Maternal dietary methionine restriction alters the expression of energy metabolism genes in the duckling liver

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Abstract

Background: In mammals, the nutritional status experienced during embryonic development shapes key metabolic pathways and influences the health and phenotype of the future individual, a phenomenon known as nutritional programming. In farmed birds as well, the quantity and quality of feed offered to the dam can impact the phenotype of the offspring. We have previously reported that a 38% reduction in the intake of the methyl donor methionine in the diet of 30 female ducks during the growing and laying periods - from 10 to 51 weeks of age - reduced the body weight of their 180 mule ducklings compared to that of 190 ducklings from 30 control females. The maternal dietary methionine restriction also altered the hepatic energy metabolism studied in 30 of their ducklings. Thus, their plasma glucose and triglyceride concentrations were higher while their plasma free fatty acid level was lower than those measured in the plasma of 30 ducklings from the control group. The objective of this new study was to better understand how maternal dietary methionine restriction affected the livers of their newly hatched male and female ducklings by investigating the hepatic expression levels of 100 genes primarily targeting energy metabolism, amino acid transport, oxidative stress, apoptotic activity and susceptibility to liver injury.

Results: Sixteen of the genes studied were differentially expressed between the ducklings from the two groups. Maternal dietary methionine restriction affected the mRNA levels of genes involved in different pathways related to energy metabolism such as glycolysis, lipogenesis or electron transport. Moreover, the mRNA levels of the nuclear receptors PPARGC1B, PPARG and RXRA were also affected.

Conclusions: Our results show that the 38% reduction in methionine intake in the diet of female ducks during the growing and egg-laying periods impacted the liver transcriptome of their offspring, which may explain the previously observed differences in their liver energy metabolism. These changes in mRNA levels, together with the observed phenotypic data, suggest an early modulation in the establishment of metabolic pathways.

Keywords: Duck, Methyl donor, Nutritional programming, Differentially expressed genes

Background

The effects of maternal nutrition on offspring phenotypes have been largely documented and reviewed in recent years in human and rodents [1–4] as well as in farmed animals [5–7] including poultry [8, 9]. In particular, the nutritional status experienced early in life, i.e. during embryonic and fetal development, interacts with major metabolic pathways and shapes the health and
phenotypes of the individual into adulthood, a phenomenon known as nutritional programming.

In birds, in ovo manipulation of nutrients by either injection of a nutrient or removal of a part of a component is a direct way to impact the offspring phenotypes [10, 11]. For example, Willems and co-authors removed a part of the albumen to explore the lasting impacts of protein under-nutrition in layer-type hens. They reported a lower hatching weight and hepatic proteome changes in chicks hatched from albumen deprived eggs [12] and differential gene expression in the hepatic transcriptome of the adult hens from albumen-deprived eggs [13]. These adult hens laid smaller eggs and had a lower laying rate and a higher number of second grade eggs, a consequence of early protein under-nutrition [14]. Altogether, this group demonstrated long-lasting effects of nutritional programming induced by early protein under-nutrition on production performances in layer-type hens. It has also been reported that early methyl donor availability plays critical roles in hepatic carbohydrate and lipid metabolism. For example, in ovo injection of betaine was shown to affect hepatic cholesterol metabolism in newly hatched chicks [15] and to protect from corticosterone-induced hepatic steatosis [16].

However, the quantity and quality of the feed offered to female birds can also have an impact on the performances of the offspring. For example, when betaine was added to the diet of hens, it altered the expression of genes in the liver of their chicks [17]. In a previous study, we investigated the effects of a reduced level of dietary methionine (Met) on laying performances of female common ducks Anas platyrhynchos and its impacts on the phenotypes of their newly hatched mule ducklings [18]. Indeed, the male mule duck is the male sterile inter-generic hybrid offspring of a female common duck and a Muscovy drake (Cairina moschata). It benefits from heterosis effects that increase the production of fatty liver (hepatic steatosis) induced by overfeeding and is therefore widely used for foie gras production in France [19]. In our previous study [18], the restricted group of females received Met-restricted diets (R group) containing 0.25% of Met whereas the control group received control diets (C group) containing 0.40% of Met that meets Met requirements, during the growing and laying periods, from 10 to 51 weeks of age. Thus, females in the R group laid eggs of lower weight and containing less albumen, thus inducing a lower availability of nutrients for embryonic development. The ducklings that were the offspring of the females from the R and C groups were subsequently assigned to R and C groups, respectively. The male and female newly hatched ducklings in the R group showed a reduced body weight when compared to those of the C group and a tendency to an increased ratio of liver weight to body weight. Moreover, their plasma alanine transaminase (ALT) activity was reduced and their plasma alkaline phosphatase (ALP) activity showed a tendency to be reduced too. Their plasma concentrations of glucose and triglycerides (TG) were higher whereas their plasma level of free fatty acids (FFA) decreased. These observations suggested altered hepatic energy metabolism in male and female newly hatched ducklings from the Met-restricted dams. Based on literature data, this alteration could be a consequence of a nutritional programming following the reduced availability of Met -that is a methyl donor- in the maternal diet and a lower availability of nutrients during embryonic development. In this context, the objective of the present study was to further explore the effects of the maternal dietary Met restriction by comparing the level of expression of 100 genes in the liver from male and female newly hatched ducklings either offspring of the Met-Restricted or Control dams. These target genes were mainly related to energy metabolism, amino acid transport, oxidative stress, apoptotic activity and susceptibility to liver injury.

Results
The objective of this study was to compare the hepatic transcript levels of 100 target genes in the livers of C group and R group ducklings to provide information on how maternal methionine restriction affected hepatic energy metabolism in male and female ducklings from the Met-restricted dams.

The normalized relative expression of the 100 target genes was analyzed in 38 duckling livers from both groups (R group versus C group) after a qnorm transformation. However, 13 of the 100 genes studied and 2 of the 38 liver cDNA samples showed more than 25% of missing data and were removed from the study (see Method section). Moreover, another cDNA sample was also removed from the data set because it showed data points that differed significantly from other observations. The results are thus given for the 87 remaining genes and the 35 remaining liver cDNA samples which are from 9 male and 8 female ducklings from the C group and 10 male and 8 female ducklings from the R group.

Liver samples were classified into four subgroups according to the maternal diet and to the sex of the ducklings
First, a hierarchical clustering was performed to define groups of genes and animals with similar expression patterns. The ducklings were roughly separated according to the two maternal diets within two clusters numbered 1 and 2 on rows in Fig. 1A. The cluster 1 was mainly composed of ducklings belonging to the R group whereas the cluster 2 was mainly composed of ducklings belonging to the
C group. In each case, only 4 ducklings were not grouped to their initial group. The cluster 1 was divided into 2 sub-clusters called 1a and 1b. The sub-cluster 1a contained 11 samples corresponding to 8 male ducklings from the R group, 2 male ducklings from the C group and 1 female duckling from the R group. The sub-cluster 1b contained 7 samples that were 3 female and 2 male ducklings from the R group and 1 male and 1 female ducklings from the C group. The cluster 2 was divided into 2 sub-clusters called 2a and 2b. The sub-cluster 2a contained 10 samples corresponding to 6 male and 4 female samples from the C group. The sub-cluster 2b contained 7 female samples, 4 from the R group and 3 from the C group.

