# Differential proteomics between unhatched male and female egg yolks reveal the molecular mechanisms of sex-allocation and sex-determination in chicken

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**ABSTRACT** There is a huge demand to identify the sex of unhatched fertilized eggs for laying industry and to understand the differences between male and female eggs as early as possible. Then the molecular mechanisms of sex determination and sex allocation in chicken were revealed. Therefore, TMT proteomic was applied to characterize the variation of molecular matrix between unhatched male and female egg yolks. A total of 411 proteins were identified and 35 differentially expressed proteins (**DEPs**), including 375332005, 015809562, 763550308 (upregulated, **UPs**) and 1337178851, 89000557, 89000581 (downregulated, **DPs**), etc. were confirmed between them. Gene ontology analyses showed that DEPs were mainly involved in response to stimulus, distributed in the extracellular region and participated in binding; KEGG analyses showed that few DPs were participated in cell growth and death, transport and catabolism, signaling molecules, interaction and were enriched in ubiquitin mediated proteolysis, endocytosis, ferroptosis, etc. metabolic pathways. Moreover, most of the DEPs and related metabolic pathways were associated with sex hormones. More importantly, this study supports maternal sex-allocation theory and extends our understanding of the molecular mechanism of sex determination and differentiation in avian. Which also provides a powerful evidence for ovo sexing of unhatched fertilized domestic chicken eggs by nondestructive approach and will be of great significance to eggs processing and production.

**Key words:** fertilized egg yolks, TMT proteomics, male, female, sex-allocation

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## INTRODUCTION

Various techniques or approaches have been used to detect the gender of avian eggs during early of incubation over the past few decades. For example, the feather color of chicks, hormone levels of embryo's allantoic ($E_{9\text{-}16}$, incubation days), morphological difference between gonads (ovary and testis) ($E_{6\text{-}7}$), the variation of fluorescent and raman spectral ($E_{3.5}$) and so on (Smith et al., 2007; Weissmann et al., 2013; Galli et al., 2018). That is to say, numerous techniques and/or studies have noticed sex difference between chicken embryos, but almost all of them require eggshell breaking or minimally invasive.

In recent years, the variation of hyperspectral spectrum ($E_0$) and odor characteristics ($E_1$) have been used to characterize (identify) the sex of avian eggs by nondestructive approach (Costanzo et al., 2016; Galli et al., 2018). Moreover, the odor differences between unhatched male and female eggs ($E_0$) have been confirmed in our previous work (Xiang et al., 2022). It's easy to understand that sex differentiation of blastocyst is very low and the differences between hermaphroditic blastocysts should not be easily recognized during $E_0$−$E_1$ of incubation. It could be hypothesized that these differences might be due to matrix composition rather than embryo in eggs, and the specific molecular mechanism needs to be further elucidated.

It has long been thought that sex determination in avian may be occurred at the time of fertilization (or meiosisⅠ) through the inheritance of sex chromosomes (ZZ vs. ZW). But the precise mechanism of sex determination in avian is still unknown. In the last few decades,



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researches on sex determination of chicken have focused on the gonads differentiation of embryo at the histological level ($E_{6.5}$), which is caused by embryonic growth rate or selective utilization of egg components (Webster et al., 2015). The gonadal rudiments formed at $E_{3.5}$ are morphologically identical between 2 sexes and sex differences in fluorescence (910 nm) and Raman spectrum of embryos (5 mm) in (hatched) eggs ($E_{3.5}$) have been supposed to be related to the protein content in blood (Galli et al., 2018; Smith et al., 2007).

Fortunately, the metabolic components (hormones, antibodies, antioxidants, vitamins) and hormone receptor genes have been known to be allocated to egg yolk (attachments) in a sex-specific manner (Saino et al., 2003; Gilbert et al., 2005; Badyaev et al., 2006). Moreover, it is noteworthy that some sex-related volatiles have been identified as a hormone-linked constituent of avian odor (Whittaker et al., 2011). Thus, the molecular composition of egg yolks should be parsed to further understand the precise mechanism between them. What's more, as we all know that proteins (known as a link between genes and phenotypes) are the essential component of yolk and participate in various metabolic activities during incubation (Zhu et al., 2020). Therefore, the protein composition of fertilized egg yolk should be resolved in detailed at the molecular level.

Admittedly, nowadays, different high-throughput omics techniques have been widely applied to explore the (differential) mechanism of food matrix and agricultural products (Wang et al., 2019, 2021; Liu et al., 2021). For example, (modified) proteomics have been successfully utilized to characterize divergent proteome patterns of egg albumen between domestic chicken, duck, goose, turkey, quail and pigeon, and sex-related proteins have been confirmed in various biological tissues (Pérez-Sánchez et al., 2008; Sun et al., 2017; Ribeiro et al., 2019). So we speculated that there should be some differences in matrix between unhatched male and female eggs and no studies have been reported on this topic, not to mention proteomics at the molecular level.

However, the above perspective or hypothesis has not been accepted by all scientists (Esther et al., 2018). Therefore, the main aim of this manuscript is to characterize the variation of proteins between unhatched male and female egg yolks using TMT-based quantitative proteomic and to study the function mechanism of gender-related proteins by bioinformatics analysis. These results may conduce to elucidate the molecular mechanisms of differences in composition between unhatched male and female eggs and provide a support for nondestructive sexing identification of unhatched fertilized eggs. More importantly, this research will also provide an evidence (insight) for maternal sex allocation, enhance our understanding of sex determination and differentiation in avian, and will be of great significance to egg processing and production.

## MATERIALS AND METHODS

### Samples and Preparations

Fertilized chicken eggs laid within 24 h were collected from Yukou poultry Co., Ltd (Jingzhou, Hubei, China) and were precooled overnight in a refrigerator (−18°C). Egg whites were removed manually and then DNA attached on the germ disc of frozen fertilized chicken eggs were extracted using 400 $\mu$L digestive buffer at 65°C for 2 h, and then mixed with 200 $\mu$L buffer PA. The mixture was centrifuged at 10,000 g/min for 5 min and the sediment was washed 3 times with 75% ethyl alcohol. The residual ethanol was naturally volatilized and DNA was dissolved by TE Buffer. After sexing, egg yolk of male and female fertilized eggs (n >15) were mixed (homogenized) and freeze-dried under vacuum conditions. Lyophilized powders were frozen at −80°C until further experiment.

