Mass spectrometry-based metabolic profiling for identification of biomarkers in serum related to the change of laying ducks in different physiological periods

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ABSTRACT
Eggs are a major animal protein source for humans. Egg formation is a complex process involved in coordinating various tissues and organs, while blood is necessary for gonad differentiation and egg formation. In this study, non-targeted metabolomics technology was employed to determine the serum metabolites at the time point of 70 days (before laying period, T0), 130 days (pre-laying period, T1), 300 days (peak laying period, T2), and 340 days (post laying period, T3) in female ducks, to provide comprehensive metabolic profiling in reflecting the overall temporal changes. The results showed significant differences in the metabolites of different comparison groups (T0 vs. T1, T2 vs. T3, and T0 & T1 vs. T2 & T3), supporting the main physiological responses in ducks are differential among laying periods. L-Aspartic Acid, Biotin, and Thyroxine can be considered the potential biomarkers related to sexual maturity. L-Lysine, Lumichrome, Thiamine, Riboflavin, and Pyridoxine may be biomarkers related to egg formation. Sorbitol, Isorhamnetin, Naringenin, and Hydroxykynurenine were the biomarkers associated with the decline of egg production. In addition, the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis showed that sexual maturity was mainly involved in the biosynthesis of secondary plant metabolites, Tricarboxylic Acid Cycle, Mineral absorption, pentose phosphate pathway, and Oxocarboxylic acid metabolism. Egg formation disturbed amino acid metabolism, vitamin metabolism, and carbohydrate metabolism. The neuroactive ligand-receptor interaction pathway may affect egg production. These findings can detect the different physiological statuses in female ducks and provide a theoretical basis for increasing egg production, and ensuring sustainable farming.

HIGHLIGHTS
• The main physiological responses in ducks are differential among laying periods.
• 3, 6, and 4 biomarkers related to sexual maturity, the formation of the egg, and the decline of egg production, respectively.
• Sexual maturity was mainly involved in the biosynthesis of secondary plant metabolites, Tricarboxylic Acid Cycle, Mineral absorption, pentose phosphate pathway, and Oxocarboxylic acid metabolism, and egg formation disturbed amino acid metabolism, vitamin metabolism, and carbohydrate metabolism. while the neuroactive ligand-receptor interaction pathway may affect egg production.

1. Introduction
Eggs have long been recognised as an excellent animal-based protein source for humans (Miranda et al. 2015). Moreover, the egg also contain carbohydrates, easily digestible fats, minerals, and vitamins (Huopalahti et al. 2007; Sugino et al. 2018). Due to the presence of these rich nutrients and biological activities, eggs play a vital role in human health, and disease prevention and treatment (Bertechini and Mazzuco 2013). In the poultry industry, egg production efficiency can be considered as most important trait as it directly influences economic benefits (Liu et al. 2019). Thus, it is a critical task for researchers and farmers to improve egg production performance in layer farming. The formation of eggs is a very complex process, which is affected by numerous factors,
including genetic (Brundage et al. 1996), environmental (Al-Saffar and Rose 2002), endocrine factors (Du et al. 2020), and the physicochemical characteristics of the diets (Pérez-Bonilla et al. 2012), and under their combined action (Yang et al. 1989).

The eggs formation requires the coordination of multiple organs and tissues that are regulated by a large number of hormones. Overall, the yolk, albumen, and eggshell are formed in the ovary, oviduct, and uterus, respectively (Kaspers 2016). The major egg yolk proteins (Speake et al. 1998) and lipids (Griffin 1992) are synthesised from the liver, while other yolk proteins (Moran 1987) and carbohydrates (Harmon and McLeod 2001) are absorbed into the blood from the intestines, and finally enter the oocyte through the blood transportation. In addition, the egg white proteins are secreted directly from the tubular gland cells into the lumen of the oviduct (Deeley et al. 2019). Before laying, hormonal changing causes gonadal maturation in the pubertal transition of female poultry. The release of Gonadotropin-releasing hormone (GnRH) promotes the maturation of the animal reproductive system and regulating hormonal secretion (Sisk and Foster 2004). Simultaneously, Follicle-stimulating hormone (FSH) can promote the proliferation of ovarian somatic cells (Sanchez-Bringas et al. 2006), which increases in the ovarian volume, and the growth rate become rapid under the action of serum luteinizing hormone (LH) (Zhu et al. 2015). In addition, when the ovary is fully developed, LH (Yoshimura et al. 1993), Progesterone (P4) (Yoshimura and Bahr 1991), Oestradiol (E2), and FSH (Ma Y et al. 2020) promote ovulation, which ultimately increase of egg production. Besides, E2 can also promote yolk formation, preparing for the deposition of yolk in follicles (Li et al. 2014). Due to these hormonal secretions, the number and size of follicles on the ovary can increase for subsequent egg-laying.

