

Plasma metabolomic profiling reveals potential onset of lay biomarkers in broiler breeders

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ABSTRACT Changes in the metabolic fingerprint of plasma during the onset of lay in broiler breeders were investigated. We used metabolomics to identify biomarkers of sexual maturity and to provide a comprehensive understanding of breeder metabolome during the pullet to hen transition period. A total of 36 pullets were used, in which 30 pullets were randomly assigned to one of 10 unique growth trajectories and 6 birds were assigned to an unrestricted group. The growth trajectories were designed using a 3-phase Gompertz growth model with 10 levels of BW gain in the prepubertal and pubertal growth phases ranging from the breeder-recommended target BW to 22.5% higher, in 2.5% increments. The BW trajectories were applied to each individual bird using a precision feeding (**PF**) system, which collected BW and feed intake data for each individual bird. The birds were classified based on age at first egg (**AFE**), and 12 pullets were chosen from the lower and upper AFE extremes (early and late onset of lay) at 18, 20, 22, 24, and 26 wk of age to run repeated blood

plasma metabolomic assays. The metabolomic profile data were collected using a direct-injection liquid chromatography-tandem mass spectrometry and steroid assays. Univariate analysis identified 87 differential metabolites between the early- and late-onset of lay groups at 24 wk of age and 104 differential metabolites between the pullet and hen groups. Further investigation of differential metabolites showed 15 potential biomarkers for pullet to hen transition by analyzing the receiver operating characteristic (**ROC**) curve, mainly consisting of carnitine and choline metabolites. Differential metabolites during the pullet to hen transition were mainly associated with lipid, energy, and amino acid metabolism pathways, which gave clues to the physiological and metabolic shifts resulting from sexual maturation. At 24 wk of age, the main pathways involved in differentiation of the early- and late-onset of lay groups were related to lipid and amino acid metabolism. These metabolites could be involved in biosynthesis of egg yolk precursors in the liver.

Key words: parent stock, sexual development, metabolomics, metabolic status, maturation

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INTRODUCTION

To successfully stimulate sexual maturity and persistent production upon photostimulation, broiler breeder pullets need to reach certain BW, body composition, and physiological thresholds within the context of metabolic balance (Sun and Coon, 2005; Sun et al., 2006; de Beer and Coon, 2009; van Emous et al., 2013). To date, most research models describing the control of the reproductive axis and sexual maturation have largely focused on the impact of environmental cues such as photoperiod (Bédécarrats, 2015), rather than incorporating the impact of growth trajectory and metabolic

status. The impact of growth pattern on metabolism acts as a trigger for onset of lay in broiler breeders (Renema et al., 2007; Hanlon et al., 2020).

Broiler breeders are subjected to feed restriction programs (quantitative and qualitative) to control excessive growth during the rearing phase. Limited everyday feed restriction and skip-a-day feeding programs are the most common quantitative methods of feed restriction in broiler breeders (Carneiro et al., 2019). Diluting the nutrient density of the feed is an example of qualitative feed restriction (Zuidhof et al., 1995). The metabolic consequences of various restriction feeding regimes have been studied in broiler breeders (Buyse et al., 2000; Kita et al., 2002; de Beer et al., 2007, 2008; Ekmay et al., 2010; Moradi et al., 2013). Nutritional status and the subsequent responses of key plasma metabolic hormones (insulin, glucagon, and triiodothyronine) are important factors that determine the level of hepatic lipogenesis in birds (Hillgartner et al., 1995), which is involved in vitellogenesis. Although the length of fasting

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period is different in various feed restriction regimes, fasting is known to influence many metabolic processes, shifting metabolism from anabolism to catabolism and from lipogenesis to lipolysis (Richards et al., 2003). Likewise, feeding frequency can affect metabolic responses and reproductive efficiency. Variations in nutrient intake and subsequent energy status are communicated to the liver and hypothalamic-pituitary axis by alterations in the plasma levels of hormones such as insulin, glucagon, triiodothyronine and metabolites such as glucose and free fatty acids (Sun et al., 2006; de Beer et al., 2008). de Beer et al. (2007) found that skip-a-day feeding of broiler breeders was less efficient than everyday feeding due to the need to deposit and remobilize nutrients during fasting period. Shortening fasting length, through increasing feeding frequency, increased feed utilization efficiency and enhanced egg production rate and egg weight, as well as reduced hepatic lipogenesis (Richards et al., 2003; Moradi et al., 2013). The liver provides amino acids, lipids, nucleotides, vitamins, and choline as essential compounds for yolk precursor synthesis (Zhu et al., 2020). The authors indicated that plasma glutathione and ascorbic acid levels were down-regulated, and choline abundance was upregulated during onset of lay in ducks, which can be used to predict sexual maturity.

Predicting sexual maturity using the changes in plasma metabolome profile can be used to evaluate nutritional and growth management in breeder flocks. Reproductive system maturation and changes in some plasma metabolites and reproductive hormones occur during the pullet to hen transition period. Sexual maturity is most commonly measured as age at first egg (AFE) in poultry (Renema et al., 2007; Wolc et al., 2010; Hadinia et al., 2020). However, development of medullary bone and ovarian follicles are initiated roughly 14 to 16 d before the first oviposition (Whitehead, 2004; Shi et al., 2020), which indicates another measure for sexual maturity. The process of sexual maturation in breeder hens embodies a major shift in their physiological status (Johnson et al., 2009). Fluctuations in plasma hormones and substrates may provide signals that link metabolic status to the activation of the reproductive system. The maturation involves activation of the hypothalamus-pituitary-gonad (HPG) axis in which is controlled by hypothalamic secretion of Gonadotropin Releasing Hormone (GnRH). Hadinia et al. (2020) increased broiler breeder dietary energy from 2,807 to 3,109 kcal/kg of diet from 22 to 26 wk of age. The percentage of birds which commenced laying by 26 wk of age was 100% in the high ME intake treatment and 30% in the low ME intake treatment. They concluded that higher ME intake advanced the activation of HPG axis, stimulated reproductive hormone levels, and increased lipid deposition in the body of high ME intake treatment group.

Previously, we investigated the effects of incremental increases in target BW gain, including nonrestricted broiler breeders, during prepubertal and pubertal growth phases on reproductive performance (Zukiwsky et al., 2021). The onset of lay depended on the degree of feed

restriction, and some of the unrestricted pullets commenced egg production 2 wk prior to photostimulation. These results strongly suggest that body composition, or metabolic status, or both have a role in triggering sexual maturation. At present, most metabolomic studies have focused on mammals, and little is known about the metabolomics of broiler breeders. Therefore, profiling the plasma metabolome may provide a new perspective for studying the metabolic response of sexual maturity in breeders, a better understanding of its biological mechanisms, and potential biomarkers for predicting the onset of lay. The ultimate goal is to design a point of care device (similar to a portable blood glucometer) to measure broiler breeder plasma metabolome in real time at the flock level and in a matter of seconds. Thereby, the poultry industry can use the extracted data to evaluate sexual development status in a flock. The objectives of the current study were to evaluate the effect of lay status (pullet vs. hen), photostimulation BW, and onset of lay timing (early vs. late) on plasma metabolomic dynamics to identify potential biomarkers of sexual development in broiler breeders.

MATERIALS AND METHODS

Animals and Management

The animal protocol for the study was approved by the University of Alberta Animal Care and Use Committee for Livestock and followed the Canadian Council on Animal Care guidelines and policies (CCAC, 2009). The experimental protocol was previously described in full detail (Zukiwsky et al., 2021). Briefly, a total of 36 (30 growth restricted and 6 unrestricted) Ross 708 broiler breeder pullets were placed in a single pen from hatch to 43 wk of age at a stocking density of 3.0 birds per m². The birds were fed using 2 precision feeding (PF) stations, which collected real-time BW and feed intake data for each individual bird. All birds were fed commercial broiler breeder diets: starter (crumble; ME 2,726 kcal/kg, 21.0% CP, 1.00% Ca, and 0.45% available P) from hatch to d 34; grower (mash; ME 2,799 kcal/kg, 15.0% CP, 0.79% Ca, and 0.44% available P) from d 35 to d 179; and laying diet (crumble; ME 2,798 kcal/kg, 15.3% CP, 3.30% Ca, and 0.38% available P) from d 180 onward. Water was provided ad libitum throughout the experiment. The photoschedule was 8L:16D (15 lx) during the rearing phase. Pullets were photostimulated at wk 22 by increasing the photoperiod to 11L:13D (20 lx); to 12L:12D (25 lx) on wk 23, then at wk 24 to 13L:11D (50 lx) for the remainder of the experiment. A nest box with 8 nesting sites equipped with radio frequency identification (RFID) readers, which identified and weighed eggs of individual hens, was installed in the room. To prevent floor eggs, sufficient additional laying space was provided as trap nests with 8 nesting sites, which also allowed the identification of each egg according to the hen that laid it. The nest systems were installed at 14 wk of age; so that the pullets had the chance to adapt to the nesting system prior to the onset of lay.