### Molecular Sexing

Amplification of DNA from the germ disc was performed using a T100 Thermal Cycler PCR (Bio-Rad, Hercule, CA) with primers SF and SR in 25 $\mu$L systems (Table S1). PCR assay conditions: The initial denaturing step was performed at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 40 s, and 7 min at 72°C for the final extension step. PCR products were separated by agarose gels (1.8%) electrophoresis (120 V, 15 mA), visualized with 4S Green Nucleic Acid Stain and UV light. Male eggs were only characterized by CHD1-Z (600−650 bp; one band), while female eggs showed CHD1-W (400−450 bp) and CHD1-Z (600−650 bp; two bands) (Steiner et al., 2011). The result of gender identification for fertilized chicken eggs was shown in Figure S1.

### Extraction of Total Protein

Egg yolk powders were transferred into a shock tube with 500 $\mu$L BPP extraction mixture and shaken 40 s using a grinder (SPEX Geno2010) for 3 time, followed by centrifugation at 12,000 g/min at 4°C for 20 min. Tris-saturated phenol (500 $\mu$L) was added to the supernatant, vortex-mixed for 10 min at 4°C and then 12,000 g/min centrifuged at 4°C for 20 min. The upper phenol phase was mixed 2.5 mL cooled ammonium acetate-methanol (0.1 mol/L) and incubated at −20°C overnight. The remaining precipitate was washed 3 times with cooled 90% acetone (methanol) and then dissolved in 8 mol/L urea. Protein concentration was determined by using a bicinchoninic acid assay kit according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Shanghai, China; Wiśniewski et al., 2009; Zhu et al., 2019a).

### Trypsin Digestion

The 100 mg protein solution was reduced with 90 $\mu$L TCEP (10 mmol/L, Thermo Fisher, Waltham, MA) at

37°C for 30 min and alkylated with 40 mmol/L iodoacetamide (final concentration) for 40 min at room temperature in darkness. Alkylated samples were incubated with 6 volumes of cooled acetone at −20°C for 4 h, and then 10,000 g/min centrifuged at 4°C for 20 min. Protein samples were diluted by adding 100 $\mu$L50 mmol/L TEAB until the concentration of urea less than 2 mol/L. Finally, trypsin (Promega) was added with 1:50 (w/w, trypsin/protein) for the first digestion at 37°C overnight and 1:100 (w/w, trypsin/protein) trypsin for a second 4 h digestion (Jia et al., 2020).

### TMT Peptide Labeling and High-pH Reversed-Phase Peptide Fractionation

TMT 10-plex isobaric label reagents were thawed at room temperature and reconstituted in acetonitrile. The peptide mixtures were labeled with TMT reagents according to the manufacturer's instructions (Thermo Fisher, Waltham, MA) and incubated for 2 h at room temperature. Subsequently, labeled peptides were pooled, desalted (Sep-Pak), and dried by vacuum centrifugation (Stryiński et al., 2019). Labeled tryptic peptides were first separated into 20 fractions using high-pH reverse-phase chromatography (Ultimate 3000, Thermo Scientific) equipped with ACQUITY BEH C18 column (1.7 $\mu$m, 2.1 mm × 150 mm, Waters, Milford, MA) and gradient of 2 to 80% acetonitrile (pH 10) for 45 min (Table S2). Then, the peptides were combined into 10 fractions and dried by vacuum centrifugation (Li et al., 2018).

### LC-MS/MS Analysis/Identification and Quantification

TMT-labeled tryptic peptides were resuspended in 0.1% formic acid (ACN), cleaned on a C18 column (75 $\mu$m × 25 cm, Thermo Fisher Scientific, Waltham, MA) and analyzed by LC-MS/MS equipped with EASY-nLC 1200 liquid chromatography system coupled to Q-Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Separation system was generated at a constant flow rate of 300 nL/min and detailed gradient program was shown in Table S2. Spray voltage of 1.95 kV and capillary temperature of 275°C were used for ionization (Stryiński et al., 2019). MS scanning was performed over the range of 350 to 1550 m/z with a resolution of 60,000 and the second-order mass scan resolution was set to 15,000. Mass spectrometer was operated in data-dependent acquisition mode, and the top 20 precursor ions were subjected to fragmentation by high-energy collision-induced decomposition with 15.0 s dynamic exclusion. Automatic gain control was set to $5 \times 10^4$ and fixed first mass was set to 100 m/z (Sun et al., 2019; Zhu et al., 2019b).

### Database Searches

Proteins identification was performed by searching against the protein database from the most relevant sequenced *Galliformes*, using Proteome Discoverer TM Software 2.1. Trypsin/P is specified as cleavage enzyme allowing up to 2 missing cleavages. The first and main search of mass tolorance for precursor ions was set as 20 and 5 ppm, respectively. Mass error tolerance of the second fragment ions was 0.02 Da and carbamidomethyl on cys was specified as a fixed modification. Oxidation on met and acetylation on the protein N-terminus were specified as variable modifications. False discovery rate was adjusted to <1%, minimum score for peptides was set >20 and *P*-values were calculated using two-sample two-tailed Student's *t*-test. Proteins with fold change (**FC**) >1.20 or <0.83 ($P < 0.05$) were defined as differentially expressed proteins (**DEPs**) (Jia et al., 2020).

### Bioinformatic and Multivariate Analysis

Functional annotation (classification) of identified proteins (gene sequences) are performed by various nucleotide and/or protein databases, including Gene Ontology (**GO**, http://www. geneontology.org/), Kyoto Encyclopedia of Genes and Genomes (**KEGG**, http://www.genome.jp/kegg/), Clusters of Orthologous Groups (**COG**, http://www.ncbi.nlm.nih.gov/COG/), Pfam (http://pfam.xfam.org/, Pfam 32.0), and Sub-Cell location (http://www.genscript.com/psort/wolf_psort.html) (Jia et al., 2020; Liu et al., 2020b; Jaina et al., 2021). For more information, detailed functional annotation, enriched pathways ($P < 0.05$), and enrichment chord, including GO and KEGG, were searched for DEPs between fertilized male and female egg yolks (Zhu et al., 2019a). Principal component analysis (**PCA**) and partial least squares discrimination analysis (**PLS-DA**) of the identified proteins were performed by MetaboAnalyst 4.0 (Li et al., 2018).

## RESULTS AND DISCUSSION

### Panorama of Proteins in Unhatched Fertilized Egg Yolks

***Identification of Global Proteins From Unhatched Fertilized Egg Yolks*** A total of 411 proteins, including ovalbumin, ovomucoid, ovotransferrin, apolipoprotein, etc. were identified and quantified via TMT proteomics in fertilized chicken egg yolk. Similar results were or obtained in (fertilized) chicken egg yolk plasma and granule (Sophie et al., 2014; Karlheinz and Matthias, 2010). There were 276 proteins belonging to *Gallus gallus* and the remaining proteins belonging to *Meleagris gallopavo* (28), *Bambusicola thoracicus* (17), *Numida meleagris* (16), *Coturnix japonica* (27), *Colinus virginianus* (13), etc. (https://www.ncbi.nlm.nih.gov/ pubmed/). The accession number, protein name, molecular weight, and sequence coverage of proteins identified were supplied in Table S3.