Based on their relative expression level, the 87 genes were grouped into 2 main clusters on the columns, named A and B. The cluster A was composed of 21 genes overexpressed in samples of cluster 1, mainly composed of ducklings belonging to the R group. The cluster B was divided into 2 sub-clusters called B1 and B2. The sub-cluster B1 was composed of 15 genes whose expression levels did not differ between the previously defined duckling clusters 1 and 2. In contrast, the large sub-cluster B2 showed 51 genes that attempted to be overexpressed in samples from cluster 2, which mainly consisted of ducklings belonging to the C group.

The score plot (distribution of individuals) of the PCA (Principal Component Analysis) along the 2 first principal components (horizontal and vertical axes) is given on Fig. 1B. The samples were first separated by the maternal diet on the horizontal axis and then by the sex of the ducklings on the vertical axis. The two first principal component summarized respectively 33% (horizontal axis) and 14% (vertical axis) of the whole variability.

Altogether, the samples were separated not only according to the maternal diet but also according to the sex of the ducklings thus defining four subgroups i.e. males from the R group (MR), females from the R group (FR), males from the C group (MC) and females from the C group (FC).

Maternal dietary Met restriction altered expression of energy metabolism genes

Of the 87 genes studied, the normalized and transformed relative expressions were used to investigate the differences in gene expression between the liver samples of the offspring of the two diet groups (C group versus R group) (Additional Table 1). Data were analyzed with a linear mixed model that included the maternal diet, the sex of the duckling, and the interaction between them as fixed effects, as well as the duckling -associated to its relationship matrix- as a random effect. The Table 1 describes the 27 genes showing a significant difference (corrected \( P \)-value < 0.05) imputable either to the maternal diet (Diet \( P \)-value (BH); 16 significant genes) or/and to the sex of the duckling (Sex \( P \)-value (BH); 15 significant genes). Four of them (\( GPAM, PGM1, ELOVL6, \) and \( PRKAA1 \)) showed significant differences for both the maternal diet and the sex of the ducklings. Finally, no gene had a significant interaction between duckling sex and maternal diet effects. Seven genes that tend to be differentially expressed (\( P \)-value (BH) between 0.05 and 0.10) between maternal diets (2 genes) or between duckling sexes (6 genes) are added in Table 1 and identified by a star. For each gene, least square means and standard deviations are given for the two groups of maternal diet (R group and C group) and for the two duckling sexes. Hereafter, the focus will be on the 16 differentially expressed genes (DEGs) for the maternal diet effect. Nine of them were down-regulated (\( NDUFA4, COX2, ENO1, MITTP, PRKAA1, RXRA, CYTB, NUDIF6 \) and \( PPARGC1B \)) whereas seven were upregulated (\( GPAM, PGM1, ELOVL6, PGK1, UGDH, BCL2A1 \) and \( PPARG \)) in the R group samples when compared the C group samples.

The Fig. 2 shows the results of the Principal Component Analysis (PCA) performed on the 16 DEGs for the maternal diet effect. The first principal component (horizontal axis) explained 53.7% of the whole variability and discriminated the samples according to the diet received by the female ducks (R group versus C group). The second principal component (vertical axis) explained 15.1% of the whole variability and discriminated the samples according to the sex of the ducklings. This is in concordance with the fact that 4 out of the 16 DEGs showed a significant effect of the sex of the ducklings and a fifth one tended to be differentially expressed according to the sex of the ducklings (Table 1). Again, the expression variability divided the samples into the four same subgroups: MR, FR, MC and FC. In addition, the PCA bi-plot showed correlations between the DEGs and the two main principal components and confirmed the opposite regulation pattern between the 9 down-regulated genes and the 7 up-regulated ones reported when compared R group and C group samples in Table 1.

(See figure on next page.)

**Fig. 1** Exploratory data analyses of the 87 genes. The ducklings from R group and C group are represented with triangles and circles, respectively. The females are in red and the males in blue. **A** Hierarchical Cluster Analysis of the gene expressions. In the heatmap, the duckling liver samples and the genes are arranged in rows and columns, respectively. The yellow, orange and red colors correspond to the low, median and high values of the qqnorm transformed normalized relative expressions of the studied genes. The clusters corresponding to duckling liver samples are named 1, 1a, 1b, 2, 2a and 2b. The clusters corresponding to genes are named A, B, B1 and B2. **B** Score plot of the PCA along the 2 first principal components. The two first principal components summarized respectively 33% (horizontal axis) and 14% (vertical axis) of the whole variability.
**A: Hierarchical clustering**

**B: PCA score plot**

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**Fig. 1** (See legend on previous page.)
### Table 1  Differentially expressed genes (DEGs) in the liver of ducklings