The PF system was used to identify individual birds using a wing band containing a RFID transponder and feed them according to how their real-time BW compared to the preprogrammed target BW (Zuidhof et al., 2019). The PF system provided access to a meal if the individual birds' real-time BW was equal to or less than its preprogrammed target BW; otherwise, the system gently ejected the birds from the PF station. All birds had access to the PF stations for 24 h/d throughout the experiment.

Photostimulation BW and Age at First Egg

Median BW of the multiple BW observations of individual birds at 154 d of age (22 wk) was considered as their photostimulation BW. These data were extracted from the PF system database. To determine AFE, the cloacae of all hens were palpated daily to detect the presence of a hard-shelled egg in the shell gland from 20 wk onward. Presence or absence of a hard-shelled egg in the shell gland was recorded for each hen to determine AFE. Eggs were collected from nest boxes, weighed, and individual hen laying records were reconciled daily.

Plasma Samples Preparation

Blood samples (3 mL) were taken from the brachial vein of each bird biweekly from 18 to 26 wk of age. A 4 mL sodium heparin vacutainer was used to collect blood from each bird between 1 and 3 h after the start of the photoperiod. The samples were immediately centrifuged at 1,244 x g at 4°C for 15 min to recover plasma. The plasma samples were stored at -20°C until metabolomic profile analysis. The metabolomic data were collected using a locally developed direct-injection liquid chromatography-tandem mass spectrometry (DI/LC-MS/MS) assay and steroid assay (Zwierzchowska et al., 2020).

Experimental Design

The experiment was a completely randomized controlled study with 30 pullets reared on one of 10 growth trajectories. A 3-phase Gompertz growth model describing the growth in prepubertal, pubertal, and postpubertal phases was fitted to the Ross 708 female broiler breeder recommended target BW to estimate the phase-specific BW gain coefficients. The growth trajectories were designed with 10 levels of BW gain in the prepubertal and pubertal growth phases ranging from the breeder-recommended target BW (CON) to 22.5% higher (CON+22.5%) in 2.5% increments. The BW trajectories were applied to each individual bird using the PF system. Therefore, each bird was an experimental unit. An additional 6 birds were assigned to an unrestricted group. The unrestricted birds were not limited to a maximum BW or a certain growth trajectory but were rather provided access to feed upon every PF station visit.

The various BW trajectories in this study caused birds to commence laying at different ages, creating a range for AFE criteria. We used AFE to create 4 experimental classifications. 1) Early vs. late onset of lay: After collecting plasma samples, the candidate plasma samples were chosen for the metabolomic assays based on the bird's AFE. More specifically, 12 birds each having the highest and lowest AFE were selected for the metabolomics study. 2) Heavy vs. standard BW: The median photostimulation BW of the candidate birds was used to define the upper (heavy BW) and lower (standard BW) extremes. 3) Pullet vs. hen: The candidate birds were categorized as either a pullet or a hen at each blood sampling age, depending on whether they had laid an egg prior to the collection of the plasma sample. 4) Mature vs. immature birds: We subtracted 15 d from AFE as an estimated time for initiation of maturity in the birds. Then the candidate birds were divided into either mature or immature at each blood sampling age. Initiation of sexual maturity including the development of medullary bone and ovarian follicles start around 14 to 16 d before the first oviposition (Whitehead, 2004; Shi et al., 2020).

Direct-Injection Liquid Chromatography-Tandem Mass Spectrometry

A targeted quantitative metabolomics approach was applied to analyze the plasma samples using a combination of direct injection mass spectrometry with a reverse-phase LC-MS/MS custom assay. This custom assay, in combination with an AB Sciex 4000 QTrap (Applied Biosystems/MDS Analytical Technologies, Foster City, CA) mass spectrometer, can be used for the targeted identification and quantification of up to 150 different endogenous metabolites including amino acids, acylcarnitine, biogenic amines and derivatives, uremic toxins, glycerophospholipids, sphingolipids, and sugars (Foroutan et al., 2019, 2020). The method combined the derivatization and extraction of analytes, and the selective mass-spectrometric detection using multiple reaction monitoring pairs. Isotope-labeled internal standards and other internal standards were used for metabolite quantification. The custom assay contained a 96 deep-well plate with a filter plate attached with sealing tape, reagents, and solvents used to prepare the plate assay. For all metabolites except organic acids, plasma samples were thawed on ice and were vortexed and centrifuged at 13,000 x g. Ten μ L of each sample was loaded onto the center of the filter on the upper 96-well plate and dried in a stream of nitrogen. Subsequently, phenyl-isothiocyanate was added for derivatization. After incubation, the filter spots were dried again using an evaporator. Extraction of the metabolites was then achieved by adding 300 μ L of extraction solvent. The extracts were obtained by centrifugation into the lower 96-deep well plate, followed by a dilution step with mass spectrometry running solvent.

For organic acid analysis, 150 μL of ice-cold methanol and 10 μL of isotope-labeled internal standard mixture were added to 50 μL of plasma samples for overnight protein precipitation. After centrifuging at 13,000 \times g for 20 min, 50 μL of supernatant was pipetted into the center of wells of a 96-deep well plate, followed by the addition of 3-nitrophenylhydrazine reagent. After incubation for 2 h, BHT stabilizer and water were added before LC-MS injection.

Mass spectrometric analysis was performed on an AB Sciex 4000 QTrap tandem mass spectrometry instrument equipped with an Agilent 1260 series UHPLC system (Agilent Technologies, Palo Alto, CA). The samples were delivered to the mass spectrometer by a LC method followed by a direct injection method. Data analysis was done using Analyst 1.6.2 (Sciex Canada, Concord, ON, Canada).

Steroid Assay

Sample Preparation Plasma samples were thawed on ice, in the dark, before use. After mixing the samples with internal standards mixture solution, they were pipetted into Eppendorf tubes. After that, 100 μL of PBS buffer was added to each tube and vortexed for 30 s. Methyl tert-butyl ether was used for extraction. Subsequently, samples were centrifuged at 13,000 \times g and 4°C for 15 min, and 750 μL of supernatants were transferred into HPLC vials and dried under nitrogen purge until completely dry. To the dried tubes, 100 μL of derivatization solution was added, followed by shaking for 15 min. All the tubes were then incubated at 60°C for 1 h, and subsequently 20 μL was injected into an UHPLC-equipped 4000 QTrap mass spectrometer for LC-MS/MS analysis.

LC-MS/MS Method An Agilent 1260 series UHPLC system (Agilent, Palo Alto, CA) was used for LC-MS/MS analysis with an AB Sciex 4000 QTrap mass spectrometer (Sciex Canada, Concord, ON). For the HPLC work, solvent A was 0.1% formic acid in water; and solvent B was 0.1% formic acid in methanol. The flow rate was 0.5 mL/min and the sample injection volume was 20 μL . The mass spectrometer was set to a positive electrospray ionization mode with multiple reaction monitoring. The Ion Spray voltage was set at 5,500 V and the temperature at 550°C. The curtain gas, ion source gas 1, ion source gas 2, and collision gas were set at 40, 60, 60 and medium, respectively. The entrance potential was set at 10 V. Likewise, the declustering potential, collision energy, collision cell exit potential, multiple reaction monitoring Q1 and Q3 were set individually for each analyte and internal standards.

Statistical Analysis

All statistical analyses were performed for each blood sampling age, separately. However, the analyses for the pullet vs. hen groups and the mature vs. immature birds' groups data were done for all blood sampling ages

together to investigate the overall metabolic status of the groups. The MetaboAnalyst software (The Metabolomics Innovation Centre, AB, Canada) was used for the statistical analyses (Xia et al., 2009). After uploading the metabolomic profile data to the software and conducting an integrity check, metabolites that were frequently (>20%) below the limit of detection or with more than 20% missing values were excluded from datasets. Otherwise, missing values were estimated using the KNN (feature-wise) option of the software. The data were then normalized either by sum or median to reach a bell-shape Gaussian distribution curve prior to statistical analyses. Univariate analysis methods including the fold change (FC) analysis, *t*-test, and volcano plot were conducted for exploratory data analysis. The univariate analyses provided a preliminary overview about compounds that were potentially significant in discriminating the effects under study. Metabolites with a FC value greater than 1.5 were considered as differential metabolites between the groups. Pairwise differences between metabolites concentrations within each group were reported as significantly different when $P \leq 0.05$. Trends were reported where $0.05 < P \leq 0.10$. Principal Component Analysis (PCA), an unsupervised pattern recognition method, was used to provide an overview of the population structure and to ensure clustering of the pooled quality controls. Additionally, Partial Least Squares - Discriminant Analysis (PLS-DA) model was employed for further robust separation of differential metabolites between 2 groups. Furthermore, the variable importance in the projection (VIP) values were used to define significantly differential metabolites (VIP > 1), that is, metabolites with significant difference in concentration, between the groups.

