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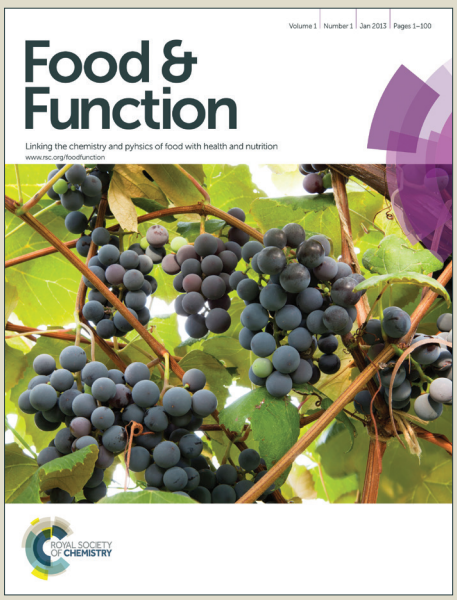

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The Impact of Fertilization on the Chicken Egg Yolk Plasma and Granule Proteome 24 Hours Post-Lay at Room Temperature: Capitalizing on High-pH / Low-pH Reverse Phase Chromatography in Conjunction with Tandem Mass Tag (TMT) Technology

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Abbreviations

CE – collision energy

DDA – data-dependent acquisition

HCD – higher-energy collision dissociation

HRG – histidine-rich glycoprotein

LC/MS – liquid chromatography / mass spectrometry

LC-MS/MS – liquid chromatography mass spectrometry / mass spectrometry

m/z = mass-to-charge ratio

RP – reverse-phase

SCX – strong-cation exchange

SSD – silver sulfadiazine

TMT – tandem mass tag

Abstract

Chicken egg yolk is a rich source of nutrients providing high quality proteins, vitamins, minerals, carotenoids and antioxidants. Chicken egg yolk, recovered from whole egg within 24 hours post-lay has been utilized as a starting material in the preparation of a dietary supplement that has been demonstrated to lead to gains in muscle mass in a human clinical study. Further, an oil derived from chicken egg yolk has been utilized as a topical agent to treat third degree burn injury. The molecular changes that take place in fertilized, chicken egg yolk during the first 24 hours post-lay are not well understood. By studying how the protein composition of egg yolk varies with fertility status, one can utilize this knowledge to develop egg yolk-based products that have been optimized for specific applications. In this study, a direct quantitative comparison was made between the proteome of fertilized chicken egg yolk with the proteome of unfertilized chicken egg yolk, both maintained at 20^{o} C and analyzed within 24 hours post-lay. Egg yolk proteins from each fertility state were digested with trypsin, labeled with distinct chemical labels (tandem mass tag reagents) and then combined in a 1:1 ratio. A TMT-labeled tryptic digest derived from chicken egg yolk proteins (fertilized and unfertilized) was separated using high-pH / low-pH reverse-phase chromatography and analyzed using mass spectrometry. 225 protein identifications were made from this TMT-labeled tryptic digest based on a minimum of 2 unique peptides observed per protein. 9 proteins increased in abundance in fertilized egg yolk relative to unfertilized egg yolk and 9 proteins decreased in abundance in fertilized egg yolk relative to unfertilized egg yolk. Some proteins that increased in abundance in fertilized egg yolk play an important role in angiogenesis (pleiotrophin, histidine rich glycoprotein) and defense against pathogens (mannose-binding lectin, β-defensin 11, serum amyloid P-component, ovostatin). Based on this study, fertilized chicken egg yolk may be more useful as a starting material relative

to unfertilized chicken egg yolk for the purpose of enriching or isolating proteins with pro-angiogenic and anti-microbial properties.

Introduction

Chicken egg yolk is a rich source of nutrients providing high quality proteins, essential vitamins (A, B6, B12, D, E, K along with folate and choline), minerals (calcium, iron, phosphorus, zinc), carotenoids (lutein, zeaxanthin) and antioxidants. These nutrients play an important role in maintaining muscle mass and strength [1], supporting pregnancy [2] as well as preserving brain [3] and eye [4] function. It has been demonstrated recently that subjects who consumed a dietary supplement prepared from chicken egg yolk (separated from whole egg within 24 hours post-lay) on a daily basis while performing resistance training gained increased muscle mass relative to the placebo group over a 3 month duration [1]. When a composition prepared from chicken egg yolk (separated from whole egg within 24 hours post-lay) was used as a topical agent to treat rodents with a third degree burn injury, enhanced re-epithelialization without the formation of scar tissue was observed in this group of rodents relative to rodents that were treated with 1% silver sulfadiazine (SSD), a topical agent commonly used to treat second and third degree skin burns [5]. Because chicken egg yolk provides health benefits beyond basic nutrition, chicken egg yolk is regarded as a functional food source [6].

Over the last decade, advances in the fields of mass spectrometry-based proteomics [7-8] and lipidomics [9] have significantly enhanced our understanding of the unique biochemical properties of chicken egg yolk. One of the earliest attempts to comprehensively characterize the chicken egg yolk proteome was performed by Mann and Mann [7]. By applying 1-D SDS-PAGE in conjunction with LC-MS/MS, Mann and Mann identified 119 proteins from chicken egg yolk. Of the 119 proteins, 86 were identified for the first time in chicken egg yolk. Some of

the most abundant proteins included serum albumin, vitellogenin cleavage products, apovitellenins, IgY, ovalbumin, and a 12 kDa serum protein with cross-reactivity to β-2-microglobulin. One year after this report by Mann and Mann, Farinazzo *et al.* [8] reported the identification of 255 proteins from chicken egg yolk by applying combinatorial hexapeptide ligand libraries. By designing and immobilizing hexapeptides onto a solid surface that can specifically capture and detect low abundance proteins, Farinazzo *et al.* [8] managed to identify more than twice as many proteins from chicken egg yolk relative to the original report from 2008. This research provided valuable insight into the bioactive properties of egg yolk [10]. Similar studies have also been performed on the egg white proteome using 1D-SDS-PAGE in conjunction with LC/MS/MS [11], the original results later augmented using combinatorial hexapeptide ligand libraries [12] followed by advancements in mass spectrometry instrumentation [13].

Quantitative proteomic technologies [14-16] have made it possible to not only study the static proteome of the chicken egg yolk but to also gain insight into the dynamic nature of the chicken egg yolk proteome. In a recent study, Réhault-Godbert *et al.*[17] applied a label-free quantitative proteomics method to study changes that occur in the chicken egg yolk proteome (plasma fraction) upon fertilization. In order to gain insight into chicken embryonic development during the first 12 days of incubation, direct comparisons were made at 12 days post-lay to unfertilized egg yolk (plasma fraction). Of the 127 proteins identified in chicken egg yolk, 69 proteins displayed changes in relative abundance among the study conditions. Following 12 days of incubation, Réhault-Godbert *et al.* [17] reported that 5 proteins displayed a major increase in relative abundance while 15 proteins showed a significant decrease in the yolks of the plasma fraction of fertilized chicken eggs.

As discussed earlier, a composition of egg yolk prepared from eggs that were maintained at room temperature prior to cracking and that were separated from whole egg within 24 hours post-lay has been utilized as a raw material to prepare products with diverse functions. These products have ranged from topical agents used to treat third degree burn injury to dietary supplements to enhance the growth and development of lean muscle mass such as Fortetropin® [1]. Although fertilized eggs are typically maintained at 37°C in hatcheries, the fertilized eggs that are used in the manufacturing process of Fortetropin® are typically maintained at room temperature for approximately 24 hours prior to cracking [18]. As there is precedent for using a fertilized egg yolk composition maintained at room temperature for approximately 24 hours prior to cracking [18] to manufacture dietary supplements, these conditions were mimicked as closely as possible in this study. By understanding how the protein composition of egg yolk varies with fertility status, one can utilize this knowledge to design egg yolk-based products with specific applications. The purpose of this study was to determine the differences at the proteomic level between fertilized chicken egg yolk (plasma and granular fractions) relative to unfertilized chicken egg yolk (plasma and granular fractions) at 24 hours post-lay while maintained at room temperature prior to cracking.