| GENE   | R group  | C group  | Females | Males | Diet | Sex | Sex*Diet |
|--------|----------|----------|---------|-------|------|-----|----------|
|        | LsMeans ± SD | LsMeans ± SD | LsMeans ± SD | LsMeans ± SD | P-value (BH) | P-value (BH) | P-value (BH) |
| ---    | ---       | ---       | ---       | ---   | ---  | --- | --- |
| GPAM   | 0.39 ± 0.16 | −0.52 ± 0.17 | −0.59 ± 0.17 | 0.47 ± 0.15 | < 0.01 | < 0.01 | 0.28 |
| NDUFA1 | −0.61 ± 0.19 | 0.64 ± 0.19 | −0.02 ± 0.2 | 0.05 ± 0.18 | < 0.01 | < 0.10 | 0.24 |
| PGM1   | 0.53 ± 0.15 | 0.65 ± 0.15 | −0.53 ± 0.16 | 0.41 ± 0.14 | < 0.01 | < 0.01 | 0.78 |
| COX2   | −0.55 ± 0.24 | 0.77 ± 0.28 | 0.01 ± 0.26 | 0.2 ± 0.25 | 0.01 | 0.03 | 0.85 |
| ELOVL6 | 0.51 ± 0.27 | −0.05 ± 0.31 | −0.46 ± 0.26 | 0.47 ± 0.25 | 0.01 | < 0.01 | 0.92 |
| ENO1   | −0.54 ± 0.24 | 0.51 ± 0.27 | −0.1 ± 0.25 | 0.07 ± 0.23 | 0.01 | 0.01 | 0.85 |
| MTTP   | −0.48 ± 0.21 | 0.55 ± 0.22 | 0.1 ± 0.23 | −0.02 ± 0.2 | 0.01 | 0.87 | 1.00 |
| PKG1   | 0.49 ± 0.25 | −0.46 ± 0.27 | −0.29 ± 0.25 | 0.32 ± 0.23 | 0.01 | 0.07 | 0.29 |
| PRKAA1 | −0.49 ± 0.2 | 0.4 ± 0.2 | −0.48 ± 0.21 | 0.39 ± 0.19 | 0.01 | 0.02 | 0.83 |
| RXRA   | −0.78 ± 0.03 | 0.51 ± 0.24 | −0.77 ± 0.31 | 0.49 ± 0.23 | 0.01 | 0.11 | 1.00 |
| UGDH   | 0.48 ± 0.21 | −0.53 ± 0.22 | −0.22 ± 0.22 | 0.15 ± 0.2 | 0.01 | 0.42 | 0.83 |
| CTYB   | −0.6 ± 0.28 | 0.52 ± 0.33 | −0.15 ± 0.29 | 0.07 ± 0.27 | 0.02 | 0.54 | 0.70 |
| LPL    | 0.34 ± 0.21 | −0.43 ± 0.21 | −0.33 ± 0.22 | 0.25 ± 0.2 | 0.03 | 0.13 | 0.70 |
| NDUF6  | −0.4 ± 0.22 | 0.42 ± 0.22 | −0.08 ± 0.23 | 0.1 ± 0.21 | 0.03 | 0.81 | 0.70 |
| PPARG1B| 0.47 ± 0.22 | −0.5 ± 0.24 | −0.1 ± 0.23 | 0.07 ± 0.22 | 0.03 | 0.81 | 0.87 |
| PPARC1B| −0.43 ± 0.23 | 0.43 ± 0.23 | 0 ± 0.23 | 0.2 ± 0.22 | 0.03 | 0.98 | 0.87 |
| BMR    | −0.48 ± 0.3 | 0.46 ± 0.34 | −0.27 ± 0.3 | 0.25 ± 0.29 | 0.06 | 0.18 | 0.78 |
| PPARA  | −0.35 ± 0.22 | 0.33 ± 0.22 | −0.29 ± 0.23 | 0.27 ± 0.21 | 0.10 | 0.23 | 0.79 |
| ELAVL1 | 0.2 ± 0.24 | −0.35 ± 0.28 | −0.67 ± 0.24 | 0.53 ± 0.24 | 0.22 | < 0.01 | 0.99 |
| HMGR1  | 0.1 ± 0.33 | −0.25 ± 0.37 | −0.67 ± 0.32 | 0.51 ± 0.31 | 0.47 | < 0.01 | 0.12 |
| MEF2C  | 0.0 ± 0.19 | −0.12 ± 0.19 | −0.71 ± 0.2 | 0.59 ± 0.18 | 0.75 | < 0.01 | 1.00 |
| SCD1   | 0.32 ± 0.26 | −0.04 ± 0.31 | −0.52 ± 0.26 | 0.4 ± 0.26 | 0.10 | < 0.01 | 0.70 |
| TALDO1 | 0.31 ± 0.25 | −0.37 ± 0.29 | −0.58 ± 0.25 | 0.51 ± 0.24 | 0.10 | < 0.01 | 0.70 |
| VLDLR  | −0.23 ± 0.3 | 0.01 ± 0.34 | 0.58 ± 0.29 | −0.8 ± 0.29 | 0.66 | < 0.01 | 0.99 |
| PCK1   | −0.28 ± 0.31 | 0.1 ± 0.35 | 0.36 ± 0.31 | −0.54 ± 0.3 | 0.47 | 0.01 | 0.85 |
| FASN   | 0.28 ± 0.27 | −0.38 ± 0.31 | −0.43 ± 0.27 | 0.33 ± 0.26 | 0.14 | 0.02 | 0.08 |
| MAPK1  | 0.19 ± 0.3 | −0.38 ± 0.35 | −0.57 ± 0.31 | 0.38 ± 0.29 | 0.32 | 0.02 | 0.85 |
| ABCA1  | 0.05 ± 0.29 | −0.27 ± 0.32 | −0.54 ± 0.29 | 0.32 ± 0.28 | 0.55 | 0.04 | 0.84 |
| DGAT2  | 0.06 ± 0.24 | −0.12 ± 0.24 | −0.49 ± 0.24 | 0.43 ± 0.23 | 0.71 | 0.04 | 0.85 |
| ALDOB  | −0.34 ± 0.25 | 0.36 ± 0.28 | 0.36 ± 0.26 | −0.35 ± 0.24 | 0.13 | 0.06 | 0.85 |
| SDHA   | −0.24 ± 0.22 | 0.16 ± 0.24 | −0.45 ± 0.24 | 0.37 ± 0.22 | 0.35 | 0.06 | 0.85 |
| DHC24  | 0.11 ± 0.2 | −0.15 ± 0.2 | −0.37 ± 0.21 | 0.33 ± 0.2 | 0.44 | 0.07 | 0.08 |
| HADH   | −0.32 ± 0.23 | 0.21 ± 0.23 | −0.44 ± 0.25 | 0.33 ± 0.21 | 0.30 | 0.07 | 0.83 |
| LDHA   | −0.13 ± 0.37 | 0.01 ± 0.43 | 0.26 ± 0.37 | −0.38 ± 0.35 | 0.86 | 0.07 | 0.28 |

Genes are listed according to whether they are differentially expressed for maternal diet (first part of the table) or for duckling sex (second part of the table and/or in bold). The corrected P-value with Benjamini-Hochberg (BH) procedure of the diet effect, the sex effect and their interaction are presented.

The star (*) indicates genes with a P-value (BH) between 0.05 and 1.0. For each gene, least square means (Ls-Means) and standard deviations (SD) are presented for the two maternal diet groups (R group and C group) and for both sexes.

**Correlations between the DEGs and the duckling phenotypic traits revealed differences in liver metabolism**

Phenotypic traits were measured in a previous study on the same newly hatched mule ducklings [18] and the main results are summarized in Table 2. These traits included body weight, liver weight, percentages of liver lipids and liver dry mater (DM), plasma activities of ALP, ALT and AST as well as plasma concentrations of glucose, TG and FFA.

The Fig. 3 shows the correlation matrices of the hepatic mRNA levels of the 16 DEGs between the two maternal diets and the phenotypic traits of the ducklings in the R and C groups and then in males and females.

The correlation matrices differed strongly between the ducklings from the R group and the ones from the C group. Briefly, the number of significant correlations is lower in the R group than in the C group. In particular, the plasma concentration of TG is only correlated...
to MTTP in the R group (−0.55) whereas it is correlated to 8 genes in the C group (6 negative correlations: −0.66 COX2, −0.49 CYTB, −0.66 ENO1, −0.71 MTTP, −0.56 PPARGC1B and −0.55 PRKAA1 and 2 positive correlations: 0.58 PGK1 and 0.75 UGDH). On the contrary the plasma activities of ALP, ALT and AST were correlated to 0 DEG in the C group and to 1 DEG for ALP (−0.61 GPAM), to 1 DEG for ALT (0.63 PPARG) and to 3 DEGs for AST (−0.47 ENO1, 0.75 PPARG and 0.58 UGDH) in the R group. The correlations of the plasmatic content of FFA are quite similar between the R group and the C group with a negative correlation with ELOVL6 (−0.77, and −0.72, in the R and C group respectively), GPAM (−0.80 and −0.58, in the R and C group respectively) and PGM1 (−0.60 and −0.70, in the R and C group respectively). But two significant correlations were detected only in the R group (−0.76 PGK1 and 0.49 PPARGC1B) and two other ones only in the C group (0.55 CYTB and 0.58 NDUFB6). The correlations between the hepatic content and the 16 hepatic DEGs were low. The hepatic lipid content was correlated to 0 DEG in both groups, the liver weight was only correlated to 2 genes in the C group (0.57 PGK1 and 0.55 UGDH) and the DM content was correlated to 3 DEGs in the R group (0.51 CYTB, 0.48 PPARGC1B and 0.47 PRKAA1) and to 1 DEG in the C group (−0.50 PPARG).