***Functional Annotation of Global Proteins From Unhatched Fertilized Egg Yolks*** GO, KEGG, COG, Pfam, and Sub-Cell location were performed to elucidate the function, pathways and distribution of proteins

identified in unhatched fertilized egg yolk (Figure S2). Genes of 361 proteins (87.83%) were annotated for 15 functions (B, C, E, F, G, J, K, L, M, O, P, Q, R, S, and Z) in COG and which were mainly related to posttranslational modification, protein turnover, chaperones and general function prediction (Figures S2a and b). Among the identified proteins, 402 (97.81%) were annotated in P fam, belonging to 20 families, including V-set, lg-3, Sushi, SRCR, I-set and so on (Figure S2a, c). Most of these proteins were distributed in cytoplasm (223), extracellular (70), endoplasmic reticulum (32), plasma membrane (23) etc. sub cell-location (Figures S2a and c; Bai et al., 2018).

A total of 206 (50.12%) identified proteins were mainly located in extracellular region (part) and organelle, etc. (*cellular components*); participated in cellular, metabolic process, biological regulation, single-organism, etc. (*biological process*), and involved in binding, catalytic activity, molecular function regulator, etc. (*molecular function*) (Figure 1A). And 262 (63.75%) of identified proteins were annotated to 200 KEGG pathways (Figure 1B). Transport and catabolism (39, Cellular Processes), signaling molecules and interactions (30, Environmental Information Processing) were the primary enrichment pathways, followed by infectious diseases: Viral in Human Diseases (24, Human Diseases) (Liu et al., 2020b; Zhang et al., 2020).

## Analysis of Differential Proteins Between Unhatched Male and Female Egg Yolks

### Identification of Differential Proteins Between Male and Female Egg Yolks

A total of 35 proteins (FC $\geq$1.2 or $\leq$0.83, $P \leq 0.05$) were found to be significantly differentially expressed between unhatched fertilized male and female egg yolks, including 18 upregulated (**UPs**) and 17 downregulated (**DPs**) differential proteins (Figure S3) (Zhu et al., 2019a; Zhong et al., 2020). That is to say, 375332005 (accession number), 1015809562, 973583714, etc. and 1337178851, 89000557, 89000581, etc. proteins were higher expressed in male (UPs) and female egg yolk (DPs), respectively (Figure S4 and Table 1). Most of these gender-specific differential proteins belong to immunoglobulin (**IG**) regions (or partial). Similar results are more common in clinical medicine, such as IG and IgG subclass concentrations differed significantly due to sex and race and age (Tyler et al., 2019; Harkness et al., 2020; Zhong et al., 2020) and IgG glycan levels changed significantly and presented pronounced gender-related differences from 6 to 12 wk in mouse (Han et al., 2020).

In addition, ovotransferrin BC type (71274079), serum amyloid A (294987919), microseminoprotein-like (733897253), vitellogenin-2-like (1201900467) and hypothetical proteins (1215469310), (1215486213),

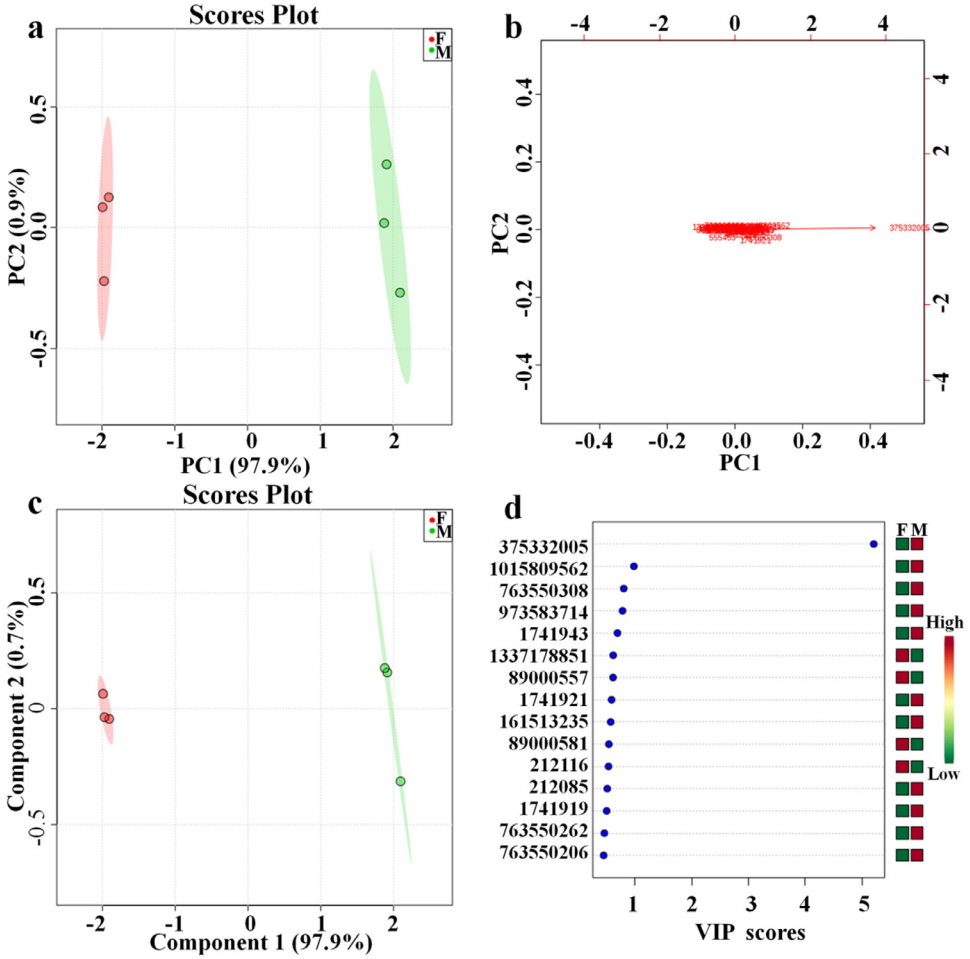


**Figure 1.** Multianalysis of DEPs between unhatched male and female egg yolks. (A, B) Scores plot and Bio-plot for PCA; (C, D) scores and VIP scores plot for PLS. Abbreviations: DEPs, differentially expressed proteins; PCA, principal component analysis; PLS, partial least squares.