One anticipated challenge in this study was overcoming the large dynamic range of the chicken egg yolk proteome as the chicken egg yolk proteome is dominated by proteins such as serum albumin, vitellogenin cleavage products, apovitellenins, IgY and ovalbumin which are present in very high abundance relative to other chicken egg yolk proteins. In order to address this dynamic range problem, protein digests prepared from unfertilized and fertilized chicken egg yolk (plasma and granule fractions) were labeled with distinguishable isotopomer tags which were then subjected to two-dimensional fractionation prior to analysis by mass spectrometry

using high-pH reverse phase chromatography in the first dimension followed by low-pH reverse phase chromatography in the second dimension. Wang and co-workers [19] demonstrated that a high-pH / low-pH reverse phase chromatography-based workflow led to an improvement of ~60% in terms of protein identifications when characterizing human MCF10A cells relative to conventional workflows such as SCX / RP chromatography.

Using an RP-RP based workflow, 225 protein identifications established with 2 unique peptides, 95% peptide probability and 95% protein probability were made from chicken egg yolk. Within approximately 24 hours post-lay at room temperature conditions, proteins that are involved in promoting angiogenesis, defense against microbial pathogens and the development of the eggshell to serve as a protective barrier until embryonic development is complete increased in abundance in fertilized chicken egg yolk relative to unfertilized chicken egg yolk. Proteins that comprise the vitelline membrane decreased in abundance in fertilized egg yolk relative to unfertilized egg yolk. This manuscript will discuss how one can capitalize on knowledge related to the variation of protein egg yolk composition with fertility status to develop egg yolk-based products that are optimized for specific applications.

Materials and Methods

Egg yolk processing, tryptic digestion of egg yolk proteins and TMT duplex labeling of tryptic peptides:

Fertilized eggs and non-fertilized eggs that were laid on a specific day between 6:00 am to 4:00 pm were collected (Goffle Road Poultry Farm, Wyckoff, NJ) and then transported back to the laboratory for immediate processing. 6 fertilized eggs (sample ‘F6’) were manually cracked, the egg white was discarded and yolks were combined and then homogenized on ice for 2 minutes using a T10 Basic hand-held homogenizer (IKA Works, Inc., Wilmington, NC). The

same process was repeated using 6 unfertilized eggs (sample 'U6'). The fertility status of each egg was confirmed by measuring total DNA content using a Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA) based on the technique described by Johnson *et al.*[20]. 1 mL of 6 M Guanidine HCl was added to 50 mg of each sample (F6 and U6) and then placed on a rocking plate overnight while maintaining a temperature of 4^{o} C. Samples F6 and U6 were then reduced by the addition of 20 mM DTT and 30 mM Tris HCl at pH7.5 and incubated at 60^{o} C for 30 minutes. This step was followed by alkylation with 40 mM iodoacetamide at 20^{o} C for 1 hour in the dark. Subsequently, samples F6 and U6 were dialyzed against a 2 L volume of 2 M Guanadine HCl, 50 mM NH_4HCO_3 solution for 1.5 hours. This was followed by two dialysis steps against 2 L of 50 mM NH_4HCO_3 for 2 hours (each step). 5 mg of each sample (F6 and U6) were digested with trypsin at 1: 100 (w:w) at 37^{o} C overnight. Digested samples were centrifuged at 25,000 g for 10 minutes. Supernatants were filtered through a 10 K molecular weight cut-off (MWCO) spin filter and dried under vacuum. 1.25 mg aliquots of F6 and U6 (~280 µg tryptic peptides) were labeled with amine-reactive TMTduplex™ reagents (Thermo Fisher Scientific, Waltham, MA) in accordance with the manufacturer's instructions. Sample F6 was labeled with the TMT-126 reagent and sample U6 was labeled with the TMT-127 reagent. Both samples were combined in a 1:1 ratio and dried under vacuum.

<u>High-pH Reverse Phase HPLC Fractionation of TMT-Labeled Tryptic Peptides Derived from Fertilized and Unfertilized Egg Yolk:</u>

Fractionation of the TMT-labeled tryptic peptide mixture was performed using a Gilson 300 series HPLC system equipped with a Gilson UV/Vis-155 detector (Gilson, Inc., Middleton, WI). The tryptic digest was solubilized in 200 µL of 20 mM ammonium formate at pH 10 and injected onto a C18 XBridge column (3.5 µm, 2.1 X 150 mm) (Waters Corporation, Milford,

MA) using a linear gradient increasing at a rate of 1% B per minute from 2 - 45% mobile phase B (A: 20 mM ammonium formate at pH 10; B: 20 mM ammonium formate, 90% acetonitrile at pH 10) at a flow rate of 250 µL/min while monitoring a UV absorbance at 214 nm. Fractions were collected every minute during the course of the run. Based on the UV trace, 22 fractions were selected, dried under vacuum and analyzed by LC/MS (fractions with UV absorbance at 214 nm).

Liquid chromatography-mass spectrometry analysis:

Each of the 22 fractions described in the previous section was analyzed in duplicate by nano LC-MS/MS using a Dionex™ UltiMate 3000 RSLCnano system (Thermo Scientific, Sunnyvale, CA) interfaced to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher, San Jose, CA). Samples were loaded onto a self-packed 100 µm x 2 cm trap column packed with Magic C18AQ, 5 µm 200 A^o particles (Michrom Bioresources Inc, Auburn, CA) and washed with Buffer A (0.2% formic acid, water) for 5 minutes at a flow rate of 10 µL/min. After the wash, the trap was brought in-line with an analytical column (Magic C18AQ, 3 µm 200 A^o, 75 µm x 50 cm) and peptides were fractionated at 300 nL/min using the following multi-step gradient: 4 to 15% Buffer B (0.16% formic acid, 80% acetonitrile) over 35 minutes, 15 to 25% B over 65 minutes and 25 to 50% B over 55 minutes). LC/MS/MS data was acquired using a data-dependent acquisition (DDA) method. One cycle was comprised of a full scan acquired using the Orbitrap mass analyzer with a resolution of 60,000 followed by MS/MS acquisition using higher-energy collision dissociation (HCD) fragmentation applied to the 10 most intense ions observed in the full scan event. A collision energy (CE) threshold of 38%, repeat count of '1' and a dynamic exclusion duration of 60 seconds were specified.

Bioinformatics:

Each of the 44 .RAW files generated from a single LC/MS experiment was searched against a *Gallus gallus* protein FASTA database (GCF_000002315.3_Gallus_gallus-4.0_protein.fasta) downloaded from ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF_000002315.3_Gallus_gallus-4.0/GCF_000002315.3_Gallus_gallus-4.0_protein.faa.gz using the Sorcerer PE search engine, version 5.1.1 implemented on a Sorcerer 2 workstation (Sage-N Research, Milpitas, CA). Trypsin was specified as the protease and two missed cleavages of trypsin were permitted. The following static modifications were specified: Lysyl TMT2plex (225.155838 amu) and N-terminal TMT2plex (225.155838 amu). The following dynamic modifications were specified: Carbamidomethylation of Cysteine (+57.021465 amu) and Oxidation of Methionine (+15.99492 amu). In addition, a precursor ion tolerance of 50 ppm was specified.

TMT quantitation and statistical analysis were performed using Scaffold Q+S, version 4.4.1 (Proteome Software, Portland, OR). Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm [21] with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [22]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Channels were corrected by the matrices [0.000,0.000,0.900,0.0960,0.00400] and [0.000,0.00900,0.898,0.0890,0.00400] in all samples according to the algorithm described in i-Tracker [23]. Using the 'Intensity-Based Normalization' scheme within Scaffold Q+S software,

normalization was performed at the spectra level, results from the spectra level were carried forward to the peptide level and then from the peptide level to the protein level using median values. The normalization scheme that was applied to the raw data is based on the normalization model developed by Oberg *et al.* [24]. Acquired intensities in the experiment were globally normalized across all acquisition runs. Individual quantitative samples were normalized within each acquisition run. Acquired intensities in the experiment were globally normalized across all acquisition runs. Intensities for each peptide identification were normalized within the assigned protein. The reference channels were normalized to produce a 1:1 fold change. All normalization calculations were performed using medians to multiplicatively normalize data. When calculating the average coefficient of variance associated with each protein fold-change, only unique peptides (after grouping peptide-spectrum matches) derived from that protein across both technical replicates were used to perform the calculation.