In addition, the number of correlations between DEGs is higher in the C group than in the R group. For example, UGDH is correlated to 12 and 3 DEGs, COX2 to 9 and 4,
Table 2  Effects of maternal dietary Met restriction on duckling traits (from Bodin et al., 2019 [18])

| Treatment       | R group | C group | P_Diet | P_sex | P_interaction |
|-----------------|---------|---------|--------|-------|---------------|
| n               | Mean ± SD | n | Mean ± SD | P_Diet | P_sex | P_interaction |
| Body weight (g) | 180 | 33.0 ± 0.9 | 190 | 35.2 ± 0.9 | <0.001 | NS | NS |
| Liver weight (g) | 28 | 1.51 ± 0.08 | 21 | 1.40 ± 0.11 | NS | 0.001 | NS |
| Liver: BW (%)    | 28 | 4.30 ± 0.17 | 21 | 3.92 ± 0.20 | 0.07 | 0.06 | NS |
| Liver lipids (%) | 28 | 17.23 ± 1.43 | 19 | 17.67 ± 1.44 | NS | NS | NS |
| Liver dry matter (%) | 28 | 41.10 ± 0.73 | 20 | 41.21 ± 1.10 | NS | NS | NS |
| Plasma FFA (mMol/L) | 28 | 0.27 ± 0.05 | 27 | -0.09 ± 0.21 | 0.01 | 0.01 | NS |
| Plasma Glucose (mMol/L) | 28 | 0.55 ± 0.05 | 27 | -0.09 ± 0.21 | 0.01 | 0.01 | NS |
| Plasma TG       | 28 | 0.55 ± 0.19 | 27 | -0.09 ± 0.21 | 0.01 | 0.01 | NS |
| Plasma ALP      | 28 | 3.32 ± 0.09 | 27 | 4.69 ± 0.21 | NS | 0.006 | NS |

Numbers, means, and standard errors of the measured traits as well as the significance of the differences between means are given. P-values < 0.05 were considered significant.

CYTB to 9 and 2, ELOVL6 to 10 and 4, ENO1 to 10 and 4, in the C group and R group, respectively.

Furthermore, the comparison of males and females reveals that the phenotypes are more correlated to the DEGs in males than in females. Actually, the plasmatic content of TG is correlated to 8 DEGs in the males (5 negative correlations: -0.53 ELOVL6, -0.48 GPAM, -0.58 PGK1, -0.52 PGM1, -0.46 UGDH and 3 positive correlations: 0.54 NDUFB6, 0.51 PRKAA1 and 0.56 RXRA) and 4 DEGs in the females (−0.61 BCL2A1, 0.59 CYTB, 0.68 ENO1 and 0.52 NDUFA4) and the liver weight is correlated to 11 DEGs in the males (7 positive correlations: 0.55 COX2, 0.67 ENO1, 0.54 NDUFA4, 0.48 NDUFB6, 0.66 PPARGC1B, 0.70 PRKAA1, 0.51 RXRA and 4 negative correlations: -0.49 ELOVL6, -0.61 GPAM, -0.47 PGM1 and -0.50 PPARG) and 0 DEG in females. On the contrary the hepatic lipid content is correlated to 0 DEG in males and to 5 DEGs in females (−0.50 COX2, −0.51 CYTB, −0.73 PRKAA1, 0.53 ELOVL6 and 0.59 PGK1). In addition, the correlation between DEGs is strong in both males and females except for BCL2A1 and NDUFB6, where the correlations with the other DEGs are stronger in males than in females.

The same correlation matrices were obtained in the four subgroups (MR, MC, FR and FC) and are presented in Additional Figure 1. Briefly, it can be noticed that the correlations between DEGs are stronger in the males from the C group (MC) compared to those in the males from the R group (MR). Moreover, the correlation matrices differ not only between groups (MR versus MC and FR versus FC) but also between sexes (MR versus FR and MC versus FC), which is consistent with the results found in the exploratory data analyses (Fig. 1) and the Principal Component Analysis (Fig. 2).

Discussion

The liver is the main tissue for lipid synthesis in birds. In adult mule ducks, the capacity to accumulate and store lipids -mainly triglycerides- in liver, is enhanced by overfeeding that leads to hepatic steatosis for fatty liver production. In the newly hatched mule ducklings, prior to the first feeding, body weight was about 6% lower while the liver to body weight ratio tended to be about 10% higher in ducklings from R group compared to those of the C group. Moreover, plasmatic parameters suggested altered hepatic energy metabolism in male and female ducklings from the Met-restricted dams (Table 2).

We therefore studied the livers of newly hatched ducklings, questioning the extent to which nutritional programming may have altered the expression level of hepatic genes in ducklings from dams fed reduced Met diets. Indeed, such a change in gene expression, if accompanied by a change in the synthesis of associated proteins, could alter metabolic pathways and lead to long-term effects on the ability of overfed animals to develop hepatic steatosis. The Fig. 4 gives a schematic representation of the role of the 16 DEGs and the 2 genes represented in the 16 DEGs and the 2 genes (PPARA and BMB, P-value (BH) ≤ 0.10) in the liver of newly hatched R group ducklings. The up-regulated and down-regulated DEGs being respectively in red and green.

Although the dry matter content and the lipid content of the livers of R group ducklings were similar to the ones of C group ducklings, the maternal dietary methionine deficiency affected the mRNA level of genes involved in
different pathways linked to energy metabolism such as glycolysis, lipogenesis, and mitochondrial electron transport. Thus, among the three DEGs involved in glycolysis, one was down-regulated (ENO1) and two were up-regulated (PGM1 and PGK1) in the R group. Moreover, COX2 (MT-CO2), NDUFA4, NDUFB6 and CYTB (MT-CYB), involved in the electron transport were all down-regulated in the R group ducklings suggesting a less active mitochondrial electron transport chain in this group.

Furthermore, ELOVL6 and GPAM were up-regulated suggesting a higher amount of TG in livers of the R group ducklings. In this group, the high content of glucose in plasma may increase the glycolysis activity and the pyruvate production. Then, in the mitochondria, the pyruvate may be converted into Acetyl-CoA that may in turn be converted in citrate by the tricarboxylic acid cycle (TCA cycle). Citrate may then be exported from the mitochondria and used as precursor of the de novo lipogenesis.
The expression level of *MTTP* whose protein controls lipoprotein assembly was down-regulated in the R group which may have led to limited lipid export from the liver. Moreover, *PRKAA1* encodes for the catalytic subunit of the 5’-prime-AMP-activated protein kinase (AMPK). In hepatocytes, PRKAA1 drives fatty acid oxidation and decreases lipogenesis, protecting against fatty liver disease [20]. In the current study, reduced *PRKAA1* mRNA levels in R group ducklings may also have contributed to higher lipogenesis. However, none of these genes showed a correlation between their expression level and the measured percentage of liver lipids.