Various reports have revealed the underlying mechanism involved in the laying process of birds. Some of them have identified genes associated with functional differences in tissues and organs during different laying periods. Upregulation of intestinal gene expression of laying hen was examined by transcriptome analysis during the post- and peak-phase of production (Wang et al. 2019), and in the geese’ ovary (Ding et al. 2015), hypothalamus (Gao et al. 2015), and hypophysis (Gao et al. 2015) during the pre-laying and laying period showed organs involvement in production performance. Similarly, transcriptome analysis showed the upregulation of the genes related to ovarian development in the early laying, peak laying, and post laying period in Muscovy ducks (Hu et al. 2021). However, these studies were based on a single organ during particular early, peak, and post laying time points. These studies were insufficient to provide overall changes from the growing to the laying period in live poultry.

Being an important and easily accessible biological fluid, blood serum is a primary carrier of small molecules in the body (Cui et al. 2013). The serums are rich in source of biochemical indicators that can reflect the overall changes in live animals (Srinivas et al. 2001; Pedlar et al. 2019), and can be used to diagnose, assess disease severity or risk, and guide other clinical interventions (Ray et al. 2010). So, the metabolites in serum can also reflect the dynamic physiological and biochemical changes of the body. From this, our research used non-targeted metabolomics to systematically profile the metabolites in serum of ducks at 70 days (before laying period, T0), 130 days (pre-laying period, T1), 300 days (peak laying period, T2), and 340 days (post laying period, T3). The different serum components were analysed with the physiological status at the same period to explore the following: (1) biomarkers associated with sex maturity; (2) biomarkers leading to the decline of egg production; and (3) biomarkers associated with nutrients in eggs. Therefore, using biomarkers in serum could provide a comprehensive view of physiological and biochemical reactions and changes in vivo. Our work may lay a foundation for the study of the molecular regulation mechanism of female ducks and promote duck egg production performance, decrease costs, improve nutrients, and ultimately contribute to the development of the duck industry.

2. Materials and methods

2.1. Sample preparation

The ducks (NongHua-GF2 strain) used in the present study were provided by the Waterfowl Breeding Experimental Farm of Sichuan Agricultural University, Ya’an, China. Total 400 ducks were fed with the same diet and management process, and the nutrient composition is provided in Supplementary Table S1. At 70, 130, 300, and 340 days of duck age, 4 mL blood samples were taken from the vein under the wings of each duck. Blood samples were subjected to centrifuged for 5 minutes at 4500 xg at 4 °C to obtain serum and stored at −20 °C for further analysis. All duck work was conducted following a protocol approved by the animal ethics and welfare committee (AEWC) of Sichuan Agricultural University, China.
2.2. Preparation of the serum samples for metabolomics assay

According to the different ages, 8 ducks were randomly selected from each group (T0, T1, T2, and T3), based on the previous grouping strategy (Figure 1). Their serum samples were thawed at 4°C from the freezer conditions. Transfer 100 μL of each sample into 2 mL centrifuge tubes, then add 400 μL of ethanol (−20°C) to each tube and vortex for 60 s; afterward, centrifuge at 12,000 xg at 4°C for 10 min, take 450 μL of supernatant to a 2 mL centrifuge tube, add 450 μL of n-hexane, and vortex for 1 min; then centrifuge at 12,000 xg at 4°C for 10 min, take all the lower layer liquid into a 2 mL centrifuge tube, and concentrate to dryness by a vacuum concentrator. Dissolve samples with 150 μL 2-chlorobenzalanine (4 ppm) 80% methanol solution, and the supernatant was filtered through 0.22 μm membrane (Jin Teng, China) to obtain the prepared samples for LC-MS. Take 20 μL from each sample to the quality control (QC) samples, and use the rest for the LC-MS analysis.