Results and Discussion

In order to understand the early changes that take place in the hen egg yolk upon fertilization within the first 24 hours post-lay in the absence of incubation, we collected unfertilized and fertilized chicken eggs and processed them within 24 hours post-lay. Egg yolks were manually separated from egg whites and the fertility status of each was confirmed by measuring total DNA content [20]. After confirming fertility status, 6 egg yolks were pooled together from fertilized eggs (sample ‘F6’) and homogenized. The same process was repeated using 6 unfertilized egg yolks (sample ‘U6’). The proteins in each egg yolk sample were digested with trypsin to give rise to tryptic peptides. Equal quantities of each tryptic peptide digest derived from fertilized and unfertilized hen egg yolk were chemically labeled using isotopomer labels, known as tandem mass tag (TMT) technology [14]. Each tandem mass tag

was carefully designed to ensure that an identical peptide labeled with a different isotopomer will co-migrate exactly in all downstream chromatographic separations.

If a given peptide X is present in the tryptic digest derived from fertilized egg yolk proteins, the peptide would be labeled with the tag, TMT-126. Any peptide labeled with TMT-126 will give rise to a characteristic reporter ion at *m/z* = 126 Da when it is subjected to fragmentation in the collision cell of the mass spectrometer. On the other hand, if peptide X is present in the tryptic digest derived from unfertilized egg yolk protein, the peptide would be labeled with the tag, TMT-127. Any peptide labeled with TMT-127 will give rise to a characteristic reporter ion at *m/z* = 127 Da when it is subjected to fragmentation in the collision cell of the mass spectrometer. If protein digests derived from fertilized and unfertilized egg yolk proteins are combined in a 1:1 ratio, one can determine the fold change associated with peptide X in the fertilized state relative to the unfertilized state by computing the peak area ratio of TMT-126/TMT-127 in the peptide fragmentation spectrum of peptide X. If the amino acid sequence of peptide X (deciphered from its peptide fragmentation spectrum using bioinformatics tools) is uniquely associated with protein X', a measurement of the aforementioned TMT-126/TMT-127 peak area ratio can be used to directly measure the fold change associated with protein X' in the fertilized state relative to the unfertilized state. When multiple tryptic peptides can be mapped to a given protein, the confidence associated with the measurement of the relative expression of a specific protein in one biological condition with respect to another biological condition generally improves. Therefore, measurements pertaining to the relative expression of a specific protein based on the observation of only a single peptide are typically less reliable and hence were not considered in this study.

In order to decipher changes in the expression of proteins present at low concentrations relative to highly abundant proteins, we fractionated each egg yolk protein digest sample (fertile and unfertile) by applying high-pH reverse phase chromatography as the first mode of separation followed by low-pH reverse phase chromatography as the second mode of separation prior to mass spectrometry analysis. Using this two-dimensional peptide separation scheme, 225 protein identifications based on 2 unique peptides, a peptide identification probability of >95% and a protein identification probability of >95% were made (Figure 1). Considering protein identifications based on 1 unique peptide, a peptide identification probability of >95%, and a protein identification probability of >95%, 471 protein identifications can be confidently assigned from this dataset. It is clear from Figure 1 that the majority of these proteins do not significantly change in terms of their expression in fertilized egg yolk relative to unfertilized egg yolk after 24 hours in the absence of incubation (maintained at room temperature). Most of the proteins that were differentially expressed in fertilized egg yolk relative to unfertilized egg yolk had a $\log_2$ fold change between -1 and 1, corresponding to fold changes between 0.5 and 2, respectively (Figure 1).

9 proteins were observed to increase in abundance in fertilized egg yolk relative to unfertilized egg yolk and 9 proteins were observed to decrease in abundance in fertilized egg yolk relative to unfertilized egg yolk when considering protein identifications using the more stringent criteria of a minimum of 2 unique peptides, >95% peptide probability and >95% protein probability. Proteins that increased in abundance in fertilized egg yolk relative to unfertilized egg yolk were involved in functions such as angiogenesis, defense against microorganisms and the promotion of egg shell formation (Table 1). Proteins that decreased in abundance in the fertilized state relative to the unfertilized state included mucins which form

fibers that comprise the vitelline membrane and the chalaza (Table 2). In the following sections, we will discuss the biological functions of proteins that changed in abundance in fertile egg yolk relative to unfertile egg yolk in detail.

Increased Abundance of Pro-angiogenic Factors in Fertile Egg Yolk after 24 Hours Post-Lay at Room Temperature (20^{o}C):

In this study, it was observed that pleiotrophin, a secreted 18 kDa heparin-binding cytokine that stimulates angiogenesis through multiple mechanisms [26] increased ~3.2 fold in abundance in fertilized egg yolk relative to unfertilized egg yolk. In order to compute this result, the relative changes associated with two unique tryptic peptides derived from pleiotrophin, ALHNADCQK and YQFQAWGECDLNTALK identified in the LC-MS/MS runs were measured (Figure 2). The peptide fragmentation MS2 spectra for ALHNADCQK (Figure 3A) and YQFQAWGECDLNTALK (Figure 3B) were assigned to their respective sequences by searching the NCBI *Gallus gallus* database using the Sorcerer PE search engine after specifying the protease and appropriate post-translational modifications indicated in the 'Bioinformatics' sub-section of the 'Materials and Methods' section. Each representative MS2 spectrum in Figure 3 contains a pair of ions with m/z = 126, 127 Da. It is clear from Figure 3 that the normalized intensity ratio of TMT-126 / TMT-127 in the representative MS2 spectrum for ALHNADCQK (Figure 3A) is approximately equal to that of the TMT-126 / TMT-127 normalized intensity ratio in the representative MS2 spectrum for YQFQAWGECDLNTALK (Figure 3B). When the expression of ALHNADCQK and YQFQAWGECDLNTALK in the fertile state relative to the unfertile state across all LC/MS runs was measured, a high correlation was observed between all observations of the relative expression of pleiotrophin in the fertile state relative to the unfertile state (Figure 4). The coefficient of variation for these measurements was only 13%, giving high confidence in the significance of differential expression. While an ~3.2 fold increase in

abundance of pleiotrophin in the fertile state relative to the unfertile state of egg yolk was observed, highly abundant proteins such as apolipoprotein B, ovotransferrin, serum albumin and vitellogenin-2 displayed very little change in expression as a function of fertility state (Figure 5). Previous mass spectrometry-based proteomic studies by D'Ambrosio *et al.* [12] and Mann *et al.* [13] have demonstrated that pleiotrophin has also been identified in the egg white. On the other hand, previous proteomic studies [7,8] did not report the identification of pleiotrophin in egg yolk (Table 1). As the newly fertilized embryo begins to develop on the vitelline membrane, the aforementioned results suggest that pleiotrophin may flow from the egg white to the egg yolk, stimulating angiogenesis in the developing chick embryo.

An important assay to assess the angiogenic or anti-angiogenic properties of a therapeutic compound is the chick chorioallantoic membrane (CAM) assay, an assay that capitalizes on the angiogenic events that take place within a fertilized chicken egg. When the CAM assay is performed to study angiogenesis, the fertilized chicken egg is typically maintained at 37^{o} C in an incubator with 60% relative humidity [27] to promote angiogenesis. Even though the temperature (20^{o} C) and relative humidity parameters employed in this study were much less favorable than those employed in the CAM assay and samples were collected after only 24 hours post-lay, the ~3.2 fold increase in abundance of pleiotrophin observed in fertilized egg yolk relative to unfertilized egg yolk is clear evidence that angiogenic events are underway relatively early. Pleiotrophin exerts its action through several cell surface receptors, such as N-syndecan, anaplastic lymphoma kinase (ALK), receptor protein tyrosine phosphatase beta/zeta (RPTPβ/ζ) and $\alpha_v\beta_3$ integrin [28]. Interestingly, Réhault-Godbert and co-workers [17] did not observe the upregulation of pleiotrophin in fertilized chicken egg yolk relative to unfertilized chicken egg yolk in their study that focused on day 12. By day 12, it is likely that angiogenesis in the

fertilized chicken egg is quite advanced. Therefore, it seems plausible that pleiotrophin is not actively secreted at day 12 relative to day 1, the focus of this study. Further, the study by Réhault-Godbert *et. al.* [17] focused on only the plasma fraction of egg yolk and this study focused on both the plasma and granule fraction which could also account for differences related to pleiotrophin expression.