The goodness of fit explains how well we were able to mathematically reproduce the data of the data set. A quantitative measure of the goodness of fit was given by the parameter R^2 (the explained variation, goodness of fit). The cross-validation method employed for this study was the 10-fold cross validation, with Q^2 as measured predictive performance (goodness of prediction). The PLS-DA model needs to be validated to confirm whether the separation is statistically significant, or due to random noise (Barberini et al., 2016). Thus, a 100 times permutation test was implemented to assess the significance of class discrimination and to validate the reliability of the PLS-DA model. More specifically in each permutation, a PLS-DA model was built between the data (X) and the permuted class labels (Y) using the optimal number of components determined by previous cross validation calculations and based on the original class assignment. The ratio of the between sum of squares and the within sum of squares (B/W-ratio) was calculated for the class assignment prediction of each model. If the B/W ratio of the original class assignment were a part of the distribution based on the permuted class assignment, the contrast between the 2 class assignments could not be considered significant.

The Orthogonal Partial Least-squares Discriminant Analysis (OPLS-DA) was performed to further

investigate and analyze the separation of the groups (Trygg et al., 2007). To determine the optimal potential biomarker for each group (the pullet and hen group and the early and late onset of lay group) receiver operating characteristic (ROC) curve and area under the curve (AUC) analyses were performed based on the cross-validation strategy. The ROC curves were generated by Monte-Carlo cross-validation using balanced subsampling. In each cross-validation, two thirds of the samples were employed to evaluate the differential compounds importance. The top 50 important compounds were then exploited to build classification models, which were validated on the remaining one-third of the samples. The procedure was replicated multiple times to calculate performance, and confidence interval of each model. The linear support vector machine algorithm was used as a classification and a feature ranking method with 2 latent variables. The following decision criteria were used: the AUC of 0.9 to 1.0 indicated excellent performance; 0.8 to 0.89, good performance; 0.7 to 0.79, fair performance; 0.6 to 0.69, poor performance; and less than 0.6, insignificant value (Haase-Fielitz et al., 2009). In addition, commercial databases including the Kyoto Encyclopedia of Genes and Genomes (KEGG) and *Gallus gallus* (chicken) metabolome database (KEGG database, 2019) were employed to further search for metabolite pathways associated with the significantly differential metabolites. These compounds were then imported into the module of pathway analysis in MetaboloAnalyst to generate the pathway topology analysis. The metabolic pathway with an impact value greater than 0.1 was characterized as the significantly relevant pathway (Liu et al., 2018).

RESULTS

Validation of Metabolomic Profile Models

Validation plots (fitting and predictive performance plot and permutation test plot) were used to validate the metabolomic models at different ages (18, 20, 22, 24, and 26 wk) and different treatments (pullet vs. hen, early vs. late onset of lay, heavy BW vs. standard photostimulation BW, mature vs. immature birds). Among all treatments and ages, only the metabolomic models resulting from the early vs. late onset of lay groups at 24 wk of age and the pullet vs. hen groups showed reliable fitting and predictive performance ($Q^2 > 0.40$; Worley and Powers, 2013; Blasco et al., 2015). As the grouping based on the heavy and standard photostimulation BW was equivalent to the grouping based on the early and late onset of lay, the metabolomic results of those groups can be interpreted together. The Q^2 values for the models resulted from the early vs. late onset of lay groups at 18 and 20 wk of age were less than 0.40 (0.002 for 18 wk and 0.005 for 20 wk of age), indicating unreliable predictive performance. Furthermore, the univariate analysis results for metabolomic data at 22 and 26 wk of age did not show any significant differences in metabolite concentrations between the early and late

onset of lay groups, indicating the same metabolic status between the groups at those ages. The average AFE in the early and late onset of lay groups were 22 and 26 wk of age, respectively. Thus, it was speculated that the lack of differential metabolites before and after 24 wk of age would indicate a relatively stable physiological state before and after sexual maturation in the breeders.

Photostimulation BW and Age at First Egg

In our previous publication, we indicated that sexual maturity advanced by 10.8 d per kg increase in photostimulation BW (Zukiwsky et al., 2021). The birds' AFE ranged from 141 to 186 d of age with a median value of 175 d of age. In the current study, 6 birds with an AFE of less than the median AFE (lower extreme of AFE) were considered as the early onset of lay group, and 6 birds with an AFE of greater than the median AFE (upper extreme of AFE) were considered as the late onset of lay group. The late onset of lay group included birds from the CON, CON+2.5%, CON+5%, CON+10%, and CON+12.5% treatments and the early onset of lay group included birds from the CON+15%, CON+17.5%, CON+20%, and unrestricted treatments. The candidate birds' photostimulation BW ranged from 2,350 g (CON group) to 4,940 g (unrestricted group) with a median value of 2,675 g.

Plasma Metabolomic Profile

Using the DI/LC-MS/MS and steroid assays, a total of 142 metabolites (134 by DI/LC-MS/MS and 8 steroids) were identified and quantified in the plasma samples. The species of metabolites measured by the DI/LC-MS/MS were classified into ten groups: amino acids (23), biogenic amines (16), organic acids (18), lyso-phosphatidylcholines acyl (14), hydroxy-sphingomyelins (10), phosphatidylcholines di-acyl (8), phosphatidylcholines acyl-alkyl (2), acyl-carnitines (40), hexose (1), and miscellaneous (2). The steroids were progesterone, corticosterone, 17-hydroxy progesterone, estrone, pregnenolone, testosterone, androstenedione, and dehydroepiandrosterone.

Pullet and Hen Groups Univariate analyses provided the FC analysis, *t*-test, and volcano plot, which was a combination of the first 2 methods. Fifteen important compounds were identified by the FC analysis and volcano plot (Table 1), and 104 important compounds were identified by the *t*-test (top 50 compounds shown in Table 2) between the pullet and hen groups. Volcano plot analysis, which combines the FC and *t*-test analyses, revealed that the abundance of all identified plasma metabolites was lower in the hen group compared to the pullet group except for the plasma acetyl carnitine, creatine, and phosphatidylcholine metabolites (PC38:6AA, PC36:0AA, and PC40:6AA), which were upregulated.

Early and Late Onset of Lay Groups at 24 wk of Age Eighteen important compounds were identified by the FC analysis and volcano plot (Table 3), and 87

Table 1. Important compounds identified by fold change (FC) analysis and volcano plot¹ between pullet and hen groups².

Compounds	FC	log2(FC)	P value	Hen/Pullet ³
LYSOC18:0	0.205	-2.280	<0.001	Down
Trimethylamine N-oxide	0.305	-1.708	0.001	Down
Citric acid	0.320	-1.640	<0.001	Down
Acetyl carnitine	2.844	1.508	<0.001	Up
LYSOC17:0	0.370	-1.431	<0.001	Down
PC38:6AA	2.684	1.424	<0.001	Up
LYSOC14:0	0.400	-1.321	<0.001	Down
LYSOC18:2	0.417	-1.261	<0.001	Down
Creatine	2.277	1.187	0.002	Up
LYSOC16:0	0.440	-1.183	<0.001	Down
LYSOC20:4	0.445	-1.167	<0.001	Down
Taurine	0.471	-1.085	<0.001	Down
PC36:0AA	2.052	1.037	<0.001	Up
Trans-hydroxyproline	0.490	-1.027	0.001	Down
PC40:6AA	2.034	1.024	<0.001	Up

¹Volcano plot analysis is a combination of the fold change and *t*-test analyses, which has provided the *P* values in the table.

²The candidate birds for metabolomics assays were categorized as either a pullet or a hen at each blood sampling age, depending on whether they had laid an egg prior to the collection of the plasma sample.

³Hen/Pullet: Change in the hen group compared to the pullet group.

important compounds identified by the *t*-test (top 50 compounds shown in Table 4) between the early and late onset of lay groups at 24 wk of age. Based on the volcano plot analysis, all the identified plasma metabolites were downregulated in the early compared to the late onset of lay group except for the plasma corticosterone and phosphatidylcholine metabolites (PC38:6AA and PC36:6AA), which were upregulated.

Principal Component Analysis of Samples

Pullet and Hen Groups The PCA score plot showed a fairly clear separation between the pullet and hen groups. First and second principal components (PC1 and PC2) explained 43.4 and 10.4% of the variation in samples, respectively (Figure 1 panel A).