The *PPARGC1B* gene (PPARG Coactivator 1 Beta, also known as *PGC1B*) encodes a nuclear protein which belongs to the peroxisome proliferator-activated receptor-γ coactivators (*PGC-1*s). They coactivate transcription factors and nuclear receptors, including PPARG, to control expression of genes involved, among other things, in electron transport, fatty acid oxidation or de novo lipogenesis. Indeed, PPARG is a master regulator of lipogenesis which has been described to promote lipid storage in the liver [21–23]. PPARA is also a member of the nuclear receptor PPAR family and is highly expressed in the liver where it is the main regulator of fatty acid catabolism by regulating the transcription of genes involved in β-oxidation. Both PPARA and PPARG are activated upon binding by fatty acids ligands. In the absence of ligand, they associate to RXR partner and bind to corepressor complex and repress target genes. RXRA (Retinoid X Receptor Alpha) is a member of the RXR family and the RXRA/PPARA heterodimer is required for PPARA transcriptional activity on fatty acid oxidation genes [24]. The coactivator PPARGC1B regulates mitochondrial energy transfers in the liver by positively regulating the expression of electron transport genes such as COX2, and mitochondrial β-oxidation [25]. In the current study, PPARGC1B (PGC1B) and RXRA were down-regulated and PPARA tended to be down-regulated too whereas PPARG was up-regulated in the liver of ducklings from the R group. Thus, in general in R group ducklings, the down-regulation of RXRA and PPARGC1B – and possibly also the one of PPARA - might have limited energy dissipation through fatty acid oxidation and thermogenesis, while the up-regulation of PPARG might have promoted energy storage through increased lipogenesis. RXRA showed no significant correlation with the traits measured in the ducklings of both groups. On the contrary PPARG was positively correlated with plasma activity of AST and ALT in the R group and negatively correlated to the liver DM content in the C group. PPARGC1B was
positively correlated with plasma FFA and liver DM content in the R group and negatively correlated with plasma TG in the C group (Fig. 3).

Apart from these results concerning the regulation of energy metabolism in the R group, other regulatory pathways were interestingly modulated by the maternal methionine supply in the offspring liver, especially concerning apoptosis. Indeed, both BMF and BCL2A1 belong to the BCL-2 protein family which governs the mitochondria-dependent pathway for apoptosis. Many Bcl-2 family proteins are localized to the membranes of mitochondria and both pro- and anti-apoptotic Bcl-2 family proteins exist. BMF encodes a protein that has been shown to bind Bcl-2 proteins and functions as pro-apoptotic protein whereas BCL2A1 reduces the release of pro-apoptotic cytochrome c from mitochondria and block caspase activation and thus functions as an anti-apoptotic protein. In our study, BMF tended to be down-regulated whereas BCL2A1 was up-regulated suggesting a metabolism turn towards a less apoptotic activity in ducklings from the R group. Moreover, UGTDH was up-regulated in the R group samples, suggesting a better detoxification activity in this group. Indeed, UGTDH encodes UDP-glucose dehydrogenase that converts UDP-glucose (UDP-Glc) to UDPglucuronic acid (UDP-GlcA). Interestingly UDP-GlcA is needed for detoxification of toxic compounds in liver via glucuronidation [26].

Overall, these results suggest a regulation of apoptotic pathways and tissue damage by the maternal diet. This is consistent with a lower activity of ALT activity and a tendency to a reduced activity of ALP activity in the plasma of ducklings from the R group suggesting a fetal programming towards a reduced susceptibility to hepatic injury.

As evocated in the Background section, the availability of methyl donors in the maternal diet and its effects on liver metabolism during embryonic development are well studied, especially in mammals where reviews highlight the interactions between one-carbon metabolism, epigenetic mechanisms and energy metabolism [27–29]. In birds, Ruqian Zhao’s team has studied the effects of one-carbon metabolism and some epigenetic mechanisms (DNA and histone methylation) in the liver of the R group ducklings, leading to altered methylation of gene promotors and inducing changes in energy metabolism pathways. In order to investigate this hypothesis, we are currently studying the impact of the maternal dietary restriction on the transcripts of genes involved in one-carbon metabolism and some epigenetic mechanisms.

Interestingly, throughout this study, the results also highlighted the extent to which the hepatic metabolism of mule ducklings is sex-dependent. A substantial proportion of the genes are differentially expressed according to the sex in many tissues and sexual dimorphism of gene expression in the liver has already been reported [32] including sex differences in PPAR signaling pathways in rodent models [33]. Thus, the application of a nutritional deficiency on metabolisms that already differ, leads to different impacts and sex differences in response to developmental programming as already reviewed by Aiken and Ozanne [34] and more recently by McCabe and coauthors [35]. In our study, some of the measured traits showed a sex effect [18] (Table 2) and, moreover, a number of the studied genes were differentially expressed depending of the sex of the ducklings (Table 1). Therefore, the liver metabolism of female ducklings differed from that of male ducklings in both groups of ducklings (R and C groups). Indeed, the correlation matrices of the two sexes differed within each group, i.e. MR versus FR and MC versus FC (Additional Fig. 1).

Conclusions

In conclusion, in the present study, we compared the hepatic expression levels of 100 target genes in ducklings from female ducks fed either a control diet or a methionine-restricted diet to look for transcriptomic evidence of early metabolic programming in R ducklings. We also sought to correlate differences in gene expression with phenotypic data already reported in a previous article [18]. The current study has shown that the transcriptome of the offspring is influenced, and therefore modifiable, by variations in the maternal diet, such as a 38% reduction in methionine content. Thus, the differential expression level of the 16 DEGs, if linked to a differential
production level of the associated proteins, may contribute to changes in energy metabolism and a reduced apoptotic activity in the livers of group R ducklings. These early changes in mRNA levels together with the observed phenotypic data, suggest modulations in the establishment of early metabolic pathways. This nutritional programming experiment was obtained on mule ducklings that benefit from heterosis from the Muscovy drake and the female common duck for the interest of mule ducks in fatty liver production. However, it would be of main interest to study the nutritional programming experiment on the pure common duck to know if the liver metabolism is impacted similarly in pure breed.

Methods

Animals and experimental design

Experimental procedures and animal care were conducted in compliance with the European Communities Council Directive 2010/63/EU. The experiment was conducted at the Ducks and Goose Experimental Facility – INRAE, UEPFG, (Bougnoun, France) that received the accreditation number B40–037-1. The protocol and procedures were approved by the French Minister of Higher Education, Research and Innovation (authorization APAFIS#1847-2015092213418825v2).

