

A comparative proteomic study of high and low semen quality seminal plasma in drakes

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ABSTRACT Semen quality is the most important indicator in evaluating drake fecundity. At present, the low semen quality has become a major factor restricting the development of artificial insemination (AI) technology in ducks. Numerous studies have indicated that seminal plasma proteins play a crucial role in semen quality, but the mechanism of seminal plasma proteins regulating semen quality of drakes remains unclear. Thus, the objective of this study was to identify seminal plasma proteins associated with semen quality by comparing the seminal plasma proteomic profile of drakes with high-quality semen (HQS) and low-quality semen (LQS). Using a label-free MS-based method, a total of 745 seminal plasma proteins were identified. Of these, 55 differentially expressed proteins (DEPs) were identified (40 up-regulated and 15 down-regulated). Gene Ontology (GO) analysis showed that the DEPs were mainly enriched in transmembrane transport, extracellular matrix structural constituent, transferase activity, transferring acyl groups other than amino-acyl groups, transmembrane transporter activity, and integral component of membrane

($P < 0.05$). Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analysis indicated that the DEPs were significantly enriched in apoptosis, tyrosine metabolism, glycerophospholipid metabolism, and sulfur metabolism pathways ($P < 0.05$). Moreover, through protein-protein interaction (PPI) network analysis, eight potential candidate proteins were identified, including P19140 (Alpha-enolase), R0KUV7 (Calreticulin), R0K3X3 (Solute carrier family 2, facilitated glucose transporter member 5), R0L6V0 (Proteasome subunit beta), R0JKW0 (Cytochrome c), R0JMC5 (Tubulin alpha chain), R0LCK1 (Cathepsin C), and R0JUP6 (Cathepsin D), which could play crucial roles in semen quality. Notably, further analysis demonstrated that key protein P19140 (Alpha-enolase) might control the semen quality of drakes by regulating the expression of proteins related to apoptosis pathway. This study is the first systematically comparing the seminal plasma proteome of drakes exhibiting high and low semen quality. These results provide novel insights into the mechanisms regulating semen quality of drakes.

Key words: drakes, semen quality, seminal plasma, proteomic analysis

2022 Poultry Science 101:102130

<https://doi.org/10.1016/j.psj.2022.102130>

INTRODUCTION

Artificial insemination (AI) technology of poultry began in the 1930s, while AI for duck due to its low fertilization rate, its development and application is far lower than chicken. In recent years, with the transformation of duck raising system from traditional floor rearing to cage rearing (Bai et al., 2022; Zhang et al., 2019), the original natural mating mode will be gradually replaced by AI (Qingyuan et al., 2021). In the process of AI, the semen

quality of drakes plays a major role (Ukaszewicz et al., 2020). Studies have shown that the seminal plasma proteins of male poultry were significantly associated with semen quality and fertilization rate (Słowińska et al., 2017; Santiago-Moreno and Blesbois, 2020). Moreover, in the production practice, there are obvious differences in the volume of seminal plasma between different drakes. So, it is necessary to study the mechanism involved.

Seminal plasma is a complex biological fluid that surrounds the sperm during ejaculation and plays an important role in the semen biology of all male animals (Mariana et al., 2018; Santiago-Moreno and Blesbois, 2020). The interaction between seminal plasma and sperm induced metabolic changes, binding of seminal plasma proteins onto the sperm cell surface and membrane remodeling, potentially affecting the sperm quality, and the zygote formation in the female genital tract,

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Received April 13, 2022.

Accepted August 2, 2022.

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which play a crucial role in reproductive mechanism (Druart and De, 2018; Archana et al., 2019). Waterfowl (e.g., ducks and geese) represent a class of animals with highly specific reproductive process (Blesbois and Brillard, 2007). The semen of waterfowl usually had exceptionally high sperm concentration with relatively little seminal plasma, but this contributed to rapid changes in sperm metabolism and function (Santiago-Moreno and Blesbois, 2020). Currently, the biochemical characteristics and physiological roles of the various seminal plasma proteins in drakes were poorly understood. Over the past few years, studies in both mammals and poultry have identified some important seminal plasma proteins related to semen quality through proteomic analysis. For instance, high concentrations of porcine seminal protein-I (PSP-I) and cathepsin B (CTSB) in boar seminal plasma were associated with decreased total and progressive sperm motility (De Lazari et al., 2019). Recently, it has been reported that zinc-alpha-2-glycoprotein (ZAG) could bind to the surface of spermatozoa and regulate the sperm motility through the cAMP/PKA pathway (Qu et al., 2007). In rabbit, the percentage of sperm with intact membrane was related to seminal plasma proteins FAM115 complex and tropomyosin (Bezerra et al., 2019). In addition, key seminal plasma proteins adhesion G-protein coupled receptor G2 (ADGRG2) and serine peptidase inhibitor Kazal-type 2 (SPINK2) played a crucial role to maintain sperm motility in indigenous chicken (Li et al., 2020). Nevertheless, the regulatory mechanism of seminal plasma proteins controlling drake semen quality remains unclear.