Early and Late Onset of Lay Groups at 24 wk of Age

The PCA score plot showed a clear separation between the early and late onset of lay groups at 24 wk of age. First and second principal components (PC1 and PC2) explained 57.9 and 8.9% of the variation in samples, respectively (Figure 1 panel B).

Partial Least-Squares Discriminant Analysis and Orthogonal Partial Least-Squares Discriminant Analysis of Plasma Samples

Pullet and Hen Groups A PLS-DA and OPLS-DA model was constructed to further investigate and analyze the separation of the pullet and hen groups. As shown in Figure 2, the pullet and hen groups were clearly separated. First and second principal components (PC1 and PC2) explained 42.3 and 10.7% of the variation in samples in the PLS-DA score plot and 23.5 and 25.9% of the variation in samples in the OPLS-DA score plot (Figure 3 panel A).

The performance scores of the PLS-DA model for the pullet and hen groups were accuracy = 0.90, $R^2 = 0.80$,

Table 2. Important compounds identified by *t*-test analysis between pullet and hen groups¹.

Compounds	FDR ²	P value	Hen/Pullet ³
Carnitine	<0.001	<0.001	Up
Acetyl carnitine	<0.001	<0.001	Up
LYSOC18:2	<0.001	<0.001	Down
PC36:0AE	<0.001	<0.001	Up
18:0SM	<0.001	<0.001	Down
PC36:0AA	<0.001	<0.001	Up
Citric acid	<0.001	<0.001	Down
LYSOC18:0	<0.001	<0.001	Down
LYSOC14:0	<0.001	<0.001	Down
PC38:6AA	<0.001	<0.001	Up
LYSOC20:4	<0.001	<0.001	Down
PC40:6AE	<0.001	<0.001	Up
PC38:0AA	<0.001	<0.001	Down
LYSOC16:0	<0.001	<0.001	Down
PC40:2AA	<0.001	<0.001	Down
PC40:6AA	<0.001	<0.001	Up
16:1SMOH	<0.001	<0.001	Down
LYSOC17:0	<0.001	<0.001	Down
22:2SMOH	<0.001	<0.001	Down
PC36:6AA	<0.001	<0.001	Up
14:1SMOH	<0.001	<0.001	Down
16:1SM	<0.001	<0.001	Down
22:1SMOH	<0.001	<0.001	Down
Hydroxy hexadecanoyl carnitine	<0.001	<0.001	Down
Malonyl carnitine	<0.001	<0.001	Down
20:2SM	<0.001	<0.001	Up
HPHPA	<0.001	<0.001	Down
Hydroxy valeryl carnitine (Methyl malonyl carnitine)	<0.001	<0.001	Down
Dodecanoyl carnitine	<0.001	<0.001	Down
Methyl glutaryl carnitine	<0.001	<0.001	Down
Tetra decadienoyl carnitine	<0.001	<0.001	Down
Dodecanedioyl carnitine	<0.001	<0.001	Down
Decenoyl carnitine	<0.001	<0.001	Down
Hexadecadienoyl carnitine	<0.001	<0.001	Down
Decadienoyl carnitine	<0.001	<0.001	Down
Glutamic acid	<0.001	<0.001	Down
Hydroxy hexadecenoyl carnitine	<0.001	<0.001	Down
Dodecenoyl carnitine	<0.001	<0.001	Down
Total-dimethyl Arginine	<0.001	<0.001	Down
LYSOC16:1	<0.001	<0.001	Down
p-Hydroxy hippuric acid	<0.001	<0.001	Down
LYSOC28:0	<0.001	<0.001	Down
Hexadecenoyl carnitine	<0.001	<0.001	Down
Carnosine	<0.001	<0.001	Down
Hydroxy octadecenoyl carnitine	<0.001	<0.001	Down
LYSOC28:1	<0.001	<0.001	Down
Betaine	<0.001	<0.001	Down
Hydroxy tetradecenoyl carnitine	<0.001	<0.001	Down
Homocysteine	<0.001	<0.001	Down
Succinic acid	<0.001	<0.001	Down

¹The candidate birds for metabolomics assays were categorized as either a pullet or a hen at each blood sampling age, depending on whether they had laid an egg prior to the collection of the plasma sample.

²FDR: false discovery rate.

³Hen/Pullet: Change in the hen group compared to the pullet group.

and $Q^2 = 0.66$ (Figure 2 panel C) and the performance scores of the OPLS-DA model were R^2Y cumulative (cum) = 0.489 and Q^2 cumulative (cum) = 0.447 (Figure 3 panel B), which were indicative of robust fit and prediction. R^2Y is the fraction of the variance of descriptor matrix (X) and class response (Y) explained by each latent variable in % representing explained variation in Y by the component. To further validate the PLSA-DA model, the permutation test (n = 100 times) was used for verification (Figure 2 panel D); the highlighted bar represents the original sample. The further to the right of the distribution, the more significant

Table 3. Important compounds identified by fold change (FC) analysis and volcano plot¹ between the early and late onset of lay groups at 24 wk of age.

Compounds	FC	log ₂ (FC)	P value	Early/Late ²
PC38:6AA	2.766	1.467	<0.001	Up
LYSOC18:0	0.230	-2.114	<0.001	Down
PC36:6AA	2.071	1.050	<0.001	Up
LYSOC14:0	0.411	-1.281	<0.001	Down
LYSOC16:0	0.463	-1.108	<0.001	Down
LYSOC18:2	0.431	-1.212	0.001	Down
LYSOC17:0	0.417	-1.261	0.001	Down
Betaine	0.494	-1.015	0.001	Down
LYSOC20:4	0.399	-1.324	0.001	Down
Citric acid	0.295	-1.760	0.001	Down
Trimethylamine N-oxide	0.424	-1.235	0.001	Down
Taurine	0.470	-1.089	0.011	Down
Carnosine	0.432	-1.207	0.011	Down
Corticosterone	2.855	1.513	0.016	Up
Sarcosine	0.390	-1.358	0.034	Down
Kynurenine	0.392	-1.347	0.038	Down
Trans-hydroxyproline	0.409	-1.287	0.056	Down

¹Volcano plot analysis is a combination of the fold change and *t*-test analyses, which has provided the *P* values in the table.

²Early/Late: Change in birds having early onset of lay compared to late onset of lay group.

is the separation between the 2 groups (Bijlsma et al., 2006). The permutation test results of the OPLS-DA model for the R²Y(cum) and Q²(cum) values were 0.801 and 0.696 between the pullet and hen groups (Figure 3 panel C). Permutation test revealed that the observed separation was not by chance and the results of cross-validation were reliable.

Early and Late Onset of Lay Groups at 24 wk of Age

The PLS-DA and OPLS-DA models showed a clear separation between the early and late onset of lay groups at 24 wk of age (Figures 4 and 5). First and second principal components (PC1 and PC2) explained 57.7 and 8.4% of the variation in samples by the PLS-DA score plot (Figure 4 panel A) and 49.2 and 16.9% of the variation in samples using the OPLS-DA score plot (Figure 5 panel A). The performance scores of the PLS-DA model for the early and late onset of lay groups at 24 wk of age were accuracy = 1.0, R² = 0.96, and Q² = 0.81 (Figure 4 panel C). The performance scores of the OPLS-DA model for those groups were R²Y(cum) = 0.837 and Q²(cum) = 0.791 (Figure 5 panel B). The permutation test results for the R²Y(cum) and Q²(cum) value were 0.964 and 0.861 between the early and late onset of lay groups at 24 wk of age (Figure 5 panel C). The results of permutation tests revealed that the PLS-DA (Figure 4 panel D) and OPLS-DA (Figure 5 panel C) models did not have overfitting issue, and the separations between the groups were real.

Identification of Significant Differential Metabolites

Pullet and Hen Groups Significant differential metabolites (Figure 2 panel B) were identified and ranked by VIP values (VIP > 1.0) based on the PLS-DA model (Wang et al., 2015). The VIP is a weighted sum of squares of the PLS loadings, taking into account the

Table 4. Important compounds identified by *t*-test analysis between the early and late onset of lay groups at 24 wk of age.