Histidine-rich glycoprotein (HRG) was found to increase ~1.6 fold in abundance in fertilized egg yolk in relation to unfertilized egg yolk (Figure 6A, Table 1). HRG along with peptides that are derived from the histidine rich region (HRR) of HRG have been reported to display both pro-angiogenic and anti-angiogenic properties depending on the experimental system studied as reviewed by Poon *et. al.* [29]. It is believed that the pro-angiogenic mechanism associated with HRG is underpinned by the strong interaction that HRG forms with thrombospondin-1 (TSP), a known inhibitor of angiogenesis. On the other hand, HRG was shown to inhibit angiogenesis through modulating various signaling events that are important for endothelial cell survival, proliferation, and cell migration [29]. The pro- and anti-angiogenic role of HRG suggests that it could act in concert with pleiotrophin to promote angiogenesis shortly after fertilization when many angiogenic events are taking place and then later act as an inhibitor of angiogenesis several days post-lay when angiogenesis is near complete. Analysis of the concentration of pleiotrophin and HRG at time-points beyond the time-scale of this study, particularly at time-points near the completion of angiogenesis may provide insight into whether HRG plays the role of an inhibitor of this process at much later time-points. If the concentration of HRG increases while the concentration of pleiotrophin decreases as angiogenesis has neared completion, one could hypothesize that the role of HRG has shifted from promoting to inhibiting angiogenesis.

Increased Abundance of Anti-Microbial Factors in Fertile Egg Yolk after 24 Hours Post-Lay at Room Temperature (20^{o} C):

In fertilized chicken egg yolk, a 2-fold increase in abundance of Gallinacin-11, also known as avian β-defensin-11 (AvBD11) with respect to unfertilized chicken egg yolk was observed within 24 hours post-lay (Figure 6B, Table 1). AvBDs are small, cationic non-glycosylated peptides (1 to 9 kDa). In mammals, β-defensins are molecules with functions in both innate and adaptive immunity, possessing a broad spectrum of anti-microbial properties [30]. Previous proteomic studies have identified Gallinacin-11 in the egg white [12,13] and the vitelline membrane [25]. The results suggest that a subtle flow of Gallinacin-11 from the egg white and vitelline membrane to the egg yolk may be taking place shortly after fertilization. As degradation of the vitelline membrane takes place following fertilization, it is possible that quantities of Gallinacin-11 are deposited from the vitelline membrane into the egg yolk. It is also possible that the increased abundance of AvBD11 observed in this study occurs in order to help defend the fertilized egg yolk against pathogens during the critical period in which it develops into an embryo. Experiments performed on fertilized egg yolk at 37^{o}C and at time-points beyond 24 hours would help further shed some light into the role of Gallinacin-11 in fertilized egg yolk.

A 1.5-fold increase in soluble mannose-binding lectin (MBL) in fertilized egg yolk with respect to unfertilized egg yolk was observed (Figure 6C, Table 1). MBL plays a vital role in the innate immune system, the body's first line of defense against invasion by microbes. Unlike responses from the adaptive immune system that rely upon clonal expansion of cells that emerge days after challenge from an antigen, the innate immune response occurs within minutes to hours following a challenge. Levels of MBL in humans have been shown to increase to ~140% during pregnancy [31]. It has been suggested that in humans, adaptive immunity declines during

pregnancy which is necessary in order for the body to tolerate the fetus as a semi-allograft [31]. It was suggested by van de Geijn *et. al.* [31] that increased levels of MBL during pregnancy may represent a shift from adaptive to innate immunity. In humans, higher levels of MBL are associated with lower disease activity and a more favorable disease outcome for autoimmune diseases such as rheumatoid arthritis and multiple sclerosis [32]. Because these diseases tend to improve during pregnancy, it has been previously speculated that increased levels of MBL may play a role in pregnancy-related amelioration of these diseases [31].

In addition to MBL, another protein that plays an important role in innate immunity is serum amyloid P-component (SAP) (Figure 6D, Table 1). In this study, an increase in abundance of ~1.6-fold in levels of serum amyloid P-component in fertilized egg yolk within 24 hours post-lay with respect to levels in unfertilized egg yolk was observed. SAP binds to bacteria, including the human pathogens *Streptococcus pyogenes* [33] and *Klebsiella rhinoscleromatis* [34], which suggests that it may play a possible role in host defense. Armstrong *et al.* [35] demonstrated in a rodent model that human SAP provides protection against the pathogen, *Escherichia coli* O157:H7 Shiga Toxin 2. Interestingly, chickens and hen eggs serve as vehicles for *Escherichia coli* O157:H7 [36].

Further, this investigation revealed an ~1.5-fold increase in abundance of the *Gallus gallus* polymeric immunoglobulin receptor (GG-pIgR) in fertilized chicken egg yolk relative to unfertilized chicken egg yolk within 24 hours post-lay (Table 1). Previous proteomic studies have identified GG-pIgR in the egg white [12,13] and the vitelline membrane [25]. As the vitelline membrane begins to degrade shortly after fertilization, it is possible that some deposits of GG-pIGR from the vitelline membrane may accumulate in the egg yolk. GG-pIgR plays an important role in mucosal immunity through its association with dimeric IgA (dIgA) to form a

complex, dIgA-pIgR which is cleaved to give rise to secretory IgA (sIgA) [37]. Wieland *et. al.* [38] characterized GG-pIGR and asserted that the presence of this receptor in chicken indicates that secretory Igs may have a prominent role in the first line of defense in chicken and perhaps other non-mammalian species. The ~1.5-fold increase in abundance of GG-pIGR in fertilized egg yolk relative to unfertilized egg yolk may warrant further investigating the role that secretory Igs play in the first line of defense against pathogens to protect the developing embryo within the chicken egg. However, such an investigation would require examining time-points beyond the first 24 hours post-lay.

An increased abundance of ~1.5-fold of ovostatin in fertilized chicken egg yolk relative to unfertilized chicken egg yolk within 24 hours post-lay was also observed in this study (Table 1). Ovostatin is a protease inhibitor that inhibits many pathogenic proteases. Examples of pathogen proteases that ovostatin can inhibit are 64K and 56K metalloproteases and cysteine protease 70K that is produced by *Serratia marcescens*, elastase expressed by *Pseudomonas aeuroginosa* or alkaline protease from *Bacillus stearothermophilus* [39-40]. The increased abundance of ovostatin in fertilized egg yolk may provide protection against pathogenic proteases to the developing embryo within the fertilized egg. Previous proteomic studies have identified ovostatin in the egg white [12,13], egg yolk [7] and the vitelline membrane [25]. Upon fertilization as the vitelline membrane begins to degrade, quantities of ovostatin may flow from deposits of the vitelline membrane and egg white and accumulate to some degree in the egg yolk.

Increased Abundance of Proteins that Promote Egg Shell Formation in Fertile Egg Yolk after 24 Hours Post-Lay at Room Temperature (20° C):

An increased abundance of ~1.5-fold of ovocalyxin-32 in fertilized egg yolk with respect to unfertilized egg yolk after 24 hours post-lay was observed in this study (Table 1). Ovocalyxin-32 is a matrix protein found in the outer layers of the eggshell [41]. The chicken egg shell is the primary barrier against microbial infection, protecting the developing embryo from external pathogens. Previous proteomic studies have also identified ovocalyxin-32 in the egg white [12, 13] and the vitelline membrane [24]. As there is an increased abundance of ovocalyxin-32 in fertilized egg yolk relative to unfertilized egg yolk, it is possible that there may be a subtle flow of ovocalyxin-32 from the egg white and vitelline membrane, particularly as the membrane degrades following fertilization into the egg yolk.

Decreased Abundance of Proteins that Comprise the Vitelline Membrane in Fertile Egg Yolk after 24 Hours Post-Lay at Room Temperature (20° C):

The vitelline membrane forms an important barrier between the egg yolk and the egg white and is comprised of mucin fibers [42]. Upon fertilization, the vitelline membrane begins to degrade as the embryo begins to develop on its surface. A ~0.6 fold decrease in abundance of two mucin proteins (mucin-5B and mucin-6) in fertilized egg yolk relative to unfertilized egg yolk after 24 hours post-lay was observed (Figures 7C and 7D, Table 2). Previous proteomic studies have identified mucin-5B in the egg white [13] and vitelline membrane [25]. As the vitelline membrane degrades upon fertilization, some of the mucin proteins that previously comprised the vitelline membrane could be deposited into the egg white compartment. Hence, such a process would account for the decreased abundance of mucin-5B and mucin-6 in fertile egg yolk relative to unfertile egg yolk as all of the experiments involving egg yolk in this study included both the egg yolk and vitelline membrane. Other examples of proteins that were found

to decrease in abundance in fertile egg yolk relative to unfertile egg yolk were alpha-2-HS glycoprotein (Figure 7A), apolipoprotein C-III (Figure 7B) and apolipoprotein A-I preproprotein (Table 2).