2.3. LC-Ms analysis

The analytical system consists of a Thermo Vanquish system equipped with an ACQUITY UPLC® HSS T3 (150 × 2.1 mm, 1.8 μm, Waters, USA) column maintained at 40°C. The temperature of the autosampler was 8°C. The mobile phase consisted of 0.1% formic acid in the water and 0.1% formic acid in acetonitrile (A1) or 5 mM ammonium formate in water and acetonitrile (A3) was carried with elution gradient as follows: 0 ~ 1 min, 2% A1/A3; 1 ~ 9 min, 13.5 ~ 14 min, 98%~2% A1/A3; 14 ~ 20 min, 2%

A1-positive model (14 ~ 17 min, 2% A3-negative model) (Sangster et al. 2006; Zelena et al. 2009; Dunn et al. 2011).

The ESI-MSn experiments were executed on the Thermo Q Exactive Plus mass spectrometer (Q Exactive HF-X, Thermo Fisher Technologies, Shanghai, China). The following parameters were employed: positive ion spray voltage 3.50 kV, negative ion spray voltage −2.5 kV, Sheath gas 30 arbitrary units, auxiliary gas 10 arbitrary units, and capillary temperature 325°C. The analyser scanned over a mass range of m/z 81-1000 for a full scan at a mass resolution of 70,000. Data-dependent acquisition (DDA) MS/MS experiments were performed with a highenergy collision dissociation (HCD) scan. The normalised collision energy was 30 eV. Dynamic exclusion was implemented to remove some unnecessary MS/MS spectra information.

2.4. Metabolomic data analysis

After the original data is converted into MZXML format, a series of operations, including peaks identification, peaks filtration, and peaks alignment, were conducted using the XCMS package of R (v3.3.2). The obtained data matrix was exported to Microsoft Excel for subsequent analysis. Ion peaks with a coefficient of variation over 30% were deleted, and the batch normalisation of the intensity of the data was carried out. Simac version 14.1 for Windows (Umetrics, Umea, Sweden) was used to perform principal component analysis (PCA) and orthogonal projections to latent structures-discriminant analysis (OPLS-DA) on the metabolic data of all serum samples to assess the metabolite diversity between and within-group samples. Metabolites were annotated using the HumanMetabolome Database (HMDB), Metlin, Massbank, Lipymaps, Mzclound, and the self-built standard product Database. The screening criteria for differential metabolites were P-value ≤ 0.05 and VIP ≥ 1. Agglomerate hierarchical clustering was performed for each data set using the PHEATMAP package in R (v3.3.2). The regularised partial correlation network of metabolites was constructed using Cytoscape (v3.9.0).

2.5. Statistical analyses

Data were analysed using the SPSS version 22.0 for Windows (SPSS, Inc., Chicago, IL, USA) and R (v3.3.2). The data were presented as means ± SEM, and differences were considered statistically significant at P < 0.05.
3. Results and analysis

3.1. Quality control and PCA analysis of metabolites

The base peak chromatogram (BPC) was found different between T0 and other groups (T1, T2, T3), and most peaks have a higher abundance for T1, T2, T3 than for the extracts from T0. The BPC results showed that the system has high detection stability and good test data reliability, depending on serum samples of different laying periods based on the positive and negative ion modes. The quality control steps were performed during the mass spectrometry-based metabolomics to obtain reliable and high-quality metabolomics data. All QC samples were gathered together through the PCA plot, and the repeatability is good, indicating that the LC-MS system was stable (Figure 2(A)). Then, QC data normalised the raw data to omit the batch effects (Supplementary Table S2).