Compounds	FDR ¹	P value	Early/Late ²
PC38:6AA	<0.001	<0.001	Up
C6 (Acyl carnitine)	<0.001	<0.001	Down
PC36:0AA	<0.001	<0.001	Up
LYSOC18:0	<0.001	<0.001	Down
PC36:0AE	<0.001	<0.001	Up
PC36:6AA	<0.001	<0.001	Up
Malonyl carnitine	<0.001	<0.001	Down
LYSOC14:0	<0.001	<0.001	Down
LYSOC16:0	<0.001	<0.001	Down
Hydroxy hexadecenoyl carnitine	<0.001	<0.001	Down
LYSOC18:2	0.001	<0.001	Down
PC40:6AA	0.001	<0.001	Up
LYSOC17:0	0.001	<0.001	Down
Betaine	0.001	<0.001	Down
PC40:2AA	0.001	<0.001	Down
LYSOC20:4	0.001	<0.001	Down
16:1SMOH	0.001	<0.001	Down
Citric acid	0.001	<0.001	Down
Hexenoyl carnitine	0.002	<0.001	Down
18:0SM	0.002	<0.001	Down
Hexadecadienoyl carnitine	0.002	<0.001	Down
Methylglutaryl carnitine	0.003	<0.001	Down
Dodecenoyl carnitine	0.003	<0.001	Down
Glutaryl carnitine (Hydroxyhexanoyl carnitine)	0.003	<0.001	Down
Hydroxy hexadecadienoyl carnitine	0.004	<0.001	Down
Tetradecenoyl carnitine	0.004	<0.001	Down
Octadecadienyl carnitine	0.004	<0.001	Down
p-Hydroxyhippuric acid	0.004	<0.001	Down
Hexadecenoyl carnitine	0.004	<0.001	Down
Hydroxy valeryl carnitine (Methylmalonyl carnitine)	0.004	<0.001	Down
Hydroxy hexadecanoyl carnitine	0.005	0.001	Down
Glutaconyl carnitine	0.005	0.001	Down
Dodecanedioyl carnitine	0.005	0.001	Down
Hippuric acid	0.005	0.001	Down
Hydroxy tetradecenoyl carnitine	0.005	0.001	Down
16:1SM	0.005	0.001	Down
Hydroxy tetradecadienoyl carnitine	0.005	0.001	Down
Butenyl carnitine	0.005	0.001	Down
Hexadecenoyl carnitine	0.005	0.001	Down
Trimethylamine N-oxide	0.005	0.001	Down
Propenoyl carnitine	0.005	0.001	Down
Octadecanoyl carnitine	0.006	0.001	Down
Tetradecadienoyl carnitine	0.006	0.002	Down
Arginine	0.006	0.002	Down
Dodecanoyl carnitine	0.006	0.002	Down
Decadienoyl carnitine	0.006	0.002	Down
14:1SMOH	0.006	0.002	Down
LYSOC26:1	0.006	0.002	Down
Nonayl carnitine	0.007	0.002	Down
22:2SMOH	0.008	0.002	Down

¹FDR: false discovery rate.

²Early/Late: Change in birds having early onset of lay compared to late onset of lay group.

amount of explained Y-variation in each dimension. The differential metabolites were divided into acyl carnitine metabolites (carnitine and acetyl carnitine), phosphatidyl choline di acyl (PC36:0AA, PC40:6AE, PC36:0AE, PC38:6AA, PC38:0AA, PC40:2AA), lysophosphatidyl cholines acyl (LYSOC20:4, LYSOC18:2, LYSOC18:0, LYSOC16:0, LYSOC14:0), hydroxy sphingomyelins (18:0SM), and organic acid metabolite (citric acid).

Early and Late Onset of Lay Groups at 24 wk of Age

The differential metabolites based on the VIP scores of the PLS-DA model (Figure 4 panel B) for the early and late onset of lay groups at 24 wk of age were divided into

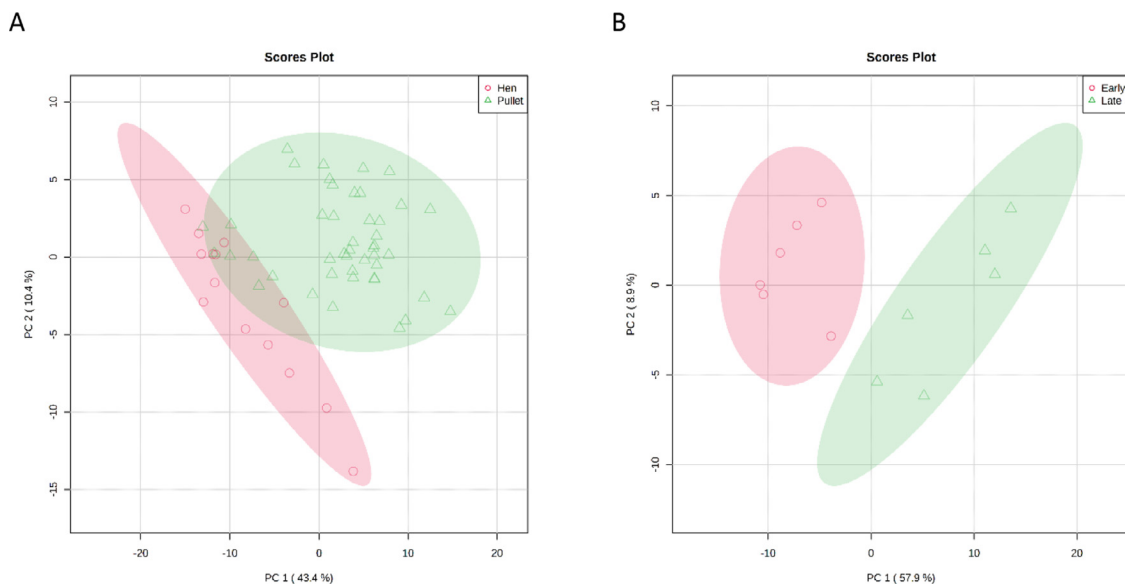


Figure 1. Principal component (PC) analysis of plasma metabolomics data at 24 wk of age shows separation of metabolomes of the pullet and hen groups (A) and the early and late onset of lay groups (B).

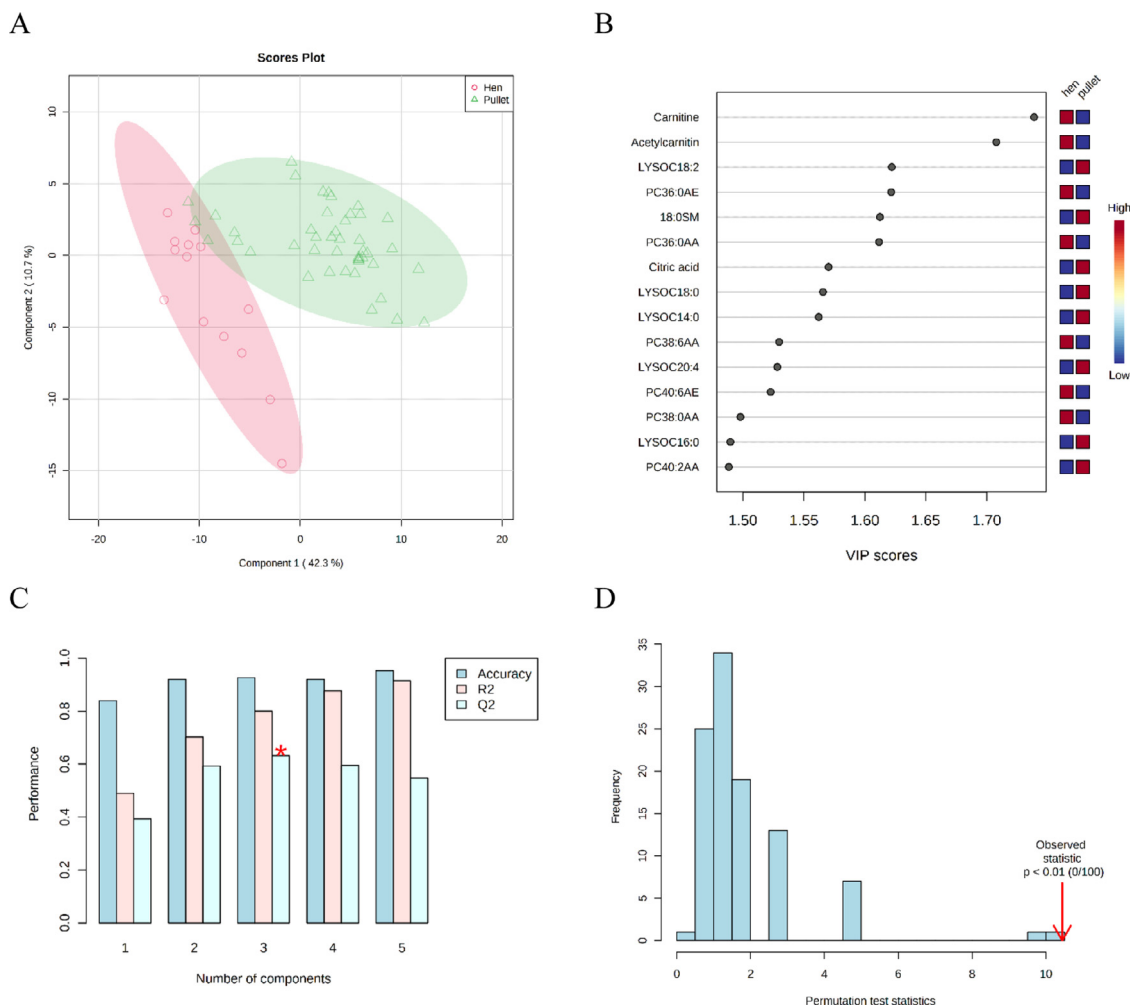


Figure 2. Partial Least-Squares Discriminant Analysis (PLS-DA) of pullet and hen plasma metabolomics data (A), variable importance in the projection (VIP) scores of the differential metabolites (B), and corresponding validation plots of the fitting and predictive performance of the model (C) and the permutation test (100 times, D) of the PLS-DA model.