Conclusions

A decrease in abundance of mucin-5B and mucin-6 in fertilized egg yolk relative to unfertilized egg yolk was observed in this study; mucin fibers comprise the vitelline membrane which degrades upon fertilization as the embryo begins to develop on its surface. Proteins that increased in abundance between ~1.5 to ~3.2 fold in fertilized egg yolk relative to unfertilized egg yolk at 24 hours post-lay while being maintained at room temperature have been observed in this study. Some of the proteins that increased in abundance in fertilized egg yolk relative to unfertilized egg play a role in angiogenesis (pleiotrophin, histidine-rich glycoprotein), defending the developing embryo against microbial pathogens (avian β-defensin 11, polymeric immunoglobulin receptor, serum amyloid P-component, ovostatin and mannose-binding ligand) and augmenting the structural integrity of the egg shell (ovocalyxin-32), necessary to provide a strong barrier against microbial infection. Angiogenesis is a very important process that must take place during wound healing. Protection against microbial infection is of paramount importance during all phases of wound healing. A topical agent to promote wound healing prepared from fertilized egg yolk would likely be enriched with proteins that promote angiogenesis and play a role in defense against microbial pathogens relative to a topical agent prepared from unfertilized egg yolk.

In the future, it would be very interesting to understand the temporal distribution of the identified proteins at 24 hours post-lay, at earlier and later time points, and at commonly used

temperatures, such as 4 ºC, 20 ºC and 37 ºC. We propose a multiplexed and orthogonal LC-MS/MS method, multiple reaction monitoring (MRM) [43] to build a panel of proteins comprising those described to be changing in this study as well as in related studies [17]. This will allow us to conduct more exact and highly quantitative studies designed to follow different post-lay time points and storage temperatures in order to find the optimum conditions for proteins relevant to angiogenesis, defense against microbial pathogens and the development of muscle tissue. Such a study may lead to further optimization of egg yolk-based products designed for specific applications.

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Figure Captions

Figure 1 - For each protein identification from the TMT-labeled chicken egg yolk sample, the degree of differential expression of a protein in the fertile state with respect to the unfertile state (if any) was determined by computing the average TMT-126/TMT-127 ratio associated with each tryptic peptide MS2 fragmentation spectrum matched to a particular protein identification and then expressing this ratio as a $\log_2$ fold change. Tryptic peptides derived from fertile and unfertile egg yolk were labeled with TMT-126 and TMT-127, respectively.

Figure 2 - The amino acid (a.a.) sequence of the pro-angiogenic factor, pleiotrophin. The two highlighted a.a. peptide sequences, ALHNADCQK and YQFQAWGECDLNTALK correspond to the two peptide identifications made from the TMT-labeled mixture of egg yolk (fertile and unfertile) that were used to identify pleiotrophin and to quantify the expression of pleiotrophin in the fertile state with respect to the unfertile state.

Figure 3 - (A) Peptide MS2 fragmentation spectrum of the pleiotrophin-derived tryptic peptide, ALHNADCQK. TMT-126 and TMT-127 reporter ions used for quantitation are displayed in the inset. (B) Peptide MS2 fragmentation spectrum of the pleiotrophin-derived tryptic peptide, YQFQAWGECDLNTALK. TMT-126 and TMT-127 reporter ions used for quantitation are displayed in the inset.

Figure 4 – Log_2 fold change of the peptides (A) ALHNADCQK and (B) YQFQAWGECDLNTALK as a function of fertility status. Log_2 fold of the protein, (C) pleiotrophin as a function of fertility status.

Figure 5 – Log_2 fold change of some of the most abundant proteins in egg yolk as a function of fertility status; (A) Apolipoprotein-B, (B) Ovotransferrin, (C) Serum albumin and (D) Vitellogenin-2.

Figure 6 - Log_2 fold change of some proteins in egg yolk that were upregulated in fertile egg yolk relative to unfertile egg yolk; (A) Histidine-rich Glycoprotein, (B) Gallinacin-11, (C) Mannose-Binding Lectin and (D) Serum Amyloid P-Component.

Figure 7 - Log_2 fold change of some proteins in egg yolk that were downregulated in fertile egg yolk relative to unfertile egg yolk; (A) Alpha-2-HS-Glycoprotein, (B) Apolipoprotein C-III, (C) Mucin-5B and (D) Mucin-6..

Figure 1

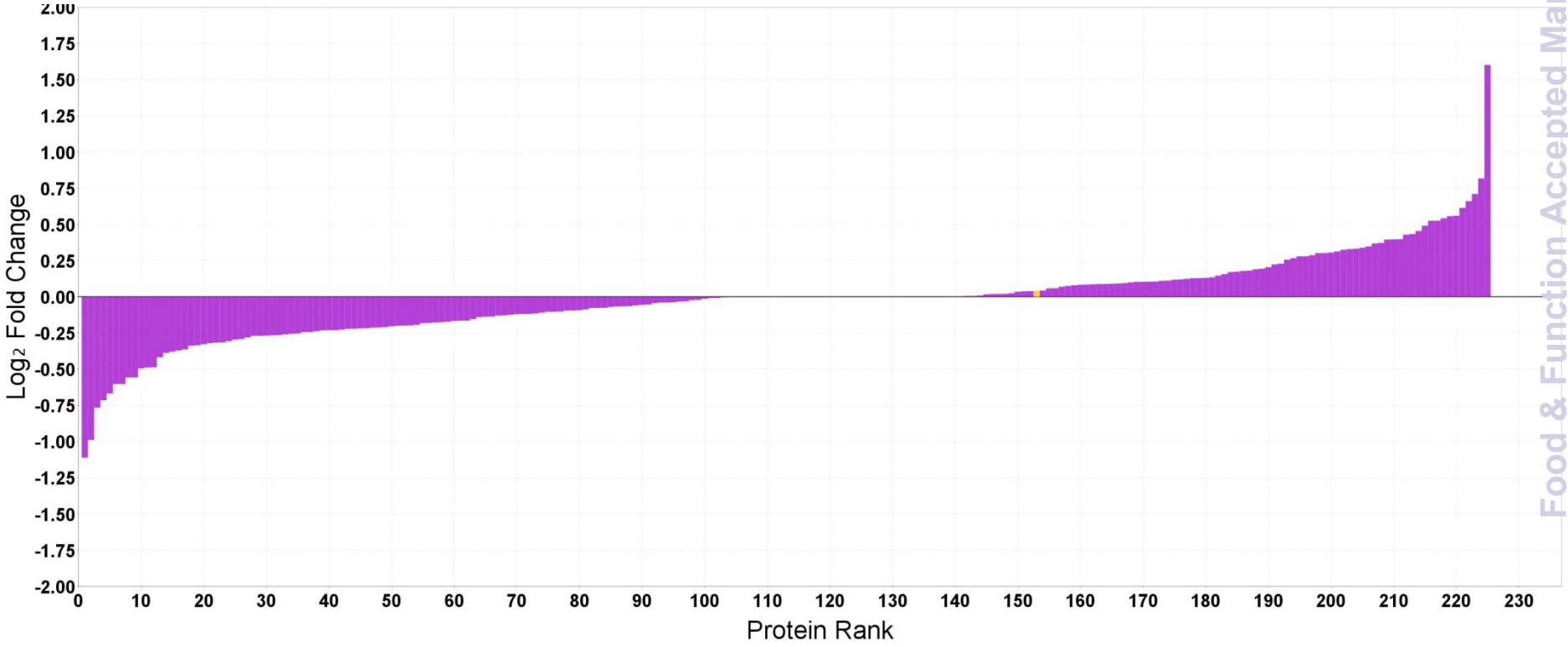

Figure 2

NP_001263291.1 (100%) 18,512.6 Da
pleiotrophin precursor [Gallus gallus]