Recently, the advances in “OMICS” techniques helped in expanding our knowledge and understanding of male livestock seminal plasma at proteomic level (Altelaar et al., 2013; Druart and De, 2018). However, changes in seminal plasma proteomic profiles related to semen quality of drakes have not been reported. Therefore, in the present study, we performed label-free proteomics analysis based on SDS-PAGE fractionation of seminal plasma proteins, aiming to reveal the seminal plasma proteomic profile between HQS and LQS drakes and identify the proteins associated with semen quality. These data were expected to help elucidate the molecular mechanism regulating semen quality of drakes.

MATERIALS AND METHODS

Ethics Approval and Consent to Participate

All drakes handling procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Sichuan Agricultural University (Chengdu campus, Sichuan, China, Permit No. DKY20170913).

Management of Experimental Drakes

For the present study, a total of 60 healthy with similar body weight (average 2.95 kg) drakes were used as experimental material. These drakes were obtained from the Sichuan Agricultural University Waterfowls Breeding

Farm (Ya'an, Sichuan, China). At the age of 140 d old, all drakes were moved to single cages and maintained under natural light and temperature condition, with lights off at 23:00 to 06:00. Feed and water were available *ad libitum*. Drakes were weighed at 190 d old and trained for semen collection by abdominal massage technique. In the present study, semen quality parameters were estimated 3 times at a 3-d interval for each drake at 210 d old and 364 d old. Semen collection during this study was performed by the skilled technicians.

Semen Quality Analysis of the Drakes

In the present study, the ejaculate volume (EV), sperm viability (SV), sperm motility (SM), sperm concentration (SC), acrosome deformity (AD), and morphological abnormal sperm (MAS) were measured on each drake semen sample. The measured methods of semen quality were as follows: 1) After artificial semen collection, the semen volume of each drake was measured by a 1 ml syringe, and then the semen was diluted with stroke-physiological saline (0.9%) according to 1:20 in the 1.5 mL EP tube; 2) Sperm motility was assessed by placing 10 uL of each sample, pre-diluted to 1:20 in the 1.5 mL EP tube described above, on a warmed (37°C) glass slide. About 200 sperm were measured using a phase contrast microscope (BM2000, NOVEL, China) at 400 ×; 3) 100 uL semen was added to 100 uL of 1% trypan blue solution (Solarbio, Beijing, China) and incubated for 15 min. Sperm viability was assessed by placing 10 uL of each sample, previously semen stained with trypan blue in the medium described above, on a warmed (37°C) glass slide. Sperm that stained blue were considered dead, and unstained sperm were considered to be viable. About 200 sperm were examined using a phase contrast microscope (BM2000, NOVEL, China) at 400 ×; 4) Gentian violet staining solution (Sangon Biotech, Shanghai, China) was used to assessed normal sperm morphology. Sperm that were linear from head to tail were considered to have normal sperm morphology. Abnormal sperm morphologies included spermatids, sperm with bent necks, and sperm with any other head deformation. Each slide contained 300 sperm; 5) Aniline blue staining solution (Sangon Biotech, Shanghai, China) was used to measure sperm acrosome. Sperm stained with aniline blue with intact acrosome or damaged acrosome. About 100 sperm were examined subjectively using a phase contrast microscope (BM2000, NOVEL, China) at 1000 × (Oil immersion); 6) Sperm concentration was determined using a hemocytometer. Each semen quality indicators were tested three times. Semen quality factor (SQF) values of each drakes were calculated according to the following equation: $SQF = \text{semen volume (for each drake evaluation; mL)} \times \text{sperm concentration (} \times 10^6/\text{mL)} \times \text{live and morphologically normal sperm (\%)} (Liu et al., 2008)$. Based on 364 d old SQF values, the measured drakes were divided into high-quality semen (HQS, n = 15) and low-quality semen (LQS, n = 15).

Seminal Plasma Collection and Preparation

At 385 d old, three drakes with similar body weight and physiological status were selected in the HQS and LQS groups, respectively. The SQF values of these selected drakes maintained stable at 210 d old and 364 d old. After artificial semen collection, seminal plasma was immediately separated by centrifugation at 2,000 g for 5 min at 4°C. The supernatant was centrifuged at 12,000 g for 20 min at 4°C for twice. The absence of sperm in the seminal plasma was confirmed by observation under a microscope, and then the seminal plasma was stored at -80°C until protein extraction.