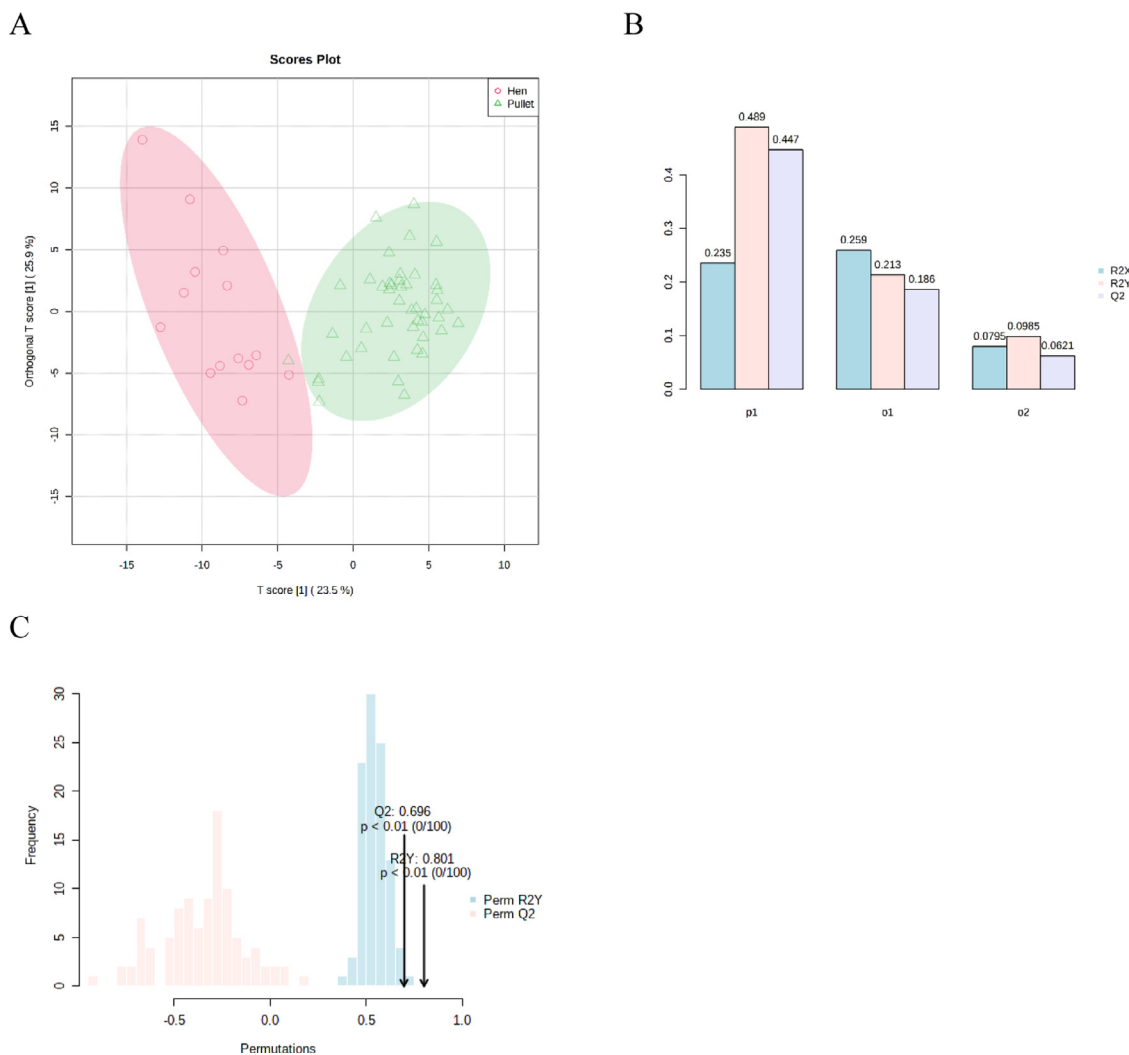


Figure 3. Orthogonal Partial Least-Squares Discriminant Analysis (OPLS-DA) of pullet and hen plasma metabolomics data (A), and corresponding validation plots of the fitting and predictive performance of the model (B) and the permutation test (100 times, C) of the OPLS-DA model.

acyl carnitine metabolites (C4OH = malonyl carnitine, C16:20H = Hydroxy hexadecadienoyl carnitine, C6 = acyl carnitine), phosphotidyl choline di acyl metabolites (PC38:6AA, PC36:0AA, PC40:6AE, PC36:0AE, PC36:6AA, PC40:2AA), lysophosphotidyl cholines acyl metabolites (LYSOC18:0, LYSOC14:0, LYSOC16:0, LYSOC18:2, LYSOC17:0), and amino acid derived metabolites (betaine).

Acquisition of Specific Potential Biomarkers by Receiver Operating Characteristic Curve Analysis

Pullet and Hen Groups The aim of the multivariate exploratory ROC curve analysis is to evaluate the performance of biomarker models created through automated important compound identification. In this study, 15 significantly differential metabolites were chosen as candidate biomarkers of the pullet to hen transition (Figure 6 panel A). The ROC curve analysis was

performed for the metabolites to clarify and estimate the maturity identification performance of the candidate biomarkers and screen potential biomarkers. A total of 13 out of 15 differential candidate biomarkers possessed an AUC more than 0.90, indicating an excellent discriminatory ability. The results indicated that acetyl carnitine, carnitine, LYSOC20:4, PC36:0AA, LYSOC18:2, LYSOC18:0, PC40:6AE, LYSOC16:0, citric acid, PC36:0AE, PC38:6AA, PC38:0AA, and LYSOC14:0 are potential biomarkers for detecting the pullet to hen transition in broiler breeders.

Early and Late Onset of Lay Groups at 24 wk of Age

A total of 15 significantly differential metabolites were chosen as the candidate biomarkers of onset of lay. The ROC curve analysis revealed that all the 15 differential metabolites had an AUC more than 0.90, representing an excellent discriminatory ability (Figure 6 panel B). The current study found the following metabolites as the potential biomarkers of the onset of lay in broiler breeders: acyl carnitine (C6), PC36:0AA, PC38:6AA, LYSOC18:0, PC36:0AE, PC36:6AA, malonyl carnitine,