MPQQQQQRRM FTAALLALVF ILAAVSTTEA GKKEKPEKKA KKSDCGEWQW
SVCVPTNGDC GLGTREGTRT GAECKQTTKT QKCKIPCNWK KQFGAECKYQ
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Figure 3

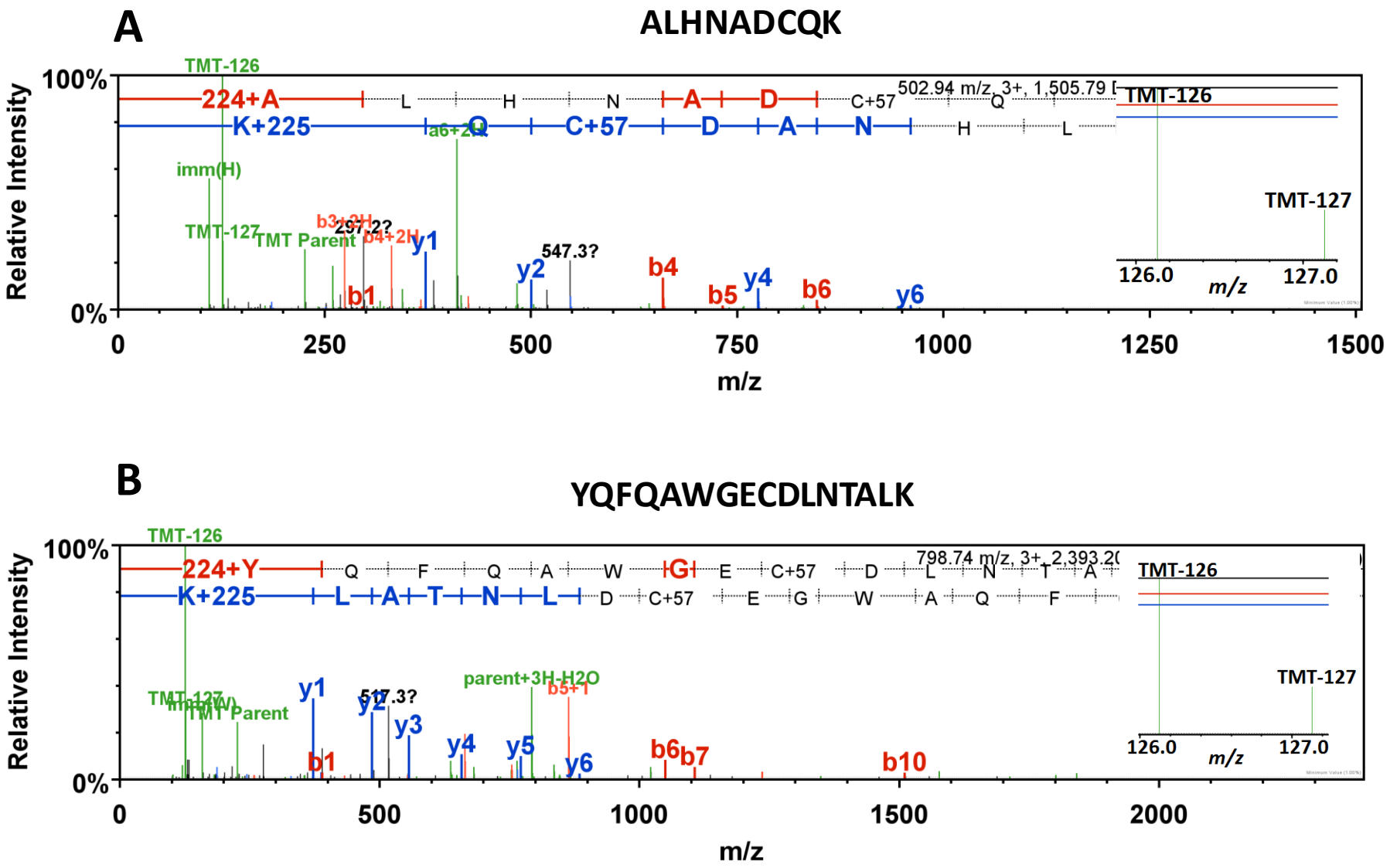
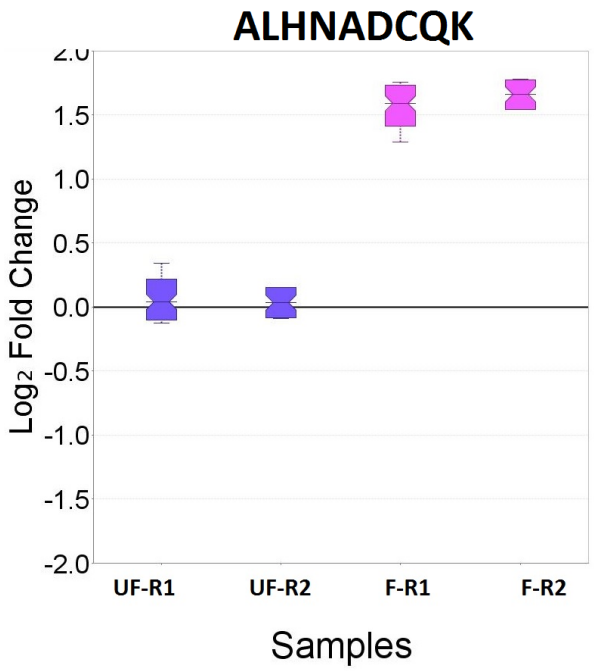


Figure 4



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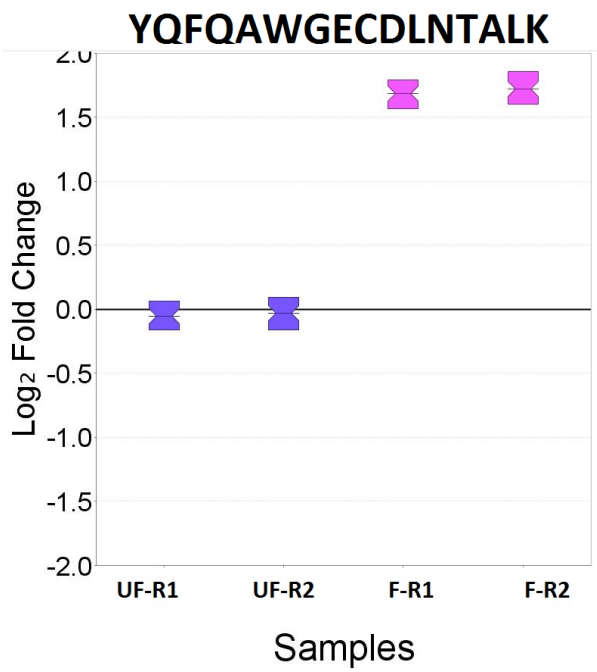

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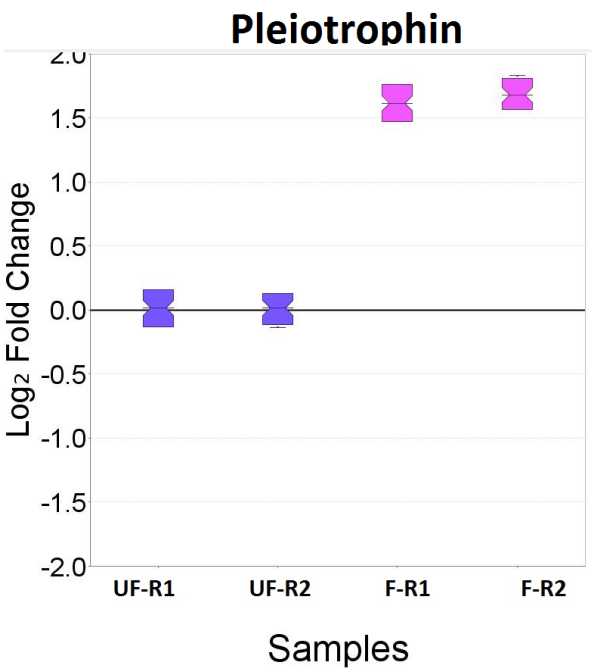