**Table 1.** Differential expressed proteins (DEPs) between unhatched male and female egg yolks.

| Accession | Description | Coverage | MW [kDa] | F (n = 3) | M (n = 3) |
|---|---|---|---|---|---|
| **212085** | **immunoglobulin light-chain VJ region, partial [Gallus gallus]** | **53.47** | **10.45** | **0.95 ± 0.03** | **1.29 ± 0.06** |
| 212116 | immunoglobulin light-chain VJ region, partial [Gallus gallus] | 53.00 | 10.10 | 1.01 ± 0.02 | 0.66 ± 0.01 |
| 555453 | Ig lambda-chain V-J region, partial [Gallus gallus] | 32.77 | 12.06 | 1.01 ± 0.04 | 0.77 ± 0.07 |
| **1741919** | **immunoglobulin heavy chain, partial [Gallus gallus]** | **38.28** | **13.48** | **0.99 ± 0.01** | **1.32 ± 0.02** |
| **1741921** | **immunoglobulin heavy chain, partial [Gallus gallus]** | **26.56** | **13.13** | **1.02 ± 0.06** | **1.41 ± 0.09** |
| **1741943** | **immunoglobulin heavy chain, partial [Gallus gallus]** | **27.27** | **13.68** | **1.00 ± 0.02** | **1.46 ± 0.01** |
| **13990800** | **immunoglobulin lambda chain, partial [Gallus gallus]** | **37.50** | **11.50** | **0.97 ± 0.02** | **1.20 ± 0.06** |
| 13990824 | immunoglobulin lambda chain, partial [Gallus gallus] | 38.60 | 11.91 | 0.91 ± 0.04 | 0.73 ± 0.05 |
| 71274079 | ovotransferrin BC type [Gallus gallus] | 60.14 | 77.76 | 0.97 ± 0.02 | 0.71 ± 0.01 |
| 89000557 | immunoglobulin heavy chain variable region, partial [Gallus gallus] | 19.01 | 14.65 | 1.01 ± 0.01 | 0.6 ± 0.05 |
| 89000581 | immunoglobulin heavy chain variable region, partial [Gallus gallus] | 18.57 | 14.22 | 0.96 ± 0.03 | 0.6 ± 0.02 |
| **89000587** | **immunoglobulin light chain variable region, partial [Gallus gallus]** | **35.29** | **12.34** | **0.95 ± 0.02** | **1.21 ± 0.01** |
| **89000591** | **immunoglobulin light chain variable region, partial [Gallus gallus]** | **47.06** | **12.25** | **0.93 ± 0.06** | **1.15 ± 0.02** |
| **161513235** | **immunoglobulin light chain variable region, partial [Gallus gallus]** | **36.11** | **10.95** | **0.99 ± 0.02** | **1.37 ± 0.02** |
| 161513259 | immunoglobulin heavy chain variable region, partial [Gallus gallus] | 37.88 | 13.68 | 0.93 ± 0.04 | 0.73 ± 0.02 |
| 161513265 | immunoglobulin light chain variable region, partial [Gallus gallus] | 27.27 | 11.19 | 0.94 ± 0.03 | 0.77 ± 0.00 |
| **161513273** | **immunoglobulin light chain variable region, partial [Gallus gallus]** | **20.18** | **11.51** | **1.00 ± 0.00** | **1.21 ± 0.06** |
| **161513281** | **immunoglobulin light chain variable region, partial [Gallus gallus]** | **26.79** | **11.62** | **0.93 ± 0.03** | **1.13 ± 0.03** |
| 161513307 | immunoglobulin heavy chain variable region, partial [Gallus gallus] | 40.32 | 13.17 | 0.97 ± 0.02 | 0.80 ± 0.02 |
| 294987919 | serum amyloid A [Gallus gallus] | 13.39 | 14.16 | 0.98 ± 0.01 | 0.75 ± 0.01 |
| **375332005** | **immunoglobulin light chain V-J-C region, partial [*Meleagris gallopavo*]** | **13.27** | **22.51** | **0.93 ± 0.04** | **4.38 ± 0.08** |
| 733897253 | PREDICTED: beta-microseminoprotein-like [*Meleagris gallopavo*] | 7.27 | 12.48 | 0.92 ± 0.06 | 0.71 ± 0.01 |
| 763550054 | immunoglobulin light chain variable region, partial [Gallus gallus] | 39.05 | 10.73 | 0.99 ± 0.02 | 0.82 ± 0.04 |
| **763550082** | **immunoglobulin light chain variable region, partial [Gallus gallus]** | **22.77** | **10.34** | **1.02 ± 0.02** | **1.32 ± 0.01** |
| 763550102 | immunoglobulin light chain variable region, partial [Gallus gallus] | 36.89 | 10.64 | 1.01 ± 0.03 | 0.75 ± 0.04 |
| **763550206** | **immunoglobulin light chain variable region, partial [Gallus gallus]** | **22.12** | **10.65** | **1.03 ± 0.02** | **1.33 ± 0.04** |
| **763550262** | **immunoglobulin light chain variable region, partial [Gallus gallus]** | **38.46** | **10.63** | **1.00 ± 0.01** | **1.31 0 ±0.01** |
| **763550308** | **immunoglobulin light chain variable region, partial [Gallus gallus]** | **37.04** | **11.07** | **1.06 ± 0.05** | **1.60 ± 0.06** |
| **763550324** | **immunoglobulin light chain variable region, partial [Gallus gallus]** | **54.37** | **10.52** | **1.03 ± 0.02** | **1.26 ± 0.03** |
| **973583714** | **anti-prostate specific antigen antibody immunoglobulin variable region, partial [Gallus gallus]** | **43.75** | **13.41** | **0.98 ± 0.02** | **1.50 ± 0.04** |
| **1015809562** | **protein TENP [Gallus gallus]** | **9.57** | **47.37** | **0.91 ± 0.05** | **1.56 ± 0.03** |
| 1201900467 | vitellogenin-2-like [*Numida meleagris*] | 21.05 | 190.4 | 0.95 ± 0.03 | 0.77 ± 0.01 |
| 1215469310 | hypothetical protein ASZ78_005511, partial [*Callipepla squamata*] | 2.17 | 55.28 | 0.96 ± 0.02 | 0.77 ± 0.04 |
| 1215486213 | hypothetical protein H355_007644 [*Colinus virginianus*] | 1.45 | 93.45 | 0.94 ± 0.03 | 0.65 ± 0.01 |
| 1337178851 | hypothetical protein CIB84_015392, partial [*Bambusicola thoracicus*] | 43.59 | 26.06 | 0.93 ± 0.05 | 0.52 ± 0.02 |

Bold words: upregulated proteins, M > F; normal words: downregulated proteins, M < F.