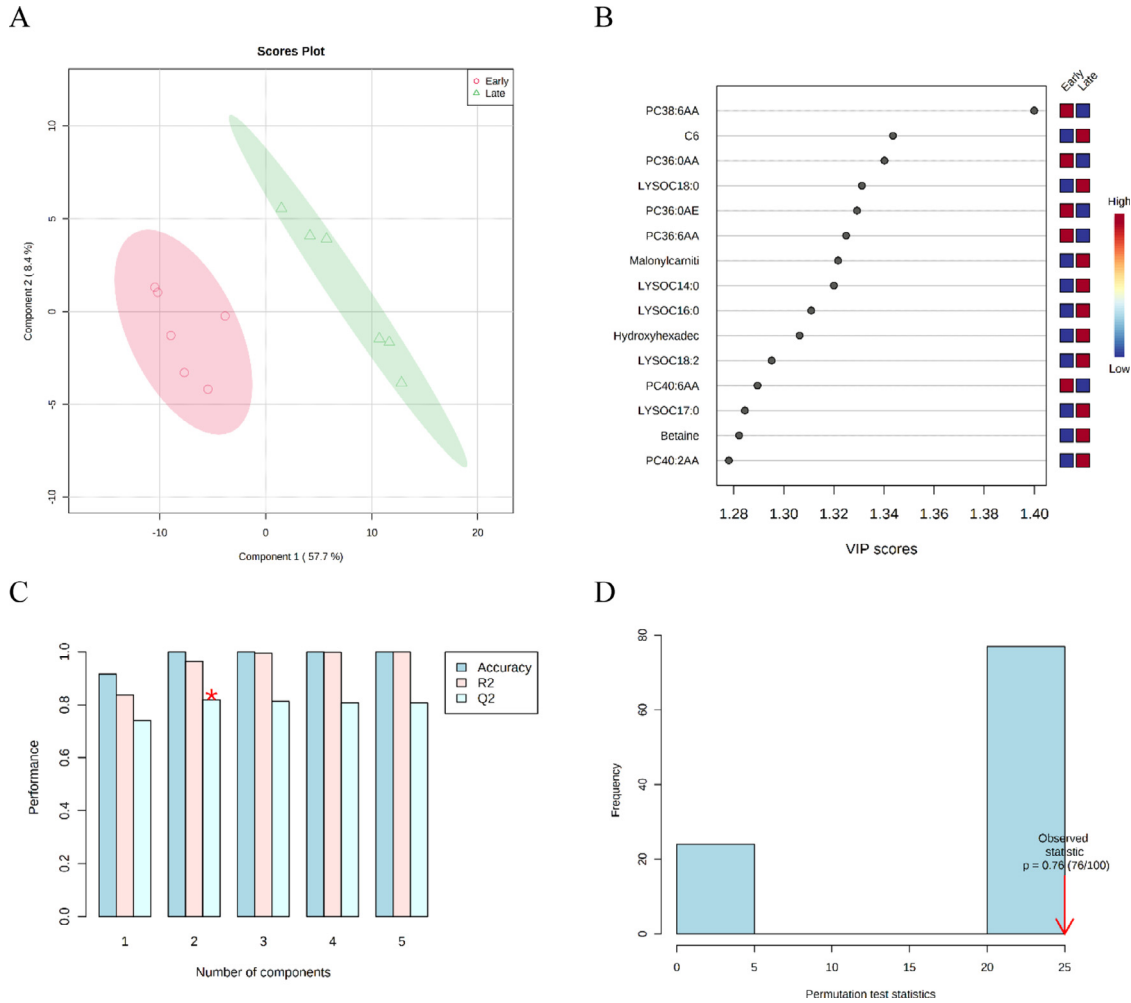


Figure 4. Partial Least-Squares Discriminant Analysis (PLS-DA) of plasma metabolomics data at 24 wk of age in the early and late onset of lay groups (A), variable importance in the projection (VIP) scores of differential metabolites (B), and corresponding validation plots of the fitting and predictive performance of the model (C) and the permutation test (100 times, D) of the PLS-DA model.

LYSOC14:0, LYSOC16:0, hydroxy hexadecadienoyl carnitine (C16:20H), LYSOC18:2, PC40:6AA, LYSOC17:0, betaine, and PC40:2AA.

Metabolic Pathway Analysis

Pullet and Hen Groups According to the significant differential metabolites in the current study, metabolomics pathway analysis was constructed to further investigate the change in metabolic pathways affected by pullet to hen transition. The analysis showed that 3 pathways had the greatest significance (Figure 7): glycerophospholipid metabolism, citrate cycle (Tricarboxylic acid; TCA cycle), and glyoxylate and dicarboxylate metabolism. Changes in these pathways might be potential targets for the pullet to hen transition in broiler breeders.

Early and Late Onset of Lay Groups at 24 wk of Age The metabolic pathway analysis for the differential metabolites between the early and late onset of lay groups at 24 wk of age revealed 2 most significance pathways (Figure 8): glycerophospholipid metabolism and Glycine, Serine, and Threonine metabolism.

DISCUSSION

Broiler breeder pullets need to reach certain BW, body composition, physiological, and metabolic thresholds to become sexually mature. Major sites involved in attaining sexual maturity include the HPG axis (maturation), liver (by formation of yolk lipids through lipogenesis), ovary (through folliculogenesis and steroidogenesis), adipose tissue (through the effect of leptin and adiponectin on HPG axis), somatotrophs (through the effects of growth hormone on insulin-like growth factor-I and the HPG axis), and thyroid axis (through modulation of effects of gonadotropins on ovarian function).

A total of 104 metabolites with different concentrations between the pullet and hen groups were screened and identified. Based on the pathway analysis for the differential metabolites identified by the VIP scores of the PLS-DA model, the main metabolic pathways associated with these differential metabolites were lipid, energy, and amino acids metabolism. The ROC curve analysis revealed that carnitine and choline metabolites could be considered as potential biomarkers of pullet to hen transition. Most of the phosphatidylcholine metabolites and carnitine metabolites were upregulated in hens

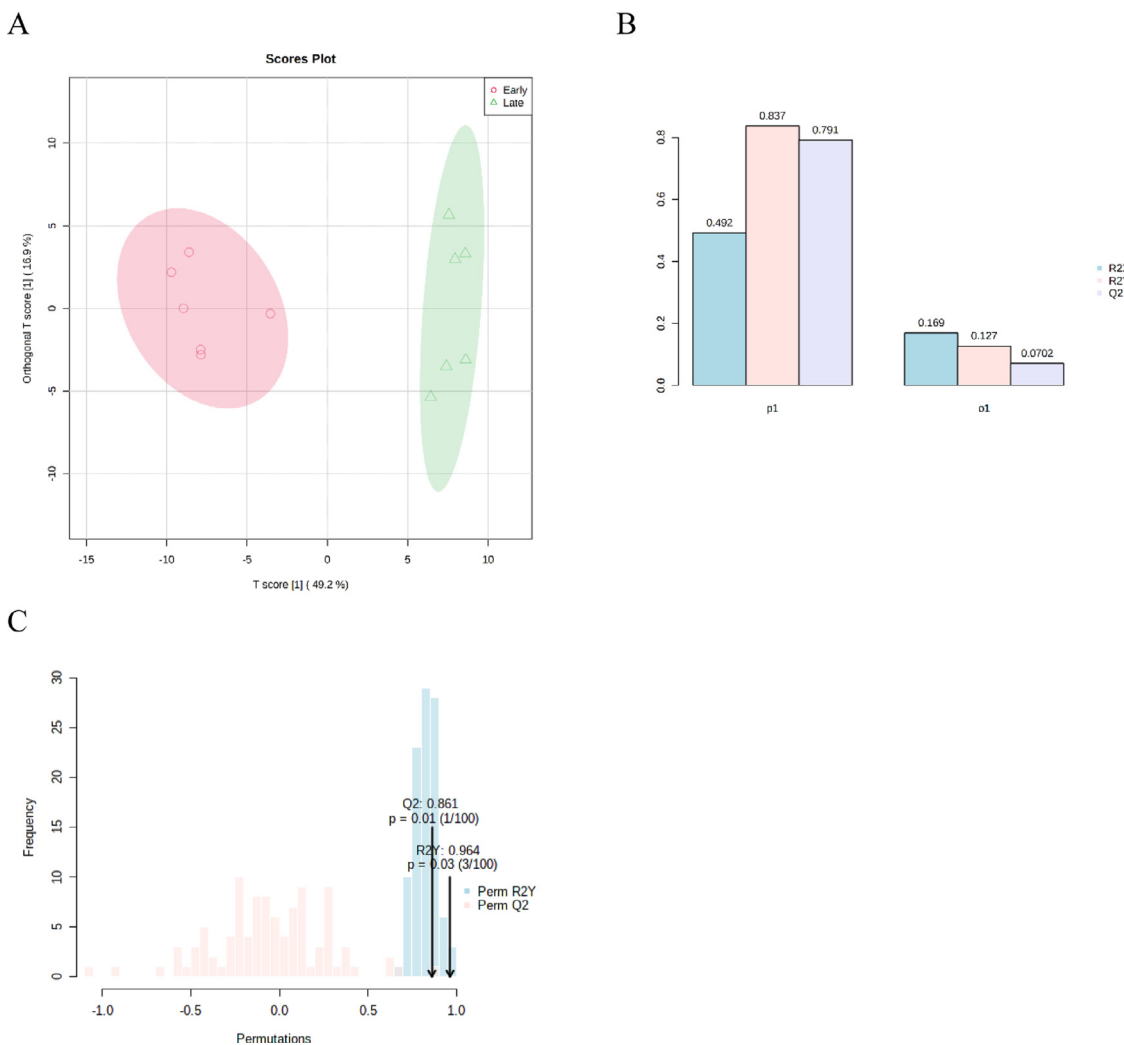


Figure 5. Orthogonal Partial Least-Squares Discriminant Analysis (OPLS-DA) of plasma metabolomics data at 24 wk of age from birds having early or late onset of lay (A), and corresponding validation plots of the fitting and predictive performance of the model (B) and the permutation test (100 times, C) of the OPLS-DA model.