The OPLS-DA of normalised data for serum samples was analysed based on positive and negative ion mode data. The results demonstrated that all 32 samples are gathered into 4 clusters, consistent with the groups (T0, T1, T2, and T3) (Figure 2(B)). The OPLS-DA showed that all groups distinguished entirely from group T0. The longer days old (Group T1, T2, and T3), the better could be distinguished from group T0, indicating that the overall trend of metabolites changes with the increased age of laying birds. Since the OPLS-DA method has grouped the samples already when building the model, the results may have been overfitting. Permutations plot can effectively evaluate whether the current model is overfitted or not (Figure 2(C)). We can observe that (1) all Q2 points are lower than the rightmost original Q2 point; (2) The regression line of Q2 is less than or equal to 0 at the intersection of ordinates. The evaluation parameters (R2, Q2) obtained from the PLS-DA model after 10 interaction cycle validations are listed in Table 1. The R2 and Q2 values usually reach the following standards. In detail, their values exceeded 0.5, and their differences were not significant. Their values were less than 1, supporting the model established was reliable, and the model has better prediction ability.

3.2. Correlation analysis between metabolites annotated

To give an overall impression, the full correlation matrix between metabolites annotated is depicted in a heatmap (Supplementary Figure S2, Supplementary Table S3). Then, the regularised partial correlation network was constructed using metabolites with a correlation greater than 0.5 (Figure 3, Supplementary Table S4). The topology of the network indicates that different metabolite groups occupied different positions; dense interactions occurred between both inter- and intra- metabolite groups.

3.3. The metabolic changes in duck serum between before laying period (T0) and pre-laying period (T1)

From before the laying period (T0) to the pre-laying period (T1), gonadal development tended to be completed, and the ovary had proper signs of fertility. Under the positive ion mode, there were a total of 7088 differential metabolites with 2374 up-regulated and 4714 down-regulated; while under the negative ion mode, there were a total of 3078 differential metabolites with 1316 up-regulated and 1762 down-regulated for the comparison groups of T0 vs. T1 (Supplementary Table S5, Figure 4(A)). Furthermore, the differences in metabolites in the negative ion mode are more prominent. After annotating, 264 differential metabolites were screened (Supplementary Table S6, Figure 4(B)), with 125 up-regulated and 139 down-regulated.

There were 201 metabolic pathways (Supplementary Table S7, Supplementary Figure S3) in group T0 vs. T1, and we listed the top 20 highest enrichment of the KEGG pathway (Figure 4(C)). The metabolic pathways of beta-Alanine metabolism and Neuroactive ligand-receptor interaction were the most abundant, and L-Aspartic acid, Uracil, Dihydrouracil, and gamma-Aminobutyric acid were enriched in beta-Alanine metabolism. In contrast, L-Aspartic acid, Taurine, gamma-Aminobutyric acid, Tyramine, and Norepinephrine were enriched in Neuroactive ligand-receptor interaction pathways.

3.4. The metabolic changes in duck serum between peak laying period (T2) and post laying period (T3)

There was a decline in egg production from the peak laying period (T2) to the post laying period (T3). Under the positive ion mode, there were 6626 differential metabolites with 3440 up-regulated and 3186 down-regulated; while under negative ion mode, there were 3177 differential metabolites with 2153 up-regulated and 1024 down-regulated in the group of T2 vs. T3, respectively (Supplementary Table S5, Figure 5(A)). Moreover, the differences in metabolites in the negative ion mode are more prominent. After being
Figure 2. Principal component analysis and partial least squares discriminant analysis (OPLS-DA) of metabolomic data. (A) Quality control under the positive and negative ion mode. (B) OPLS-DA is based on the normalized metabolomics data under the positive and negative ion modes. (C) The permutations plot for the test of the OPLS-DA model under positive and negative ion mode. The abscissa represents the first principal component, and the ordinate represents the second. Each point in the graph represents a sample, and the samples from the same group were depicted in the same color. The distance between each point indicates the difference or similarity between the metabolites in the sample.
annotated, there were 282 differential metabolites screened (Supplementary Table S6, Figure 5(B)), with 199 up-regulated and 83 down-regulated.

There were 217 metabolic pathways (Supplementary Table S8, Supplementary Figure S4) in group T2 vs. T3, and we listed the top 20 highest enrichment of the KEGG pathway (Figure 5(C)). The Protein digestion and absorption and Biosynthesis of plant secondary metabolites pathways were the most abundant, and L-Glutamic acid, L-Lysine, L-Aspartic acid, L-Glutamine, L-Methionine, L-Tryptophan were enriched in the Protein digestion and absorption pathway. In contrast, L-Glutamic acid, Oxoglutaric acid, L-Lysine, L-Aspartic acid, and L-Arginine were enriched in the Biosynthesis of plant secondary metabolites pathway.