(1337178851) were found higher expressed in female eggs. Similarly, compared to serum glucose and triglycerides in male chickens, ovotransferrin in females has been reported to be affected by location (Metzler-Zebeli et al., 2017). Female Alzheimer's disease mice were found exhibited significantly greater $\beta$-amyloid burden than age-matched males and these differences may be mediated by sex steroid hormones (Carroll et al., 2010). Serum testosterone seems to inhibit the early pathological accumulation of amyloid-$\beta$ in females (Lee et al., 2017). *Loligo*-microseminoproteins were synthesized in female reproductive exocrine glands and embed protein in the outer tunic of egg capsules, which were regarded as pheromone to attract male fighting with nearby males (Cummins et al., 2011). Vitellogenin (precursor protein of egg yolk) has been reported as a promising molecular marker (female-specific protein) for sex identification in *P. anguinus* and geoducks (Kim et al., 2018; Gredar et al., 2019).

***Multivariate and Correlation Analysis of Differentially Expressed Proteins*** The DEPs between unhatched fertilized male and female fertilized egg yolks were well separated using PCA and PLS-DA (Figures 2A and 2C). Meanwhile, immunoglobulin light chain V-J-C region (375332005), protein TENP (1015809562), immunoglobulin heavy chain, partial (763550308), antiprostate specific antigen antibody immunoglobulin variable region (973583714), immunoglobulin heavy chain, partial (1741943), etc. upregulated proteins (UPs) and hypothetical protein CIB84_015392 (1337178851), immunoglobulin heavy chain variable region (89000557) etc. downregulated proteins (DPs) contributed most to the distinction of male and female eggs (Figures 2B and 2D).

In addition, DEPs between male (green) and female (red) egg yolks were clustered with each other and proteins 763550054-161513259 and 763550206-161513281 were higher expressed in male and female egg yolks, respectively (Figure S5b). Similar results were obtained by intuitive and multivariate comparative analysis (Figure S4 and Figure 2). More importantly, positive and negative correlations were found among intragroup and intergroup of UPs and DPs, respectively (Figure S5a). It means that the UPs and DPs could perform some gender-related functions and/or properties in a synergistic (intragroup) and antagonistic (intergroup) manner.

***Functional Classification and Enrichment of Differential Expressed Proteins*** *GO Annotation and Enrichment of Differential Expressed Proteins* GO analyses (classification and enrichment) of DEPs between unhatched male and female egg yolks were presented in Figures 3 and 4. DEPs were annotated into 17

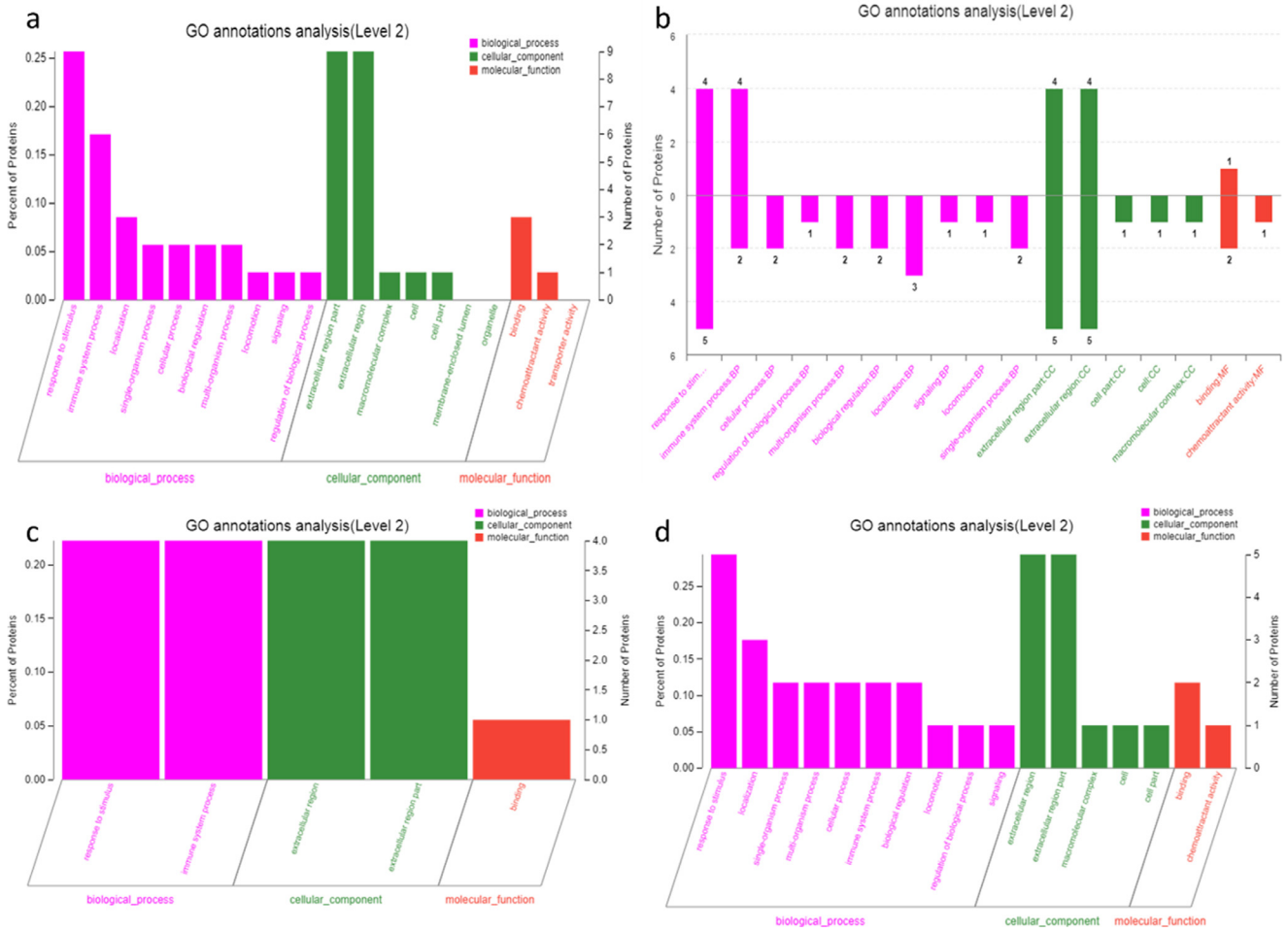


**Figure 2.** GO annotation of DEPs between unhatched fertilized male and female egg yolks. (A, B) Unidirectional and bidirectional histogram of differential proteins; (C, D) histogram for upregulated and downregulated differential proteins; abscissa: secondary classification of GO annotation; left and right ordinate: proportion and quantity of differential proteins. Abbreviations: DEPs, differentially expressed proteins; GO, Gene Ontology.