Total Protein Extraction, Quality Test and Trypsin Treatment

Bovine Serum Albumin (BSA) standard protein solution was prepared according to the instructions of Bradford protein quantitative kit (Tiangen Biotechnology, Beijing, China), with gradient concentration ranged from 0 to 0.5 g/L. BSA standard protein solutions and sample solutions with different dilution multiples were added into 96-well plate to fill up the volume to 20 μ L, respectively. Each gradient was repeated three times. The plate was added 180 μ L G250 dye solution quickly and placed at room temperature for 5 min, the absorbance at 595 nm was measured. The standard curve was drawn with the absorbance of standard protein solution and the protein concentration of the sample was calculated. Twenty microgram of the protein sample was loaded on a 12% SDS-PAGE gel electrophoresis, where the concentrated gel was performed at 80 V for 20 min, and the separation gel was performed at 120 V for 90 min. The gel was stained by Coomassie brilliant blue R-250 and decolorized until the bands were visualized clearly.

Then, trypsin treatment was performed on each protein sample. In briefly, each protein sample was taken and the volume was made up to 100 μ L with DB lysis buffer (8 M Urea, 100 mM TEAB, pH = 8.5). Trypsin and 100 mM TEAB buffer were added, the sample was mixed and digested at 37°C for 4 h. Then, the trypsin and CaCl₂ were added and digested overnight. Formic acid was mixed with digested sample, adjusted pH < 3, and centrifuged at 12,000 \times g for 5 min under room temperature. The supernatant was slowly loaded to the C18 desalting column, washed with washing buffer (0.1% formic acid, 3% acetonitrile) 3 times, then added elution buffer (0.1% formic acid, 70% acetonitrile). The eluents of each sample were collected and lyophilized.

Liquid Chromatography Coupled with Tandem Mass Spectrometry (LC-MS/MS) Analysis

Mobile phase A (100% water, 0.1% formic acid) and B solution (80% acetonitrile, 0.1% formic acid) were prepared. The lyophilized powder was dissolved in 10 μ L of solution A, centrifuged at 14,000 \times g for 20 min at 4°C, and 1 μ g of the supernatant was injected into a home-

made C18 Nano-Trap column (4.5 cm \times 75 μ m, 3 μ m). The peptides were separated using a linear gradient elution in a home-made analytical column (15 cm \times 150 μ m, 1.9 μ m). The separated peptides were analyzed by Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific, Inc.) with a Nanospray Flex (ESI) ion source, spray voltage of 2.1 kV and ion transport capillary temperature of 320°C. Full scan range from m/z 350 to 1500 with resolution of 60,000 (at m/z 200), an automatic gain control (AGC) target value was 3×10^6 and a maximum ion injection time was 20 ms. The top 40 precursors of the highest abundant in the full scan were selected and fragmented by higher energy collisional dissociation (HCD) and analyzed in MS/MS, where resolution was 15,000 (at m/z 200), the AGC target value was 1×10^5 , the maximum ion injection time was 45 ms, a normalized collision energy was set as 27%, an intensity threshold was 2.2×10^4 , and the dynamic exclusion parameter was 20 s.

The Identification and Quantitation of Proteins

Raw data were used for label-free quantitation of proteins analyzation by Proteome Discoverer (PD 2.2, Thermo Fisher Scientific, Inc.) against the *Anas platyrhynchos* reference proteome (Anas_platyrhynchos_uniprot_2021_7_15.fasta). The search parameters were set as follows: the mass tolerance for precursor ion was 10 ppm and the mass tolerance for product ion was 0.02 Da. In PD 2.2 software, carbamidomethyl was specified as fixed modifications, oxidation of methionine (M) was specified as dynamic modification, and acetylation was specified as N-Terminal modification. A maximum of 2 missed cleavage sites were allowed. In order to improve the quality of analysis results, the software PD 2.2 was further used to filtered the retrieval results: Peptide Spectrum Matches (PSMs) with a credibility of more than 99% were identified PSMs. The identified protein contains at least one unique peptide. The identified PSMs and proteins were retained and performed with FDR less than 1.0%. The protein quantitation results were statistically analyzed by t-test. The DEPs were filtered based on $P < 0.05$ and $|\log_2FC| > 1$.

The Functional Analysis of Protein and DEPs

Gene Ontology (GO) and InterPro (IPR) functional analysis were conducted using the InterProScan program against the non-redundant protein database (including Pfam, PRINTS, ProDom, SMART, ProSite, and PANTHER) (Jones et al., 2014), and the databases of Clusters of Orthologous Groups (COG) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to analyze the protein family and function pathway. DEPs were used for Volcanic map analysis, cluster heat map analysis and enrichment analysis of GO and KEGG (Huang da et al., 2009). The STRING 10 database (<http://string-db.org/>) was employed to identify the relationship between the DEPs identified in this

Table 1. Semen quality parameters of drakes in high and low semen groups.