The experimental design has already been described [18]. Briefly, 60 female common ducks received adequate level of Met until the age of 10 weeks and were then divided into two groups. They were fed experimental diets from 10 to 51 weeks of age. Met is the first main limiting amino acid in a typical corn–soybean diet for poultry [36]. In laying ducks, the recommendations vary from 0.40 to 0.45% [37–40]. Thus, two levels of total Met were used for the experimental diets: 0.25% for Met-restricted diets (R Group) and 0.40% for control diets (C Group) that meets Met requirements for female laying ducks (Additional Table 2). The mule duckling production was performed by 2 artificial inseminations per week between 34 and 36 weeks of age, with the semen of 15 Muscovy drakes not subjected to any dietary treatment and fed commercial diets. The eggs were incubated for 28 days at 37.6 °C and 60% average relative humidity. Ducklings traits were recorded at hatching. The study targeted 100 genes selected from the literature for being related to energy metabolism as well as to amino acid transport, oxidative stress, apoptotic activity and susceptibility to liver damage. Sequences were obtained either from *Anas platyrhynchos* when available, or from *Gallus gallus* on NCBI [45] and/or Ensembl [46] databases. The two primers used for each gene (Additional Table 3) were designed in exons, but each on either side of an intron, and for an annealing temperature of 60 °C, using either Primer3Plus [47] or

RNA extraction and reverse transcription

Frozen liver samples from newly hatched ducklings of both sexes and of both control and restricted maternal diet groups (10 males and 8 females in the C group and 10 males and 10 females in the R group) were ground using a Retsch grinder at 30 Hz for 45 seconds, in liquid nitrogen. Next, 80 to 100 mg of tissue powder were processed as previously described [41] for RNA extraction and purification using the TRIzol® method (Invitrogen, California, USA) followed by a column from Nucleospin RNA kit (Macherey Nagel, France) and following the manufacturer’s instructions. The on-column DNase treatment was done with 20 μl of rDNase (Macherey Nagel) and 80 μl of reaction buffer for 20 min to avoid DNA contamination as recommended [42, 43]. The total RNA was quantified using a NanoDrop 8000 spectrophotometer (Thermo Fisher, Illkirch, France) and stored at −80 °C. Its integrity was controlled by electrophoresis and using an Agilent 2100 Bioanalyzer, with the RNA 6000 Nano Lab Chip Kit (Agilent Technologies, Massy, France). Reverse transcription was carried out immediately after the quality control evaluation, and the same amount of total RNA was used for all experimental samples, in accordance with recommendations [44]. The reaction used the RNase H-MMLV reverse transcriptase SuperScriptTM II (Invitrogen, California, USA), RNasin® Ribonuclease inhibitor (Promega Corporation, USA) and oligo (dT)15 (Sigma Aldrich, France). The cDNAs were then diluted in RNase free water and stored at −80 °C.

Primers design and qPCR validation

The study targeted 100 genes selected from the literature for being related to energy metabolism as well as to amino acid transport, oxidative stress, apoptotic activity and susceptibility to liver damage. Sequences were obtained either from *Anas platyrhynchos* when available, or from *Gallus gallus* on NCBI [45] and/or Ensembl [46] databases. The two primers used for each gene (Additional Table 3) were designed in exons, but each on either side of an intron, and for an annealing temperature of 60 °C, using either Primer3Plus [47] or
LightCycler® Probe Design Software 2.0 (Roche Applied Science). The primer sequences were blasted to the databases to confirm that they were specific to the gene in question. PCR products were first subjected to 2% agarose gel electrophoresis to confirm amplicon size. Next, the primer pairs showing a specific band and no primer dimers were selected to be tested by qPCR, using SYBR green fluorescence detection (Applied Biosystems) and a QuantStudio6 (Thermo Fisher Scientific). Each primer pair was tested on four serial dilutions of a pool of cDNA (cDNA of all the animals used in the study) to obtain a standard curve and check the PCR efficiency, each point being done in duplicate. The conditions were: 50 °C for 2 min, denaturation at 95 °C for 10 min, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. A gradual temperature increasing from 60 °C to 95 °C was added to analyze the melting curves and detect primer dimers. The Cq values and the PCR efficiency were obtained directly from the QuantStudio Real-Time PCR software v1.3.

### Identification of potential reference genes

The expressions of 9 potential reference genes were tested for their stability in livers of newly hatched mule ducklings. Primer sequences were either from Chapman et al., 2016 [48] (designed in Anas platyrhynchos: ALB, GAPDH, NDUFA10 and RPS13), from Staines et al., 2016 [49] (designed in Gallus gallus: HMBS and TBP) or newly designed in Anas platyrhynchos (HPRT1, POLA1 and TUBA1C). These 9 primer pairs were tested by qPCR on liver cDNA from 8 ducklings of both sexes and of both maternal diet groups (C group and R group) with SYBR green fluorescence detection, on a QuantStudio6. The selection of the 5 most stable genes (GAPDH, HMBS, NDUFA10, RPS13, TBP), highlighted in dark grey in Additional Table 3 was done with the package SLqPCR on RStudio [50].

### Quantitative PCR and gene expression analysis

The quantification of gene expression was performed using the 96.96 Dynamic Array integrated fluidic circuits (IFCs) and the BioMark HD system from Fluidigm as previously described [51].

In this paper we present the study of transcripts of genes involved in energy metabolism in the liver of newly hatched ducklings but the full experiment was conducted on 168 liver samples (38 samples of newly hatched ducklings and 130 samples of older duck livers) and a total of 170 genes, either targeting energy metabolism for this study (100 genes) or playing a role in one-carbon metabolism (70 genes), as well as five reference genes identified in the previous step. As the technology used did not allow all samples and genes to be analyzed at the same time, care was taken to randomize the samples onto two arrays and the genes into each specific target amplification (STA). Thus, a total of four chips were made. In this study, we focus our interest on the 38 liver samples from newly hatched ducklings and 100 genes involved in energy metabolism.

For each chip, the first step consisted of a STA with 14 cycles performed on the cDNA samples, a calibrator sample (a pool of the 38 cDNA samples), a cDNA pool of the 168 cDNA samples in fivefold serial dilutions (to determine the PCR amplification efficiency), a duck genomic DNA control, an internal control (human genomic DNA) and a negative control (TE). The first STA was performed with a pre-amplification primer mix that contained primers for the five potential reference genes and 85 target genes (among those 53 are some of this study). A second STA contained primers for the five potential reference genes and other 85 target genes (among those 47 are some of this study). The resulting STA cDNA samples were treated according to Bonnet et al., 2013 [51].

Data were analyzed with the Fluidigm Digital PCR Analysis software (version 4) using the linear (derivative) baseline correction method and the auto (global) cycle threshold (Cq) method. The data were pre-processed before expression analysis. Indeed, the cycle threshold values (Ct) recorded from amplifications whose melting curves showed either abnormal Tm (melting temperature) or double peaks (corresponding to a mixture of expected and aberrant PCR products) or a high baseline, were removed. To measure the efficiency (E) of PCR for each gene, the slope of the standard curve obtained with serial dilutions of the 168 cDNA pool was used and genes with less than three dilution points were eliminated. Finally, all genes with an efficiency greater than 2.2 or less than 1.7 were removed from the analysis.

The relative expression (\( RE_{ij} \)) for each gene (i) and each sample (j) was calculated as proposed by Pfaffl [52]:

\[
RE_{ij} = \frac{E_{ij}}{E_{ij_{cal}}} = \frac{Cq_{ij_{cal}} - Cq_{ij}}{Cq_{ij_{cal}}}
\]

where \( Cq_{ij_{cal}} \) is the Cq for the gene (i) determined for the calibrator sample (a pool of the 38 cDNA samples). To determine the reference genes, the stability of the five potential genes was tested using the GeNorm (version 3.4) algorithm [50]. The three most stable genes were chosen as references genes (GAPDH, RPS13 and TBP). Finally, the relative expression (\( RE_{ij} \)) for each gene (i) and each sample (j) was normalized with the geometric average expression of the three most stable reference genes as proposed by Vandesompele et al. [50].