functional groups, including 10 biological processes, 5 cellular components and 2 molecular functions (Figures 3A and 3B; Table S3). These proteins were mainly involved in stimulus response (9/35), immune system process (6/35) (BP), extracellular region part (9/35), extracellular region (9/35) (CC) and binding (3/35) (MF). Furthermore, 4 of 18 UPs were commented into response to stimulus, immune system process (BP), extracellular region part, extracellular region (CC) and 1 UP was commented into binding (MF) (Figure 3C); DPs were mainly commented into response to stimulus, localization (BP), extracellular region, extracellular region part (CC), binding, chemoattractant activity (MF) (Figure 3D and Table S3; Wang et al., 2020).

Moreover, DEPs were enriched into 19 functions, including 16 biological processes, 2 cellular components, and 1 molecular function (Figures 4A and 4B; Table S4). For example, part of these DEPs were highly enriched by GO terminology associated with positive chemotaxis (GO: 0050918, CC) and chemoattractant activity (GO: 0042056, MF) (Booms et al., 2006). Most of the DEPs were enriched in extracellular region part (protein number: 9, CC), extracellular region part (8, CC), response to stimulus (9, BP), immunoglobulin production (6, BP), production of molecular mediator of immune response (6, BP) and immune system process (6, BP) (Cao et al., 2017). UPs were enriched into immunoglobulin production (4, BP), production of molecular mediator of immune response (4, BP), immune response (4, BP), immune system process (4, BP), response to stimulus (4, BP) (Li et al., 2015). Meanwhile, DEPs were enriched into 25 biological processes (response to stimulus, acute inflammatory response, acute-phase response, inflammatory response, defense response, response to stress), 2 cellular components (extracellular matrix and intracellular) and 1 molecular function (chemoattractant activity) (Konvalinka et al., 2013).

*KEGG Annotation and Enrichment of Differential Expressed Proteins* In addition, KEGG classification showed that 6 DPs were annotated as 5 KEGG pathways, including folding, sorting and degradation (genetic information processing), signaling molecules and interaction (environmental information processing), cell growth and death (cellular processes), transport and catabolism (cellular processes), infectious diseases: viral (human diseases) (Figure 5A). KEGG enrichment showed that DPs were related to 6 KEGG pathways, including ferroptosis (gga

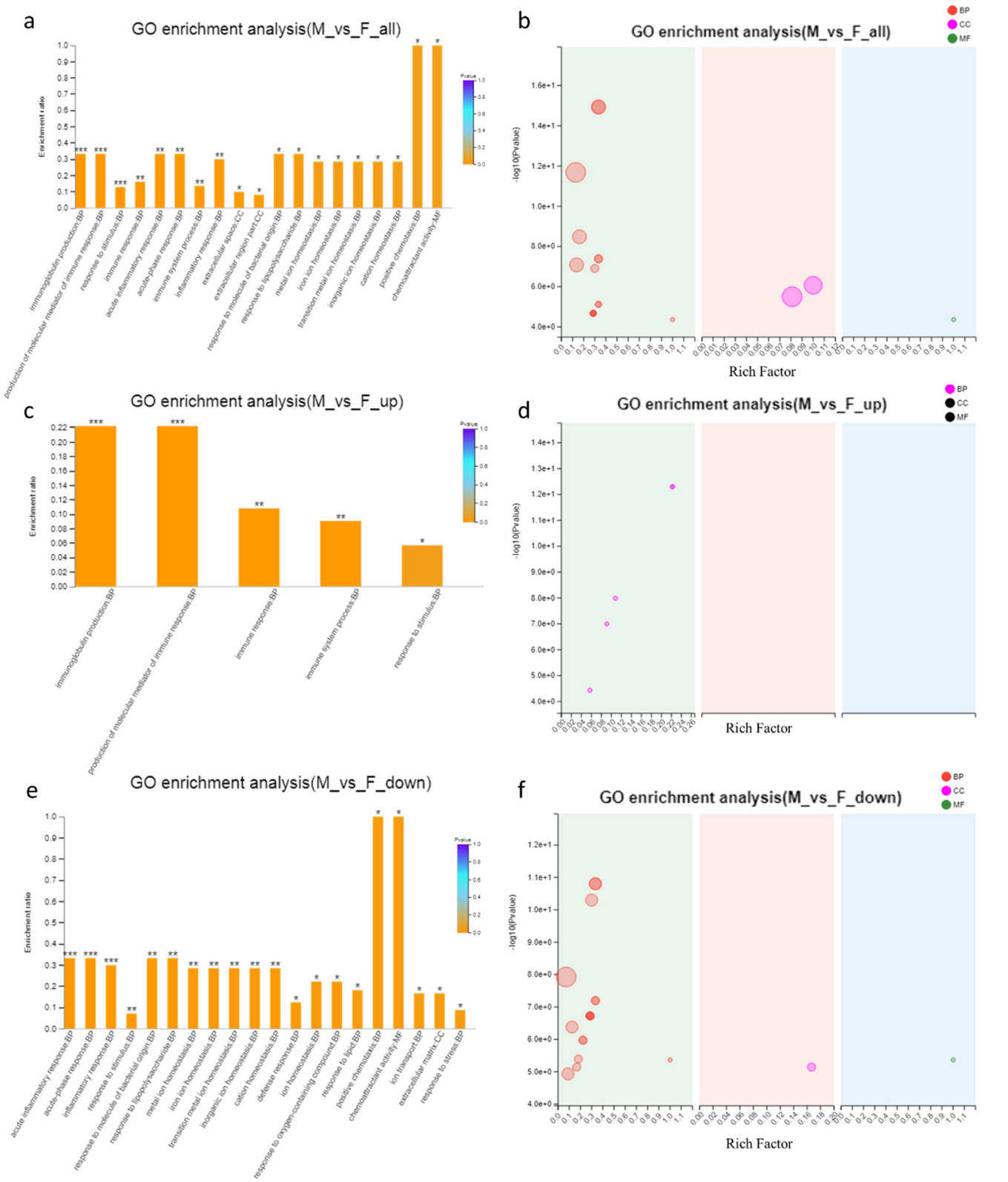


**Figure 3.** GO enrichment of DEPs between unhatched male and female egg yolks (A, B) differential proteins; (C, D) upregulated differential proteins; (E, F) downregulated differential proteins; abscissa and ordinate in A, C, E: GO classification and enrichment ratio, respectively; abscissa and ordinate in B, D, F: rich factor and *P* value, respectively. Abbreviations: DEPs, differentially expressed proteins; GO, Gene Ontology.