Items	Semen variables						
	EV (ml)	SV (%)	SM (%)	SC (10^9 /mL)	MAS (%)	AD (%)	SQF values
HQS (n = 15)	0.60 ± 0.15	94.40 ± 0.60	91.55 ± 1.02	4.01 ± 0.79	5.79 ± 0.69	5.07 ± 1.08	2083.79 ± 543.82
LQS (n = 15)	0.28 ± 0.10	94.59 ± 0.64	90.49 ± 1.65	3.46 ± 0.21	7.05 ± 0.91	5.50 ± 2.71	767.60 ± 204.01
<i>P</i> value	< 0.01	0.352	0.037	0.027	0.037	0.53	< 0.01

AD, acrosome deformity; EV, ejaculate volume; HQS, high-quality semen; LQS, low-quality semen; MAS, morphological abnormal sperm; SC, sperm concentration; SM, sperm motility; SV, sperm viability.

study (Franceschini et al., 2013). All the network visualization was performed using Cytoscape (version 3.2.1) (Smoot et al., 2011).

Statistics Analysis

Statistical analysis was performed using the SPSS 23.0 software (IBM, USA). The means of the volume, sperm viability, sperm motility, sperm concentration, acrosome deformity, morphological abnormal sperm and SQF values between HQS-vs-LQS were subjected to ANOVA testing, the means were assessed for significance by Tukey's test, and t-test was used to analyze the significance between the two groups. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Semen Quality Analysis between HQS-vs-LQS

As shown in Table 1, drakes in the HQS had higher ejaculate volume ($P < 0.01$), sperm motility ($P = 0.037$), and sperm concentration ($P = 0.027$), whereas those in the LQS had higher morphological abnormal sperm ($P = 0.037$). In addition, compared with the LQS, the SQF value was significantly higher in the HQS ($P < 0.01$).

Seminal Plasma Protein Parameters between HQS-vs-LQS

Compared to the LQS, drakes in the HQS had higher protein solution volume but lower total protein and protein concentration (Table 2). Strips of the SDS-PAGE gel were clear (Supplementary Figure 1), which indicated that the gel could be used for in-gel digestion and peptide extraction.

Annotation and Analysis of Identified Seminal Plasma Proteins between HQS-vs-LQS

In the present study, a total of 4,779 peptides belonging to 745 proteins were identified after filtering with stated criteria (Figure 1A). The results showed that identified proteins localized in a wide variety of subcellular structures, mainly including the extracellular (151 proteins, accounting for 32.54%), cytoplasm (79 proteins, accounting for 17.03%), plasma membrane (70

Table 2. Seminal plasma protein parameters of drakes in high and low semen groups.

Group	Protein solution volume (μ L)	Total protein (μ g)	Protein concentration (μ g/ μ L)
HQS (n = 3)	746.7	983.7	1.28
LQS (n = 3)	480	1530	2.96

HQS, high-quality semen; LQS, low-quality semen.

proteins, accounting for 15.09%), nucleus (53 proteins, accounting for 11.42%), lysosome (35 proteins, accounting for 7.54%), and endoplasmic reticulum (27 proteins, accounting for 5.82%) (Figure 1B). Moreover, the results of protein function annotation showed that 584 proteins identified in HQS-vs-LQS were annotated (Figure 1C; Supplementary Table S1). Of these, 351 (~47.1%) proteins were common expressed in all 6 samples. The identified proteins were annotated and classified into three categories through GO database, including the biological process (BP), cellular component (CC), and molecular function (MF). In the HQS-vs-LQS, most of identified proteins were enriched in proteolysis (BP), membrane (CC), protein binding (MF), and carbohydrate metabolic process (BP) (Figure 1D). Subsequently, KEGG enrichment analysis showed that the identified proteins were mainly enriched in transport and catabolism, cellular community-eukaryotes, cell growth and death, signaling molecules and interaction, folding, sorting and degradation, global and overview maps, and carbohydrate metabolism pathways (Figure 1E).

Identification of the DEPs between HQS-vs-LQS

In the present study, PCA score plots showed that HQS and LQS samples were clustered well (Figure 2A). In the HQS-vs-LQS, 55 DEPs were identified. Among them, 40 DEPs were up-regulated and 15 DEPs were down-regulated (Figure 2B; Supplementary Table S2). The hierarchical clustering map also recapitulated the different protein expression patterns between HQS-vs-LQS (Figure 2C).

Comprehensive Functional Analysis of DEPs Identified between HQS-vs-LQS

To determine the function of DEPs, we annotated them into 3 main categories according to terms of the