C

Figure 5

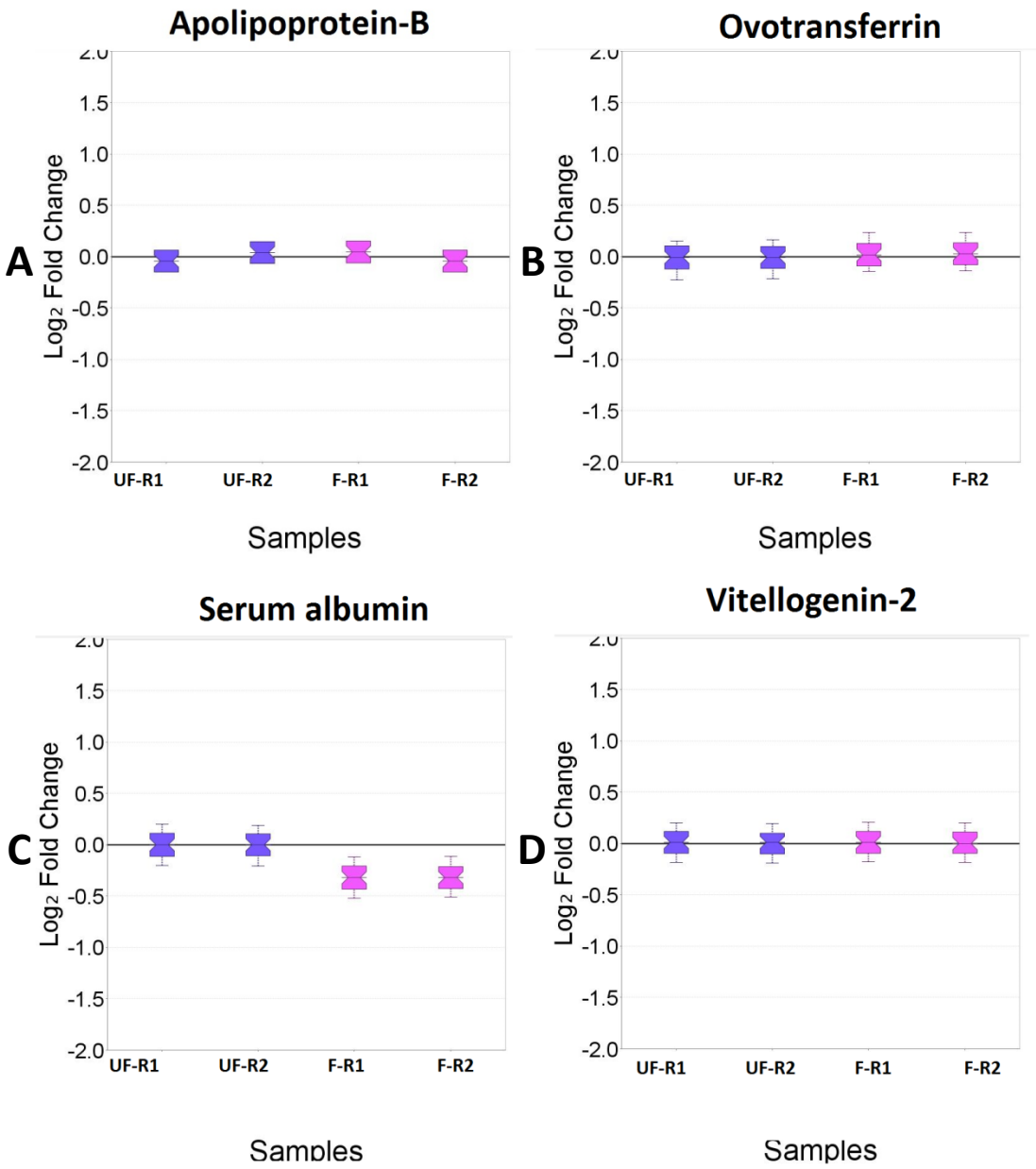
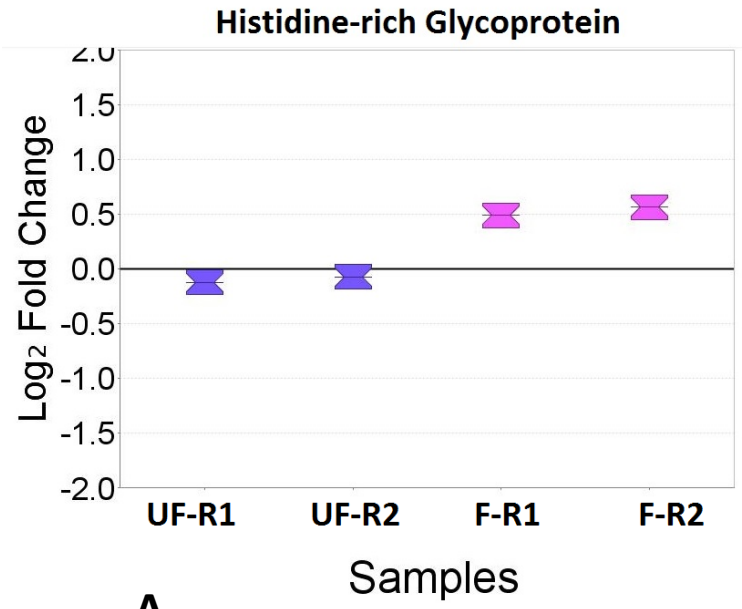
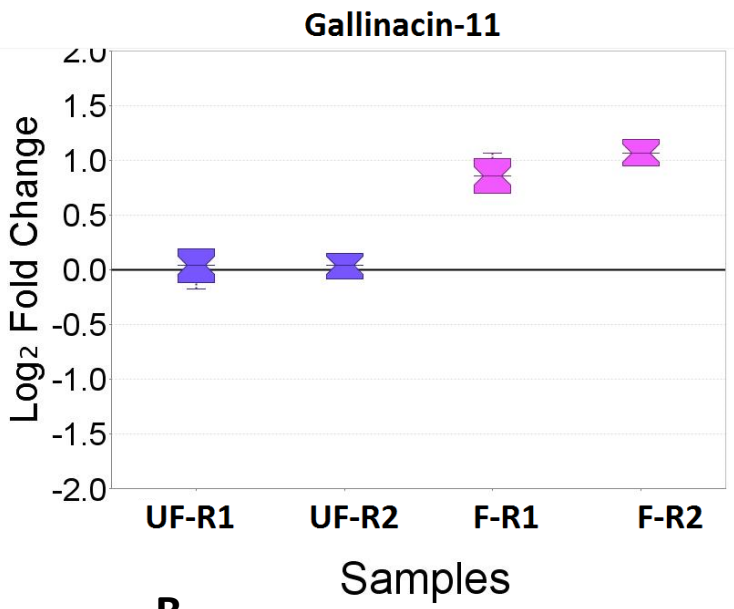
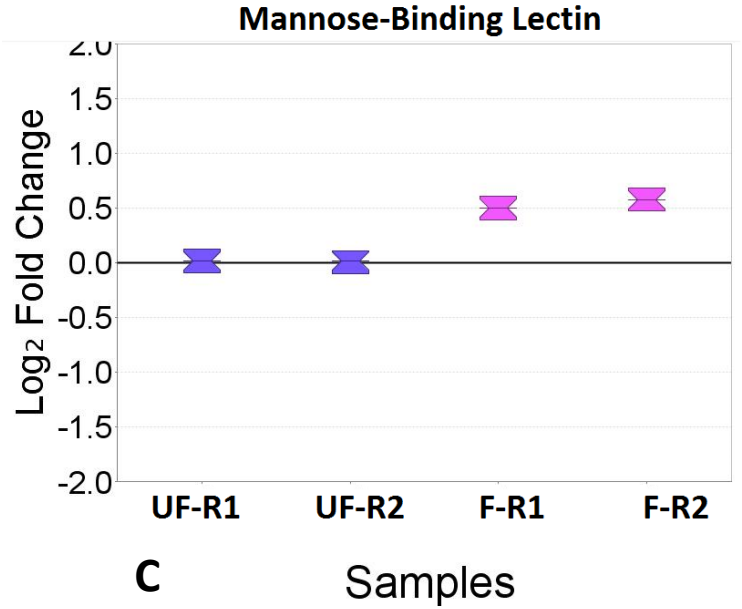