04216), ubiquitin mediated proteolysis (gga 04120), cell adhesion molecules (gga 04514), endocytosis (gga 04144), herpes simplex infection (gga 05168), phagosome (gga 04145) (Figure 5B). It could be concluded/inferred those differential proteins (female-specific) that were higher expressed in female egg yolk were annotated or enriched in KEGG database, whereas the function and metabolic pathways of male-specific proteins have been rarely reported or noticed. Similar results (more KEGG pathways were solely repressed in the low-dose and median-dose triclosan in females) were obtained in female chicken embryos (Guo et al., 2018).

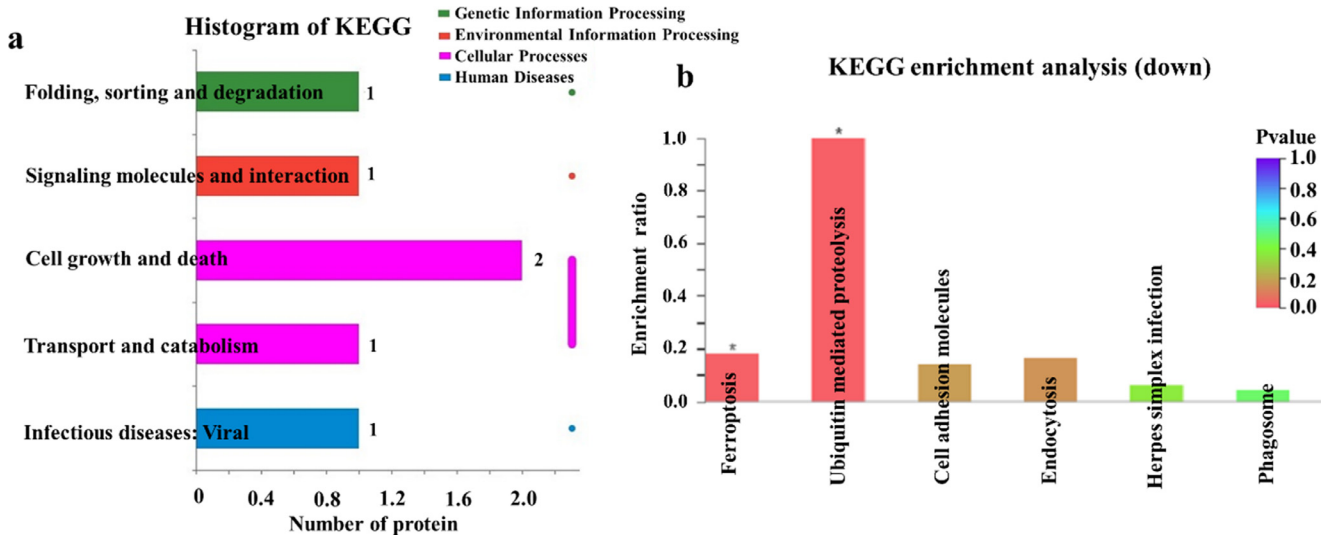


**Figure 4.** KEGG analysis of DEPs between unhatched male and female egg yolks. (A) KEGG annotation, abscissa and ordinate: pathways and number of protein; (B) KEGG enrichment, abscissa, and ordinate: function classification and enrichment ratio.

For further (GO and KEGG) enrichment chord analyses of DEPs were presented in Figure S6 and Table S5 (Liu et al., 2020a). Nine DEPs (294987919, 89000587, 71274079, 89000591, 763550262, 555453, 1337178851, 763550102, 375332005) were related to 15 pathways (acute inflammatory, acute-phase inflammatory, immune and production of molecular mediator of immune responses; response to molecule of bacterial origin and lipopolysaccharide; metal, iron, inorganic, transition metal ion, and cation homeostasis; positive chemotaxis, immunoglobulin production, extracellular space) (Figure S6c). In which, 375332005, 89000587,