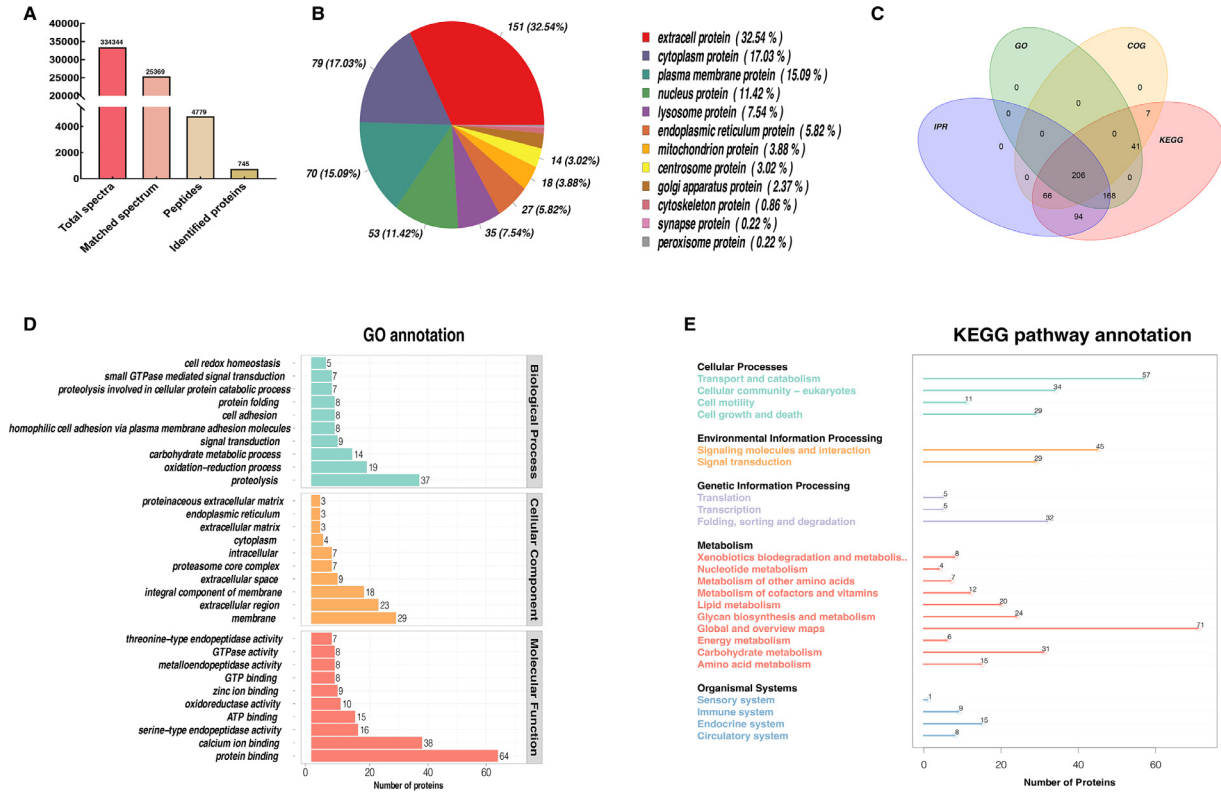


Figure 1. Statistics and functional annotation of the identified seminal plasma proteins between HQS-vs-LQS. (A) Statistical information from tandem mass spectrometry and proteins identification. (B) Subcellular localization of the identified proteins. (C) Functional annotations of proteins in public databases. (D) GO enrichment analysis of the identified proteins. (E) KEGG analysis of the identified proteins.

GO database, including the BP, CC, and MF (Supplementary Table S3). In the HQS-vs-LQS, most of DEPs were enriched in transmembrane transport (BP), extracellular matrix structural constituent (MF), transferase activity, transferring acyl groups other than amino-acyl groups (MF), transmembrane transporter activity (MF), and integral component of membrane (CC) ($P < 0.05$) (Figure 3A). Furthermore, KEGG enrichment analysis was conducted to obtain more detailed information (Supplementary Table S4). The top 20 enriched KEGG pathways were listed in Figure 3B. In the

HQS-vs-LQS, the most enriched KEGG pathways were apoptosis, tyrosine metabolism, glycerophospholipid metabolism, and sulfur metabolism pathways.

Network Analysis of the DEPs Involved in Regulating the Semen Quality

To further identify the potential key proteins regulating semen quality, the DEPs from the HQS-vs-LQS were merged to construct the PPI network (Figure 4A). The