Figure 6







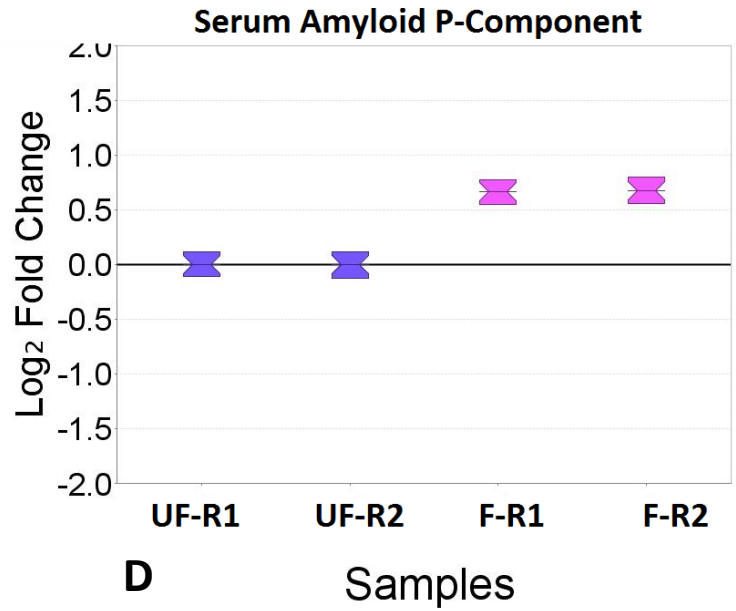


Figure 7

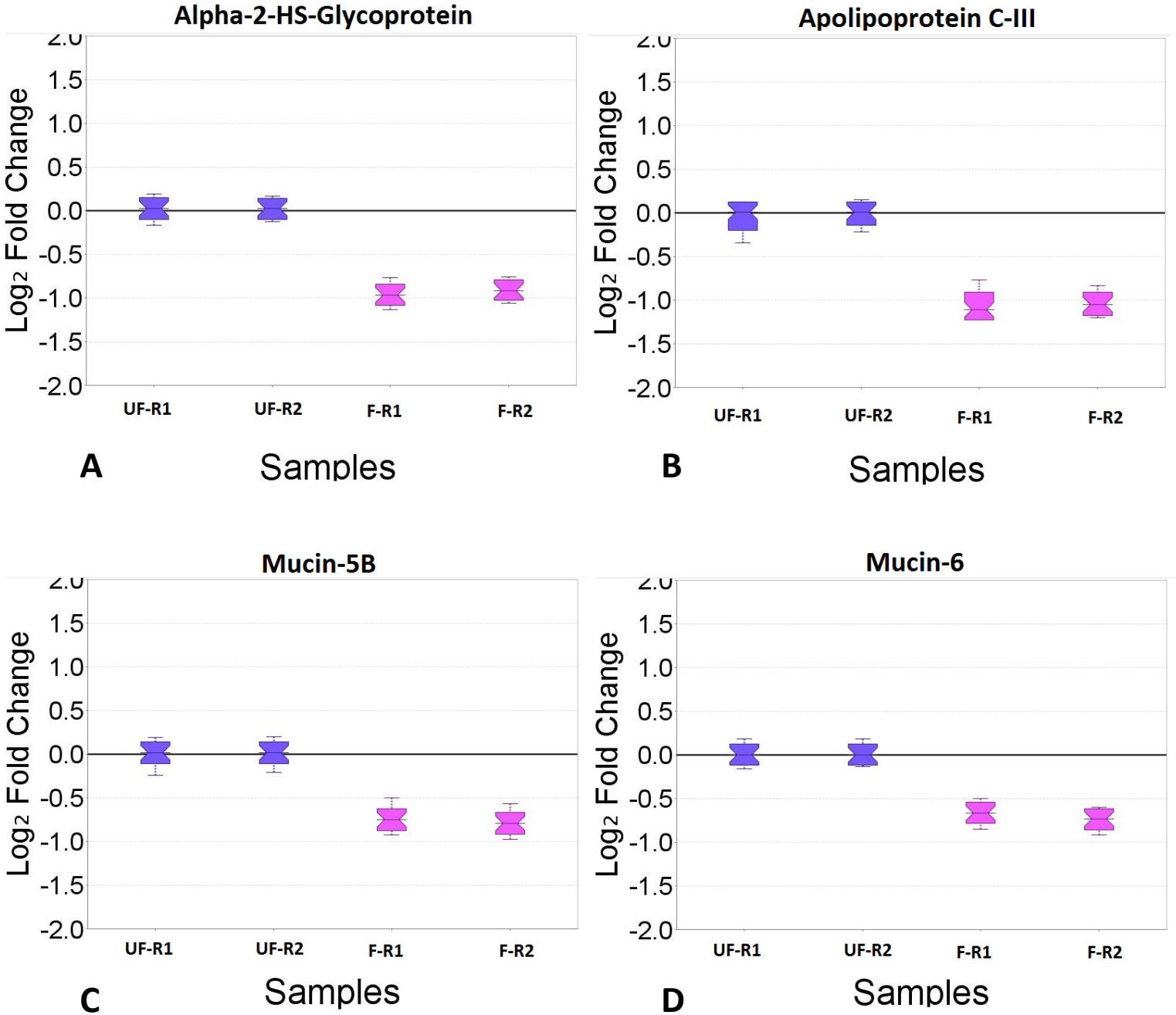


Table 1: Proteins that Increased in Abundance after 24 Hours in Fertilized Chicken Egg Yolk Relative to Unfertilized Chicken Egg Yolk at 20° C

Protein	NCBI Accession Number	Molecular Weight	Fold Change	Average %CV	Mann-Whitney Test (p-value)	Localization of Protein Within Egg Based on Previous Studies
Pleiotrophin precursor	NP_001263291.1	19 kDa	3.2	13%	0.021	Egg White [12,13]
Gallinacin-11 precursor	NP_001001779.1	12 kDa	2.0	13%	0.009	Egg White [11,12,13] Vitelline Membrane [25]
Histidine-rich glycoprotein	XP_001233925.2	80 kDa	1.6	16%	0.004	
Polymeric immunoglobulin receptor precursor	NP_001038109.1	71 kDa	1.6	18%	0.0013	Egg White [11,12,13] Vitelline Membrane [25]
Serum amyloid P-component isoform X2	XP_004948360.1	35 kDa	1.6	13%	0.021	
α-2-Macroglobulin	XP_004938162.1	162 kDa	1.5	12%	<0.0001	Egg White [11,13] Egg Yolk [7,8]
Ovostatin precursor	NP_990557.1	164 kDa	1.5	12%	<0.0001	Egg White [11,12,13] Egg Yolk [7] Vitelline Membrane [25]
Ovocalyxin-32 precursor	NP_989865.1	31 kDa	1.5	11%	0.009	Egg White [12,13] Vitelline Membrane [25]
Soluble mannose binding-lectin precursor	NP_989680.2	27 kDa	1.5	11%	0.021	

Table 2: Proteins that Decreased in Abundance after 24 Hours in Fertilized Chicken Egg Yolk Relative to Unfertilized Chicken Egg Yolk at 20° C

Protein	NCBI Accession Number	Molecular Weight	Fold Change	Average %CV	Mann-Whitney Test (p-value)	Localization of Protein Within Egg Based on Previous Studies
α-2-HS-glycoprotein	XP_422764.1	37 kDa	0.5	11%	0.0039	Egg Yolk [7]
Apolipoprotein C-III	XP_004948134.1	15 kDa	0.5	15%	0.0039	Egg Yolk [7]
Dickkopf-related protein 3 precursor	NP_990456.1	39 kDa	0.6	12%	0.00016	Egg White [11,12,13] Vitelline Membrane [25]
Mucin-5B isoform X1 precursor	XP_003641415.1	234 kDa	0.6	14%	<0.0001	Egg White [13] Vitelline Membrane [25]
Mucin-6	XP_426405.4	291 kDa	0.6	13%	<0.0001	
Apolipoprotein A-I preproprotein	NP_990856.1	31 kDa	0.7	11%	<0.0001	Egg White [11,12,13] Egg Yolk [7,8] Vitelline Membrane [25]
Avidin-like isoform X2	XP_429212.2	16 kDa	0.7	11%	0.12	Egg White [11,12,13] Egg Yolk [7,8] Vitelline Membrane [25]
C4b-binding protein α chain precursor	NP_001028814.1	79 kDa	0.7	12%	0.021	
Pantetheinase precursor	NP_001034377.1	55 kDa	0.7	12%	<0.0001	Egg Yolk [7]