**	**0.5% whey protein**	**0.5% HESM**
IgG (mg/mL)	1.20 ± 0.07^{a}	1.07 ± 0.06^{a}	0.82 ± 0.07^{b}
IgM (mg/mL)	2.69 ± 0.24^{a}	2.54 ± 0.30^{a}	2.60 ± 0.22^{a}
Corticosterone (ng/mL)	5.75 ± 0.77^{a}	$4.26\pm0.33^{a,b}$	$3.81\pm0.41^{b,c}$

Values with different superscripts in a row are significantly different ($P \leq 0.05$).

Supplementary Figure 1. Histology of intestine sections of control and HESM fed birds (magnification X400)



Control

HESM

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VI. CONCLUSION

The immune system of a newly hatched chick is functionally immature and plastic. It can be primed and modulated by numerous ways, which can make it more resilient to fight infections in later life. Pre and postnatal life is critical time for the immune system for epigenetic programming for development of resistance and tolerance and the nutritional modulation can be a major option to this end. In mammals for example, the mother's milk that contains a plethora of defense proteins, peptides, and growth factors provide such programming boosting resistance against microbes, provide probiotic factors for microbiome, and anti-infective benefits to the immature gut. The diets in birds living in wild may consist of fresh worms, insects, fish or food which can provide such programming which the domesticated poultry raised under controlled conditions may not have that exposure. Using eggshell membrane matrix and following its composition and biological effects our data suggest that the factors associated with this physiological modulation that provides protection of chicks against LPS induced changes.

Using ESM from both fresh unfertilized as well as from hatchery egg membranes, we found that it contain more than 270 proteins, which are not only antimicrobial in nature, but they are also associated with cell signaling, development process, and immune system regulation processes (chapter2/4) largely of embryonic and hematological origins. The rejuvenation potential of body fluid factors of young animals was shown using parabiotic mice ( ) suggesting that these factors can influence physiology. In extrapolating the concept we think that ESM factors which contain many antimicrobial, cell organizing, and cell signaling proteins perhaps produce the effects through neuroendocrine immune organizational pathways (chapters 3 and 5).

These experiments can be foundational to explore the idea that post hatch modulation of physiology and immunity using allogeneic or xenogeneic bioactive factors and microbes may confer life time resistance and tolerance to poultry against pathogens.

With respect to ESM, this byproduct can be reusable and saleable without causing any biosecurity issues. This research would help for utilization of hatchery waste eggshell membranes in a potentially valuable and profitable manner.