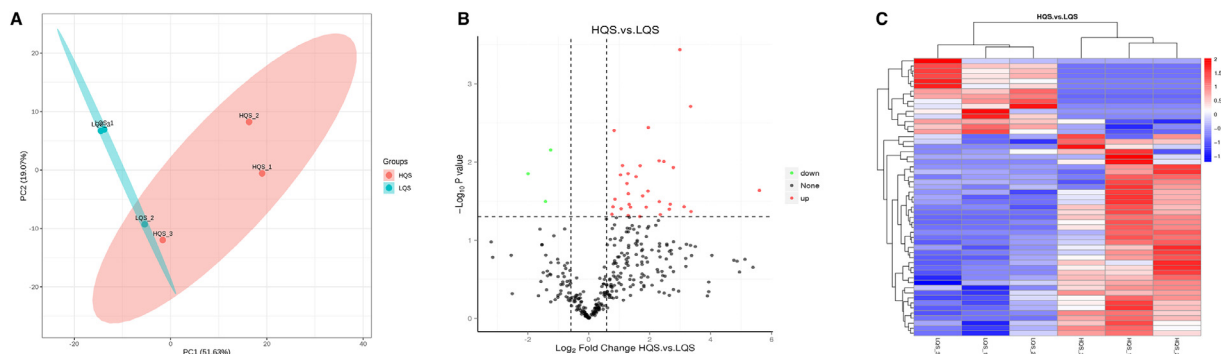


Figure 2. Principal component analysis and DEPs analysis for seminal plasma proteins between HQS-vs-LQS. (A) According to the individual's distribution of PC1 and PC2, individual points were grouped into ellipses. PC1, principal component 1; PC2, principal component 2; HQS1-3, samples of high-quality semen group; LQS1-3, samples of low-quality semen group. (B) Volcano diagram of DEPs between HQS-vs-LQS. The abscissa represents the fold change of protein expression. The ordinate represents the statistical significance of the difference in protein expression. The red dot in the figure indicates the up-regulated proteins with significant differential expression, and the green dot indicates the down-regulated proteins with significant differential expression. The dotted lines represent the cutoffs that determine significance of protein abundance. (C) Hierarchical clustering of DEPs, the abundance values were log2-transformed and normalized by Z-score transformation.

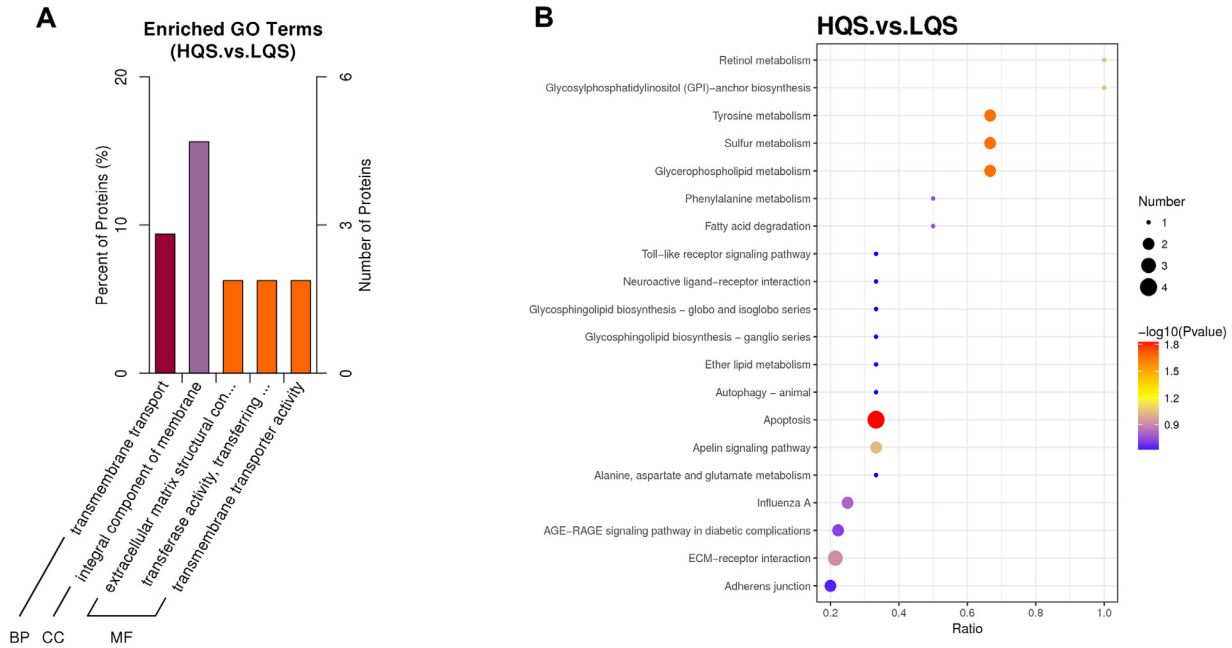


Figure 3. GO terms and KEGG pathways enriched by DEPs between HQS-vs-LQS. (A) Enriched GO terms between HQS-vs-LQS. The results were summarized in three main categories: biological process (BP), cellular component (CC), and molecular function (MF). The x-axis indicates the second-level GO term, and the y-axis indicates the number of proteins. (B) Top 20 enriched KEGG pathways of DEPs. The Ratio is the ratio of the number of DEPs in the pathway and the total number of proteins in the pathway. The higher the Ratio, the higher is the degree of enrichment.

PPI network consisted of 43 nodes and 67 edges. The top highest degree proteins included P19140 (Alpha-enolase), R0KUV7 (Calreticulin), and R0K3X3 (Solute carrier family 2, facilitated glucose transporter member 5) (Figure 4B). P19140 (Alpha-enolase) (Degree = 15) showed higher degrees of connection degrees than other DEPs and was considered core protein regulating semen quality. Notably, it was postulated that key protein P19140 (Alpha-enolase) might can control the semen quality of drakes by regulating the expression of proteins related to apoptosis pathway (Figure 4C).