In the end, 13 of the 100 genes studied and 2 of the 38 liver cDNA samples showed more that 25% of missing data and were removed from the study. Moreover, another cDNA sample was also removed from the data set because it showed data points that differed significantly from other observations. The 13 removed genes...
are highlighted in light grey in Additional Table 3. The 35 remaining liver cDNA samples are from 9 male and 8 female ducklings from the C group and 10 male and 8 female ducklings from the R group.

Statistical analyses
For the 87 remaining genes, the few missing values were imputed inside each group with a sex and maternal diet using the function imputPCAm with 3 principal components of the missMDA package of the R software [53]. These normalized and imputed relative expressions were then transformed using the function qZnorm(Y)$x to make the data follow a centered reduced normal distribution. These transformed data were then used to describe the whole dataset.

First a heatmap was obtained by using the R gplots package thus defining groups of animals and genes with similar expression patterns and a Principal Component Analysis (PCA) was performed with the package MixOmics of R software [54] on the 87 genes. The individuals were plotted on the two first principal components of the PCA score plot.

Then, ANOVAs were conducted on the transformed normalized relative expressions of the 87 genes using a linear mixed model fitted with the ASReml software [55] that included the maternal diet, the sex of the duckling, and the interaction between them as fixed effects, as well as the duckling associated to its relationship matrix as a random effect. Genes with a significant difference - diet $P$-value < 0.05 assessed after a Benjamini-Hochberg (1995) [56] correction, in order to account for multiple tests and named (diet $P$-Value BH) - were selected and considered as differentially expressed genes (DEGs). The effect of sex on gene expression was also evaluated (sex $P$-value BH), as well as the interaction between sex and diet effects (sex$\times$diet $P$-value BH). For each gene, least square means and standard deviations were calculated for the 2 groups of maternal diet (R group and C group), for the 2 sexes, and finally for the four subgroups of interest, i.e., males in the R group and C group and females in the R group and C group (Additional Table 1).

Further analyses were performed on the 16 DEGs for the diet effect. Another PCA was performed on the 16 DEGs with the package MixOmics of R software [54], using the qnorm transformed normalized relative expressions. The biplot of variables and samples from the two first principal components was obtained with the package Factoextra of R. Concentration ellipses were plotted around each group mean points with a confidence level of 0.75 using the package ellipse.

Then, correlation matrices were performed using phenotypic traits of the ducklings and the 16 DEGs for the diet effect, using the imputed normalized relative expressions to be consistent with the phenotypic data which were also not transformed.

The correlation matrices were plotted with the functions corrcov and corrplots [57, 58] and only the correlations with a $P$-value < 0.05 were reported.

Lastly, this study was conducted and is reported in accordance with the ARRIVE guidelines [59].

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12864-022-08634-1.

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**Authors’ contributions**
MIM conceived and designed the study and wrote the manuscript. XM and A. Cornuez were responsible for animal breeding. AS was responsible for sample and data traceability and performed the quantitative PCR and gene expression analysis. LEB designed most of the primers with the help of LG, performed RNA extraction, qPCR validation and conducted the step for identification of potential reference genes. AS, HC, CMB and AB carried out the statistical analyses with the help of LB. A. Collin revised the manuscript and contributed to the discussion section. HC, CMB, AB and LB revised the manuscript. All authors have read and approved the final version of the manuscript.

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**Availability of data and materials**
The datasets supporting the conclusions of this article are included within the article and its three Additional Tables and one Additional Figure.

**Declarations**

**Ethics approval and consent to participate**
Experimental procedures and animal care were conducted in compliance with the European Communities Council Directive 2010/63/EU. The experiment was conducted at Ducks and Goose Experimental Facility – INRAE, UEPFG, (Bouquen, France) that received the accreditation number B40–037-1. The protocol and procedures were approved by the French Minister of Higher Education, Research and Innovation (authorization APAFIS#1847-2015092213418825v2).

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare no competing interests.