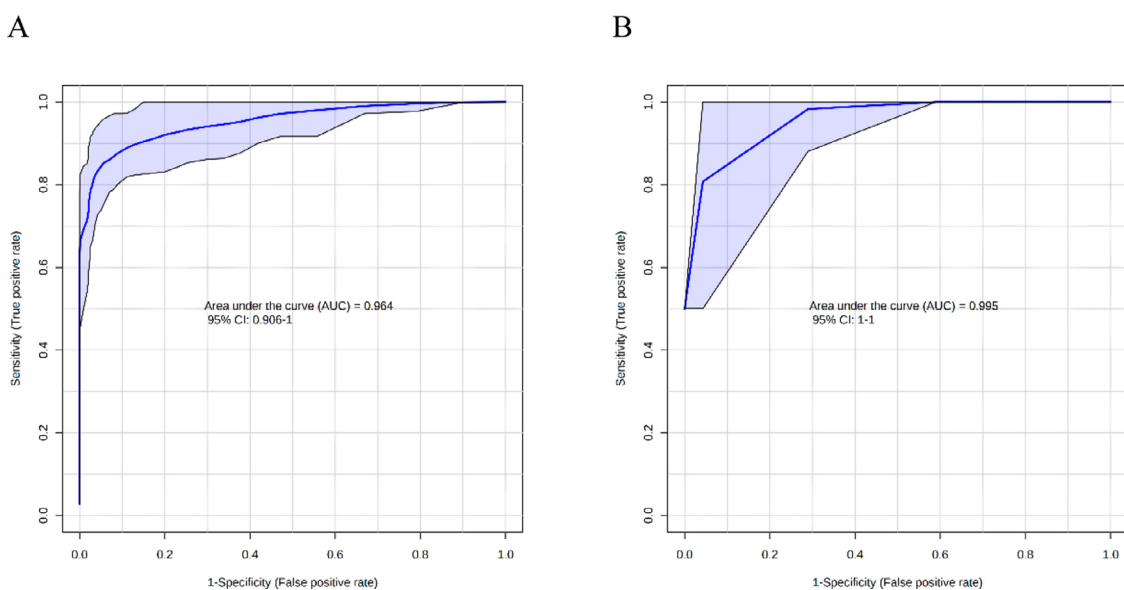


Figure 6. Receiver Operator Characteristic (ROC) curve analysis to evaluate the performance of biomarker models created through automated important compound identification for top 15 differential metabolites at 24 wk of age between the pullet and hen (A) and the early and late onset of lay groups (B).

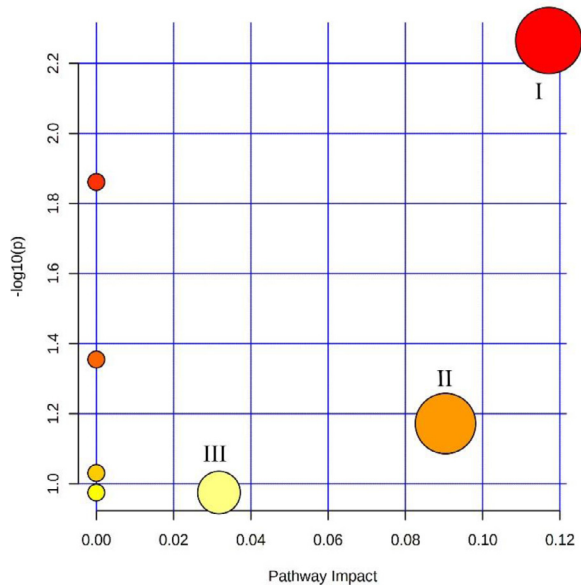


Figure 7. Pathway analysis of differential metabolites between the pullet and hen groups as shown in bubble plots. Bubble size is proportional to the impact of each pathway, and bubble color represents the degree of significance, from the highest (red) to the lowest (yellow). I = glycerophospholipid metabolism; II = citrate cycle (TCA cycle); III = glyoxylate and dicarboxylate metabolism.

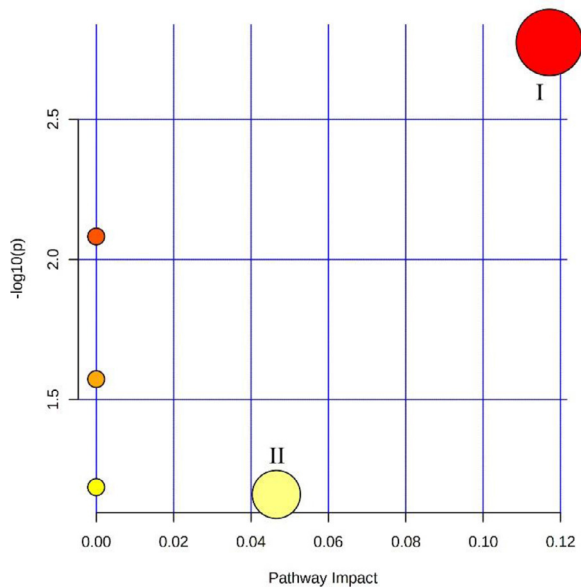


Figure 8. Pathway analysis of differential metabolites between the early and late onset of lay groups at 24 wk of age as shown in bubble plots. Bubble size is proportional to the impact of each pathway, and bubble color represents the degree of significance, from the highest (red) to the lowest (yellow). I = glycerophospholipid metabolism; II = glycine, serine, and threonine metabolism.

whereas lyso-phosphatidylcholine metabolites and citric acid were downregulated. [Zhu et al. \(2020\)](#) performed a comparative analysis of metabolites in the liver of Muscovy ducks at different egg laying stages and indicated that the glutathione and ascorbic acid abundances were downregulated, and the choline abundance was upregulated during egg laying. The metabolomic profile changes were related to the role of the liver in fat metabolism ([Cieřlik et al., 2011](#); [He et al., 2014](#)) and yolk precursor synthesis ([Wood et al., 2021](#)). The TCA cycle is

the core center of energy metabolism. Citric acid is an important intermediate metabolite of the TCA cycle. The decrease in plasma citric acid content reflected the inhibition of glycolysis in a hamster hyperlipidemia model ([Jiang et al., 2013](#)). The authors indicated that as liver lipid content increased, the levels of TCA cycle intermediates, including plasma citrate and succinate, decreased in hamsters. In the current study, downregulation of citric acid in the hens might be due to increase in lipogenesis in the liver ([Pearce, 1971](#)) at cost of reduced glycolysis, which could be reflected by reduction in plasma citric acid. Furthermore, the increase in carnitine metabolites (e.g., acetyl carnitine) suggests mitochondrial oxidation of fatty acids, especially in the liver. Carnitine is required for the transport of fatty acids through the inner mitochondrial membrane where fatty acid oxidation takes place ([Jia et al., 2014](#)). In the current study, thus, it could be hypothesized that upregulation of carnitine metabolites might be associated with the increased lipogenesis in the liver during the pullet to hen transition phase. In addition, carnitine affects the HPG axis to promote reproductive hormones secretion (GnRH, LH, FSH, and estradiol) in female rats and human ([Krsmanovic et al., 1992](#); [Genazzani et al., 2011](#); [Agarwal et al., 2018](#)). This mechanism needs to be investigated in further broiler breeder studies.

A total of 87 metabolites with different concentrations between the early and late onset of lay groups at 24 wk of age were identified. Pathway analysis of the differential metabolites identified based on the VIP scores of the PLS-DA model suggested that the main pathways involved in differentiation of the early- and late-onset of lay groups at 24 wk of age were related to lipid and amino acids metabolism. The ROC curve analysis showed that carnitine metabolites, choline metabolites, and betaine could be used as potential biomarkers to predict the timing of onset of lay in broiler breeders. Phosphatidylcholine metabolites were upregulated in the early onset of lay group at 24 wk of age whereas lyso-phosphatidylcholine metabolites, carnitine metabolites, and betaine were downregulated. In the early onset of lay group at 24 wk of age, betaine might have been used for the synthesis of phospholipids or phosphatidylcholine ([Eklund et al., 2005](#)). Phosphatidylcholine is a glycerophospholipid and a principal component of the plasma VLDL monolayer. Production of phosphatidylcholine metabolites might have been upregulated to be used as the precursors of egg yolk when a hen matures and commences egg laying. [Cui et al. \(2020\)](#) demonstrated that as Rohman layer pullets approached sexual maturity from 125 d onward, reproductive hormonal changes (mainly estrogen) directly increased the expression of genes related to lipogenesis (fatty acid synthase) and yolk precursor (very low density apolipoprotein-II and vitellogenin-II) synthesis, which increased serum concentration of phospholipid, triacylglycerol, vitellogenin, very low density lipoprotein y (**VLDLy**), lecithin, total cholesterol, and triglyceride.

In the current study, increasing prepubertal and pubertal BW gains by more than 15% of the breeder-

recommended target BW triggered fat metabolism and yolk precursors synthesis, which consequently advanced sexual maturity. In conclusion, this study indicated that metabolic transition during the onset of lay in broiler breeders is accompanied by certain metabolic signatures that can be used to predict the metabolic status linked to the bird's maturity. More research is warranted to investigate the complex interactions of all the differential metabolites and reproductive axis (HPG axis) in maturing broiler breeders.

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DISCLOSURES

The authors declare that there is no conflict of interest.

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