DISCUSSION

With the widespread application of AI technology in the duck industry, the demand for high-quality semen of drakes is increasing. In the present study, significant differences in semen quality parameters (ejaculate volume, sperm motility, concentration, morphological abnormal sperm, and SQF values) and semen plasma protein parameters of drakes were observed between the HQS-vs-LQS, suggested that the semen quality of drakes was closely related to seminal plasma proteins. A previous study showed that 1,141 seminal fluid proteins were identified in Red junglefowls (Borziak et al., 2016). In the present study, 745 proteins were identified in the seminal plasma between the HQS-vs-LQS based on proteomic analysis, and functionally annotated 584 proteins, which were mainly localized in the extracellular (32.54%), cytoplasm (17.03%), and plasma membrane (15.09%), indicating that they mainly had functions in the above cell regions. Furthermore, 55 DEPs were identified in the seminal plasma between the HQS-vs-LQS. Recently, proteomic studies in mammals and poultry

seminal plasma have identified some important proteins associated with the semen quality (Gaitskell-Phillips et al., 2021; Jia et al., 2021; Słowińska et al., 2021). These results indicated that the seminal plasma proteins play a major role in regulating semen quality of drakes.

To further reveal the biological implications of these identified DEPs, functional analyses were performed. Our results showed that the DEPs between the HQS-vs-LQS were mainly enriched in the GO terms related to the transmembrane transport, extracellular matrix structural constituent, transferase activity, transferring acyl groups other than amino-acyl groups, transmembrane transporter activity, and integral component of membrane, which were corresponded to the seminal plasma proteins functions of transport, membrane structure, enzymatic activity, and protection. Similar findings were reported in other species like humans (Dias et al., 2019), pigs (Recuero et al., 2019), and chickens (Li et al., 2020). In addition, the DEPs in seminal plasma between HQS-vs-LQS were mainly enriched into four KEGG pathways, including apoptosis, tyrosine metabolism, glycerophospholipid metabolism, and sulfur metabolism pathways. Previous studies have shown that the apoptosis pathway plays an important role in the regulation of semen quality (Luna et al., 2017; Mehdi-pour et al., 2020). Further analysis showed that R0L8P5 (S-(hydroxymethyl) glutathione dehydrogenase), R0KCV3 (Peroxiredoxin-4), R0JKW0 (Cytochrome c), and P19140 (Alpha-enolase) were found to be significant up-regulated in LQS, which suggested these proteins might play an important role in regulating drake semen quality.

When we tried to explain how these DEPs regulates semen quality of drakes, results of network analysis suggested that protein P19140 (Alpha-enolase) and