3.5. The metabolic changes in duck serum between the growing period (T0&T1) and laying period (T2&T3)

From the growing period (T0&T1) to the laying period (T2&T3), ducks’ physiological and biochemical conditions have undergone tremendous changes in the process, and there was the accumulation of nutrients in

| Mode | pre | R^2X(cum) | R^2Y(cum) | Q^2(cum) |
|------|-----|-----------|-----------|----------|
| ESI+ | 2   | 0.423     | 0.989     | 0.871    |
| ESI- | 2   | 0.250     | 0.987     | 0.927    |

ESI, electrospray ionisation.

OPLS-DA, partial least squares discriminant analysis.

R^2X, the interpretability of model X variable (independent variable).

R^2Y, the interpretability of model Y variable (dependent variable).

Q^2, the predictability of the model.

Figure 3. Regularized partial correlation network of compounds with the absolute value of correlative coefficient greater than 0.5. Each node represents a compound, and different color represents different metabolites group. The size of the node represents the number of metabolites, and the larger the dot, the more metabolites correlative. Each edge represents the strength of partial correlation between two compounds after conditioning on all other compounds in the datasets, and the solid represents positive correlation while the broken line represent negative correlation.
Figure 4. Differential metabolites and corresponding pathways between T0 and T1. (A) The volcano maps exhibited the differential metabolites between T0 vs. T1. The upper panel results from positive ion mode and the down panel are results from negative ion mode. Moreover, the X-axis represents -Log10 (p), and the Y-axis represents log2(fold change). Every point represents one metabolite. Furthermore, black points represent metabolites with no significant difference, red ones represent metabolites with an up-regulated significantly, and blue ones represent metabolites with a significantly down-regulated. (B) The number of annotated differential metabolites changes between T0 and T1. The blue block represents the up-regulated metabolites, and the orange one describes the down-regulated metabolites. (C) The top 20 significant enrichment KEGG pathways are based on the differential metabolites between T0 and T1. The point represents the number of differential metabolites in the pathway. The larger the point, the more differential metabolites are in the pathway.
Figure 5. Differential metabolites and corresponding pathways between T2 and T3. (A) The volcano maps exhibited the differential metabolites between T2 vs. T3. The upper panel results from positive ion mode and the down panel are results from negative ion mode. Moreover, the X-axis represents -Log10(p), and the Y-axis represents log2 (fold change). Every point represents one metabolite. Furthermore, black points represent metabolites with no significant difference, red ones represent metabolites with an up-regulated significantly, and blue ones represent metabolites with a significantly down-regulated. (B) The number of annotated differential metabolites changes between T0 and T1. The blue block represents the up-regulated metabolites, and the orange one describes the down-regulated metabolites. (C) The top 20 significant enrichment KEGG pathways are based on the differential metabolites between T2 and T3. The point represents the number of differential metabolites in the pathway. The larger the point, the more differential metabolites are in the pathway.
Figure 6. Differential metabolites and corresponding pathways between T0& T1 and T2& T3. (A) The volcano maps exhibited the differential metabolites between T0& T1 and T2& T3. The upper panel results from positive ion mode and the down panel are results from negative ion mode. Moreover, the X-axis represents -Log10(P), and the Y-axis represents log2 (fold change). Every point represents one metabolite. Furthermore, black points represent metabolites with no significant difference, red ones represent metabolites with an up-regulated significantly, and blue ones represent metabolites with a significantly down-regulated. (B) The number of annotated differential metabolites changes between T0& T1 and T2& T3. The blue block represents the up-regulated metabolites, and the orange one describes the down-regulated metabolites. (C) The top 20 significant enrichment KEGG pathways are based on the differential metabolites between T0& 1 and T2& T3. The point represents the number of differential metabolites in the pathway. The larger the point, the more differential metabolites are in the pathway.
the egg. Under the positive ion mode, there were 7084 differential metabolites, with 4433 up-regulated and 2651 down-regulated; under the negative ion mode, there were 3433 differential metabolites, with 1270 up-regulated and 2163 down-regulated (Supplementary Table S4, Figure 6(A)). Moreover, the differences in metabolites in the positive ion mode are more prominent. After annotating, 290 differential metabolites were screened in group T0& T1 vs. T2& T3 (Supplementary Table S6, Figure 6(B)), with 124 up-regulated and 166 down-regulated.