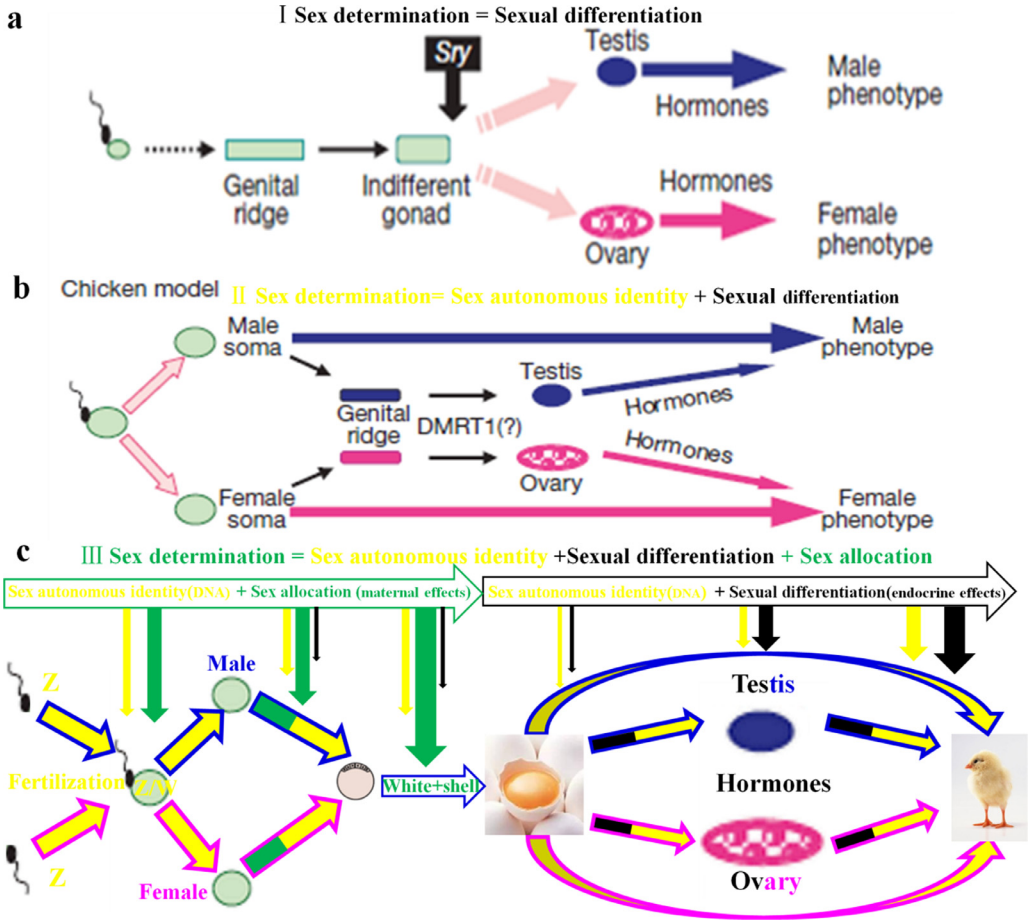


**Figure 5.** Potential mechanism of sex determination, differentiation and allocation in chicken and their relative contribution for sexual phenotype. (Blue line: male, red line: female; yellow: sex autonomous identity, green: sex autonomous identity, black: sexual differentiation; width of arrows: representative the importance of each item, wide > narrow).

89000591, and 763550262 (UPs) were related to immunoglobulin production, production of molecular mediator of immune response and immune response (Figure S6a). But for DPs, 294987919, 133717885, and 71274079 were all related to acute inflammatory response, acute-phase response, defense, and inflammatory responses; both 133717885 and 71274079 were both related to various ion homeostasis and response to molecule of lipid, lipopolysaccharide oxygen-containing compound and bacterial origin (Figure S6b).

Further, 4 DPs (1215469310, 1215486213, 71274079, 1337178851) were related to 6 KEGG pathways (gga 04216, gga 04120, gga 04514, gga 04144, gga 05168 and gga 04145). In which, protein 1215469310 was related to cell adhesion molecules (gga 04514), endocytosis (gga 04144), herpes simplex infection (gga 05168), and phagosome (gga 04145); proteins 71274079 and 1337178851 were both related to ferroptosis (Figure S6d). Fortunately, differentially expressed genes between male and female brains (Olive flounder) were involved in herpes simplex infection pathways (Zou et al., 2020). Besides, sex-related (differentially expressed) genes between primary growth follicles to pre-vitellogenic follicles transition in the ovary (females) during sexual maturation were enriched to endocytosis in KEGG analysis (Zhu et al, 2018).

It is very interesting that most of the DPs (higher expressed in female egg yolks) were commented (classified and enriched) in GO and KEGG databases. Therefore, it could be inferred that the functions and pathways of these DPs (M < F) were identified and/or studied in other species before. Coincidentally, most of the DEPs were related to sex-related diseases (Lee et al., 2017) and metabolic pathways (Jones et al., 2019), which were regulated by sex hormones directly or indirectly (Keyvanshokooh et al., 2009; Lee et al., 2017; Jones et al., 2019). Moreover, sex hormones have been reported as gender markers for hatched eggs and played an important role in gender differentiation for chicks (embryos) (Weissmann et al., 2013; Zhao et al., 2010). Not accidentally, it could be speculated that there may be a strong association between sex-related proteins and hormones.

### *Potential Mechanisms of Sex Determination, Differentiation, and Allocation in Avian (Chicken)*

As expected, our results indicate that there are certain differences in molecular composition between unhatched male and female egg yolks at the proteome level. These observations support and extend the molecular basis and mechanism of sex determination, differentiation, and allocation in avian. It is true that the phenotype differences between male and female cells or organizations in avian have been supposed largely dependent on hormones (*before 2010*, stage I) and cell autonomous sex identity has been proposed by Zhao (2010, stage II) as shown in Figure 5a & b (Zhao et al., 2010). These theories only focus on sex differences in embryonic growth rate and selective utilization of egg components, while the effects of maternal sex-allocation have been ignored.

Fortunately, this study demonstrate that maternal investment may play an important role in the mechanisms of sexual phenotype in avian (Attila Salamon 2015) and the sex of them were jointly regulated by hormones, cell autonomous sex identity and sex-allocation (III) (Figure 5c). We propose that cell autonomous sex identity and/or DNA play vital roles in sex determination, differentiation, and sexual phenotype throughout the whole process of chick embryo development, including fertilization and incubation (**CASI**, yellow part). Furthermore, sex-allocation (**SA**, green part) and sex-differentiation (**SD**, black part) play an important role in sex determination or sexual phenotype before and after hatching, respectively.

The detailed potential mechanism of sex determination (phenotype) is presented as follows: 1) During the process of (from meiosis I to) fertilization, the sex was depended critically on Z or W chromosome in haploid oocytes (CASI) and the molecular components (proteome and lipids) of matrix in vitellus were intelligently manipulated or identified by the hens (SA) (Uller et al., 2009). 2) During the formation of (from fertilization to) shellegg, zygote cells begin to grow, divide and partially slightly differentiate (SD); more importantly, it is worth mentioning that the contribution of sex-allocation, egg white, and shell were self-assembled and wrapped on the surface of vitellus according to its sex, gradually increasing until shell egg was formed (SA). 3) The effect of SA has disappeared completely during the period of incubation (from fertilized eggs to chicks) and the contribution of CASI and SD gradually increase over time.

Previous evidences have shown that the levels of hormones differ significantly between male and female avian eggs maybe caused by species-specific differences in maternal allocation and secondary sex-specific processes during early development (from fertilization to fertilized eggs) (Petrie et al.,2001; Wendt et al., 2020). These phenomena or evidences are consistent with the results of this study. Moreover, the differential deposition of matrix does take place in relation to both the sex of eggs and the social rank of their mothers. Which might enhance the mechanisms of adaptive maternal investment, in line with the expectations of the sex-allocation theory.

## CONCLUSIONS

The variation between (fertilized) male and female egg yolks has been confirmed at the level of TMT proteome and DEPs were mainly distributed in extracellular region (part), involved in immune system process and response to stimulus, participated in binding and chemoattractant activity. These results demonstrate that maternal investment may play an important role in sex determination, differentiation and sexual phenotype in

chicken. Compared with CASI, this research provides a novel insight into molecular mechanism of sex determination or phenotype, enhance the expectations of the sex-allocation theory in avian and the relationship between them should be deciphered in future. Moreover, this study provides powerful evidence for ovo sexing of unhatched fertilized domestic chicken eggs by nondestructive approach and will be of great significance to egg processing and production.

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Informed consent: Informed consent is not applicable for this study.

## DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2022.101906.

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