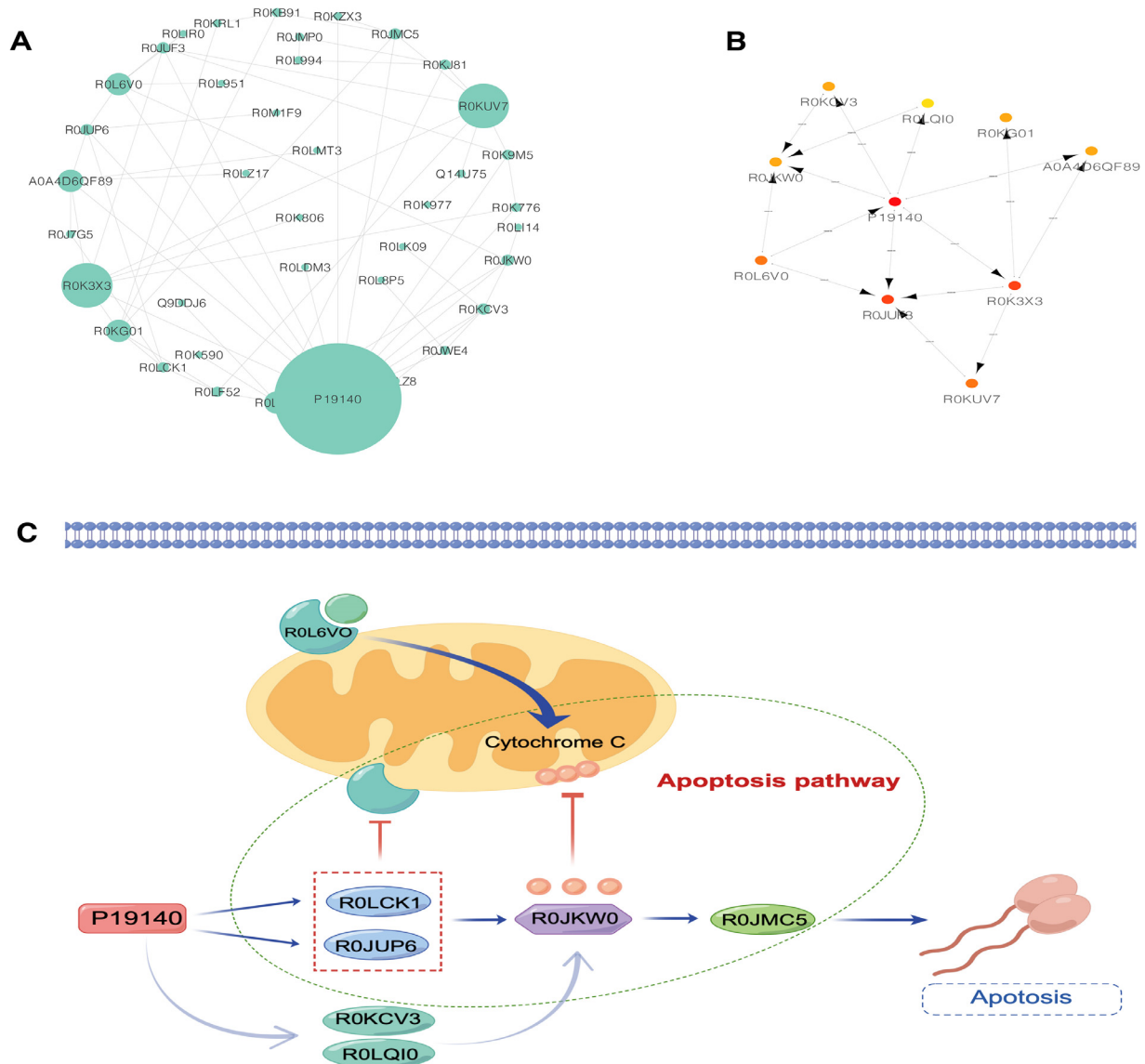


Figure 4. Network analyses of DEPs between HQS-vs-LQS. (A) PPI enrichment analysis showing physical interactions formed among DEPs in the HQS-vs-LQS. (B) Analysis of subnet modules in PPI. (C) Regulation network construction involved in semen quality of drakes, including P19140 (Alpha-enolase), R0LCK1 (Cathepsin C), R0JUP6 (Cathepsin D), R0JKW0 (Cytochrome c), R0JMC5 (Tubulin alpha chain), R0L6V0 (Proteasome subunit beta), R0KCV3 (Peroxiredoxin-4), and R0LQIO (Activation peptide).

apoptosis signaling pathway could play a critical role in affecting semen quality of drakes. In the present study, the DEPs identified in seminal plasma between HQS-vs-LQS were significantly enriched in apoptosis pathway. In recent years, studies in goat (Liu et al., 2019), male rabbit (Abdelatty et al., 2020), and roosters (Du et al., 2021) have also demonstrated the important role of this pathway in the control of semen quality. Furthermore, almost all DEPs enriched in this pathway, including R0JKW0 (Cytochrome c), R0JMC5 (Tubulin alpha chain), R0LCK1 (Cathepsin C), and R0JUP6 (Cathepsin D), were significantly up-regulated in the seminal plasma of LQS group, which indicated that apoptosis pathway-related functions were promoted. One recent report supported the notion that the R0JMC5 (Tubulin alpha chain) was critical for regulating the sperm motility (Bhagwat et al., 2014). Moreover, study have shown that protein levels of R0JKW0 (Cytochrome c) was significantly associated with sperm quality in rat

(Meneghini et al., 2022). Notably, we found that the key protein P19140 (Alpha-enolase) could control the semen quality of drakes by regulating the expression of apoptosis-related proteins. A previous study has shown that downregulating Alpha-enolase (*ENO1*) expression *via* small RNA interference resulted in the disturbance of spermatogenesis through apoptosis (Xiong et al., 2021). In another research, the finding showed that Alpha-enolase expression (*ENO1*) was correlated with sperm motility (Li et al., 2016). In addition, increasing evidence in male species demonstrated that Alpha-enolase can be used as a marker to evaluate semen quality (He et al., 2014; Jiang et al., 2015). These results suggested that the overactivation of apoptosis signaling pathway by protein P19140 (Alpha-enolase) in seminal plasma of drakes may be one of the reasons for the decreased semen quality.

In conclusion, we constructed the first protein profiles of seminal plasma of drakes in high and low-quality

semen groups. A total of 55 DEPs were identified, including 40 up- and 15 down-regulated proteins, between high and low-quality semen drakes. Furthermore, bioinformatic analysis suggested that these identified DEPs in the apoptosis pathway was crucial for drake semen quality. Notably, the key protein P19140 (Alpha-enolase) might can control the semen quality of drakes by regulating the expression of proteins related to apoptosis pathway, indicating that it may be a candidate protein related to drake's semen quality. These results provide novel insights into the mechanisms regulating the semen quality of drakes.

ACKNOWLEDGMENTS

Funding: This study was supported by China Agricultural Research System of MOF and MARA (CARS-42-4) and Key Technology Support Program of Sichuan Province (2021YFYZ0014) for the financial support.

DISCLOSURES

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102130.

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