There were 200 metabolic pathways (Supplementary Table S9, Supplementary Figure S5) in group T0& T1 vs. T2& T3, and we listed the top 20 highest enrichment of the KEGG pathway (Figure 6(C)). ABC transporters and Biosynthesis of plant secondary metabolites pathways were abundant, and L-Lysine, L-Aspartic acid, L-Glutamine, Choline, and Biotin were enriched in the ABC transporters pathway, while L-Methionine, L-Tryptophan, N-Acetyl-D-glucosamine, and L-Proline were enriched in the Biosynthesis of plant secondary metabolites pathways.

Overall, the variation of identified metabolites and Associated KEGG Pathways above was irregular, indicating that different physiological responses may occur in different groups.

4. Discussion

In the present study, non-targeted metabolomics was used to detect the metabolites in the serum of different age group ducks. In contrast to targeted metabolomics, non-targeted metabolomics offers the potential to determine novel biomarkers (Ribbenstedt et al. 2018), which will lead to unexpected discoveries (Sévin et al. 2015) and are widely used for unknown analyte identification without prior knowledge about compounds. In this study, a total of 14,973 and 8,261 metabolites were detected in the positive ion mode and negative ion mode, respectively. After being annotated, 547 metabolites were mainly concentrated in amino acid, lipid, carbohydrate, and nucleotide, which provided the basis for the subsequent study in revealing the biomarkers associated with different physiological periods of ducks.

Biomarkers are quantifiable characteristics of biological processes (Strimbu and Tavel 2010) and may be used for diagnoses purposes, assess disease severity or risk, or guide other clinical interventions (Ray et al. 2010), and are widely used in the prediction of disease (mReynolds et al. 2003; Chan and Ng 2010; Cardinale et al. 2011), monitoring of environment (Lam and Management 2009; Peakall 2012) and the detection of the physiological statuses (Simm et al. 2008). Blood is generally regarded as the best body fluid with rich content, which is highly sensitive and specific (Hanash et al. 2011) for evaluation of systemic processes, and so it is significant to detect the biomarkers in blood. In this study, these characteristics of biomarkers were utilised to analyse metabolites detected in blood and related physiological states. There were 3, 6, and 4 biomarkers related to sexual maturity, the formation of the egg, and the decline of egg production, respectively.

Sexual maturity is affected by environmental and genetic factors and regulated by metabolites. In the present study, most of the biomarkers screened were amino acids, peptides, fatty acids, carbohydrates, and alcohols. Researchers (Brann and Mahesh 1997; Estienne et al. 2000) demonstrated that excitatory amino acids, such as Aspartate, could control the reproduction and anterior pituitary hormone secretion. Moreover, we found that L-Aspartic acid is the Isomer of Aspartate, and L-Aspartic acid (Barb et al. 1992) has been proved to promote PRL, growth hormone, and cortisol secretion. In addition, an abundance of Biotin (Taniguchi et al. 2007) in the yolk is greatly required for maintaining reproductive functions, and Thyroxine (Taniguchi and Watanabe 2007) can promote follicular development. These results indicate that L-Aspartic acid, Biotin, and Thyroxine can be used as biomarkers of the sexual maturation period in female ducks. To further understand the mechanisms of these metabolites, enrichment analysis was conducted of all metabolites, which was focussed on the top 20 metabolic pathways. Zhang et al (Zhang et al. 2020) mentioned that the biosynthesis of plant secondary metabolites was a dominant role in ovarian development. Besides, a vital pathway that the Tricarboxylic Acid Cycle and fatty acid production were essential for gonad development (Zhang et al. 2020). The pathways above may affect the sexual maturity of female ducks.

In this study, the nutritional metabolites from the growing (T0& T1) and laying (T2& T3) periods were investigated. Most of the metabolites were amino acids and fatty acids, and majority of metabolites screened were the precursor and isomer of the above compounds. Normally, there are various kinds of proteins are present in eggs (Mann and Mann 2008), and Nimalaratne et al (Nimalaratne et al. 2011) also mentioned many free amino acids in egg yolk, including essential amino acids, aromatic amino acids, and L-Lysine. Beyond that, in the metabolites with a significant difference, Lumichrome was reported as a kind of natural antioxidant (Ghafoor
Figure 7. ROC analysis predicting the response of biomarkers. ROC curve analysis was performed to compare the predictive power of biomarkers. Sensitivity and specificity corresponded to the true positive rate and the false positive rate, respectively. All data were provided in Supplementary Table S6.
and Rashid 2006) in eggs, which could restrain the multiplication of cancer. Thiamine, Riboflavin, and Pyridoxine are rich in egg white (Mine 2008), and Thiamine is the precursor of Thiamine aldehyde. These compounds are abundant in eggs and may be transported by the blood to the ovary. Moreover, an enrichment analysis of some metabolites was conducted that may be related to the egg formation. Most pathways were related to amino acid metabolism, vitamin metabolism, and carbohydrate metabolism, which are associated to the formation of nutrient in egg.

During the production decline period, most metabolites were increased. Most metabolites were amino acids, fatty acids, carbohydrates, amines, and linoleic acids. A previous study (Kuo et al. 2005) reported that egg production was determined by the number of follicles destined for ovulation and the capacity of the oviduct to transform the ova into a hard-shelled egg. Moreover, these processes were strictly controlled by hormones and other physiological conditions, including energy metabolism and the control of apoptosis of oocyte and follicle-associated cells. Hydroxykynurenine (Schuck et al. 2007) can impair energy metabolism in the rat cerebral cortex, which was up-regulated in the current study. Besides, several studies have shown that Sorbitol (Teramachi and Izawa 2000), Isorhamnetin (Ma G et al. 2007), Naringenin (Arul et al. 2013), and Hydroxykynurenine (Okuda et al. 1998) will lead to apoptosis. Thus, they may reduce energy metabolism or lead to apoptosis, and then the egg production will decrease during the laying period. Enrichment analysis of all metabolites in this group was conducted, and focussed on the top 20 metabolic pathways. Goff (Goff 2018) reported that the mineral in the diet must be absorbed across the gastrointestinal mucosa and enter the blood, and the eggshell formation requires many minerals. Furthermore, the pentose phosphate pathway (Stincone et al. 2015) is a fundamental component of cellular metabolism, which is essential to maintain carbon homeostasis, to provide precursors for nucleotide and amino acid biosynthesis. A study has found that Oxocarboxylic acid metabolism is significantly enriched in the pituitary gland in high- and low-producing hens (Wang C and Ma 2019). The hormone released by the pituitary gland affects egg production. The neuroactive ligand-receptor interaction pathway may affect egg production. Therefore, our findings are helpful to detect the different physiological statuses in female ducks and provided a theoretical basis for increasing egg production, reducing feed costs, and ensuring the poultry is healthy.

5. Conclusion

In summary, L-Aspartic acid, Biotin, and Thyroxine were biomarkers of sexual maturity of female duck. L-Lysine, Lumichrome, Thiamine aldehyde, Riboflavin, and Pyridoxine may be biomarkers related to egg formation. Simultaneously, Hydroxykynurenine, Sorbitol, Isorhamnetin, and Naringenin may be the biomarkers related to the decline of egg production. In addition, sexual maturity was mainly involved in the biosynthesis of secondary plant metabolites, TCA cycle, Mineral absorption, pentose phosphate pathway, and Oxocarboxylic acid metabolism. Egg formation disturbed amino acid metabolism, vitamin metabolism, and carbohydrate metabolism. Moreover, the neuroactive ligand-receptor interaction pathway may affect egg production. Therefore, our findings are helpful to detect the different physiological statuses in female ducks and provided a theoretical basis for increasing egg production, reducing feed costs, and ensuring the poultry is healthy.

Disclosure statement

The authors declare no conflict of interest.

Ethical approval statement

The animal study was reviewed and approved by the Animal Ethics Monitoring Committee of Sichuan Agriculture University. Written informed consent was obtained from the owners for the participation of their animals in this study.

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Data availability statement

All data generated and analysed during this study are included in this published article.
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