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**References**
1. Lee HS. Impact of Maternal Diet on the Epigenome during In Utero Life and the Developmental Programming of Diseases in Childhood and Adulthood. Nutrients. 2015;7:9492–507.
2. Langley-Evans SC. Nutrition in early life and the programming of adult disease: a review. J Hum Nutr Diet. 2015;28(Suppl 1):1–14.
3. Koletsko B, et al. Nutrition During Pregnancy, Lactation and Early Childhood and its Implications for Maternal and Long-Term Child Health: The Early Nutrition Project Recommendations. Ann Nutr Metab. 2019;74:93–106.
4. Safi-Stibler S, Gabory A. Epigenetics and the Developmental Origins of Health and Disease: Parental environmental signalling to the epigenome, critical time windows and sculpting the adult phenotype. Semin Cell Dev Biol. 2020;97:172–80.
5. Chavatte-Palmer P, Taradde A, Rousseau-Ralliard D. Diet before and during Pregnancy and Offspring Health: The Importance of Animal Models and What Can Be Learned from Them. Int J Environ Res Public Health. 2016;13(5):586. https://doi.org/10.3390/ijerph13050586.
6. Sinclair KD, et al. Epigenetics and developmental programming of welfare and production traits in farm animals. Reprod Fertil Dev. 2016. https://doi.org/10.1071/RD16102.
7. Khanal P, Nielsen MO. Impacts of prenatal nutrition on animal production and performance: a focus on growth and metabolic and endocrine function in sheep. J Anim Sci Biotechnol. 2017;75.
8. Dixon LM, Sparks NHC, Rutherford KMD. Early experiences matter: a review of the effects of prenatal environment on offspring characteristics in poultry. Poul Sci. 2016;95:489–99.
9. Morisson M, et al. Nutritional Programming and Effect of Ancestor Diet in Birds. In: Handbook of Nutrition, Diet, and Epigenetics: Springer International Publishing; 2017. p. 1–18.
10. Jha R, Singh AK, Yadav S, Berrocoso JFD, Mishra B. Early Nutrition Programming (in ovo and Post-hatch Feeding) as a Strategy to Modulate Gut Health of Poultry. Front Vet Sci. 2019;6:82.
11. Calini F, Sini R. Breeder Nutrition and Offspring Performance. Braz J Poultry Sci. 2017;9:7–83.
12. Willems E, et al. Embryonic protein undernutrition by albumen removal programs the hepatic amino acid and glucose metabolism during the perinatal period in an avian model. PLoS One. 2014;9:e94902.
13. Willems E, et al. Differential Expression of Genes and DNA Methylation associated with Prenatal Protein Undernutrition by Albumen Removal in an avian model. Sci Rep. 2016;6:20837.
14. Willems E, et al. Partial albumen removal early during embryonic development of layer-type chickens has negative consequences on laying performance in adult life. Poul Sci. 2013;92:1905–15.
15. Hu Y, et al. In Ovo injection of betaine affects hepatic cholesterol metabolism through epigenetic gene regulation in newly hatched chicks. PLoS One. 2015;10:e0122543.
16. Hu Y, et al. In ovo injection of betaine alleviates corticosterone-induced fatty liver in chickens through epigenetic modifications. Sci Rep. 2017;7:40251.
17. Hou Z, et al. Maternal betaine administration modulates hepatic type 1 iodothyronine deiodinase (Dio1) expression in chicken offspring through epigenetic modifications. Comp Biochem Physiol B Biochem Mol Biol. 2018;218:30–4.
18. Bodin L, et al. Dietary methionine deficiency reduces laying performances of female common ducks and impacts traits of interest of their mule ducklings. Poul Sci. 2019;98:S590–600.
19. Marie-Etancelin C, et al. Genetics and selection of mule ducks in France: A review. World’s Poultry Sci J. 2008;64:187–207.
20. Boudaba N, et al. AMPK Re-Activation Suppresses Hepatic Steatosis but its Downregulation Does Not Promote Fatty Liver Development. EBioMedicine. 2018;28:194–209.
21. Ahmadian M, et al. PPARγ signalling and metabolism: the good, the bad and the future. Nat Med. 2013;19:557–66.
22. Steensels S, Qiao J, Ensoy BA. Transcriptional Regulation in Non-Alcoholic Fatty Liver Disease. Metabolites. 2020;10(7):283.
23. Pei K, et al. An Overview of Lipid Metabolism and Nonalcoholic Fatty Liver Disease. Biomed Res Int. 2020;2020:4020249.
24. Hardwick JP, Osei-Hyiaman D, Wiland H, Abdelmegeed MA, Song B-J. PPAR/RXR Regulation of Fatty Acid Metabolism and Fatty Acid omega-Hydroxylation (CYP4) Isozymes: Implications for Prevention of Lipotoxicity in Fatty Liver Disease. PPAR Res. 2009;2009:952734.
25. Sonoda J, Mehl IR, Chong L-W, Nofsinger RR, Evans RM. PGC-1beta controls mitochondrial metabolism to modulate circadian activity, adaptive thermogenesis, and hepatic steatosis. Proc Natl Acad Sci U S A. 2007;104:5223–8.
26. Vatsyayan J, Lin C-T, Peng H-L, Chang H-Y. Identification of a cis-Acting Element Responsible for Negative Regulation of the Human UDP-Glucose Dehydrogenase Gene Expression. Biosci Biotechnol Biochem. 2006;70:401–10.
27. Cai D, Liu H, Hu Y, Jiang Y, Zhao R. Gestational Betaine, Liver Metabolism, and Performance: a Focus on Growth and Metabolic and Endocrine Function. J Anim Sci Biotechnol. 2017;8:40251.
28. Chango A, Pogribny IP. Considering maternal dietary modulators for epigenetic regulation and programming of the fetal epigenome. Nutrients. 2015;7:2748–70.
29. Clare CE, Brassington AH, Kwong WY, Sinclair KD. One-Carbon Metabolism: Linking Nutritional Biochemistry to Epigenetic Programming of Long-Term Development. Annu Rev Anim Biosci. 2019;7:263–87.
30. Hu Y, et al. Maternal betaine supplementation decreases hepatic cholesterol deposition in chicken offspring with epigenetic modulation of SREBP2 and CYP7A1 genes. Poult Sci. 2020;99:1111–20.
31. Hu Y, et al. Corticosterone-Induced Lipogenesis Activation and Lipophagy Inhibition in Chicken Liver Are Alleviated by Maternal Betaine Supplementation. J Nutr. 2018;148:316–25.
32. Hochberg Z, et al. Child health, developmental plasticity, and epigenetic programming. Endocr Rev. 2011;32:159–224.
33. Berthier A, Johanns M, Zumbo PF, Lefebvre P, Staels B. PPARs in liver physiology. Biochim Biophys Acta Mol basis Dis. 2021;1867:166097.
34. Aiken CE, Ozanne SE. Sex differences in developmental programming models. Reproduction. 2013;145:R1–13.
35. McCabe C, Anderson OS, Montrose L, Neier K, Dolinoy DC. Sexually Dimorphic Effects of Early-Life Exposures to Endocrine Disruptors: Sex-Specific Epigenetic Reprogramming as a Potential Mechanism. Curr Environ Health Rep. 2017;4:426–38.
36. Bunchasak C. Role of Dietary Methionine in Poultry Production. J Poult Sci. 2009;46:169–79.
37. He JH, et al. Dietary methionine requirement of the Chinese egg-laying duck. Br Poult Sci. 2003;44:741–5.
38. Fouad AM, et al. Nutritional requirements of meat-type and egg-type ducks: what do we know? J Anim Sci Biotechnol. 2018;9:1.
39. Fouad AM, et al. Effects of dietary methionine on performance, egg quality and glutathione redox system in egg-laying ducks. Br Poult Sci. 2016;57:818–23.
40. Ruan D, et al. Effects of dietary methionine on productivity, reproductive performance, antioxidant capacity, ovalbumin and antioxidant-related gene expression in laying duck breeders. Br J Nutr. 2018;119:121–30.
41. Voillet V, et al. Integrated Analysis of Proteomic and Transcriptomic Data Highlights Late Fetal Muscle Maturation Process. Mol Cell Proteomics. 2018;17:672–93.
42. Bustin SA, et al. MIQE précis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. BMC Mol Biol. 2010;11:74.
43. Bustin SA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009;55:611–22.
44. Taylor S, Wakem M, Dijkman G, Alsarraj M, Nguyen M. A practical approach to RT-qPCR-Publishing data that conform to the MIQE guidelines. Methods. 2010;50:51–5.
45. The National Center for Biotechnology Information (NCBI). Bethesda. https://www.ncbi.nlm.nih.gov/.
46. The Ensembl genome database project (Ensembl). Cambridgeshire: European Bioinformatics Institute (EMBL-EBI). https://www.ensembl.org/index.html.
47. Undergasser A, et al. Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res. 2007;35:W71–4.
48. Chapman JR, et al. A Panel of Stably Expressed Reference Genes for Real-Time qPCR Gene Expression Studies of Mallards (Anas platyrhynchos). PLoS One. 2016;11:e0149454.
49. Staines K, et al. A Versatile Panel of Reference Gene Assays for the Measurement of Chicken mRNA by Quantitative PCR. PLoS One. 2016;11:e0160173.
50. Vandesompele J, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002;3:RESEARCH0034.
51. Bonnet A, et al. An overview of gene expression dynamics during early ovarian folliculogenesis: specificity of follicular compartments and bi-directional dialog. BMC Genomics. 2013;14:904.
52. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001;29:e45.
53. Josse J, Husson F. missMDA: A Package for Handling Missing Values in Multivariate Data Analysis. J Stat Soft. 2016;70:1–31.
54. Rohart F, Gautier B, Singh A, Lê Cao K-A. An R package for omics feature selection and multiple data integration. PLoS Comput Biol. 2017;13:e1005752.
55. Gilmour A, Gogel B, Cullis B, Welham S, Thompson R. ASReml User Guide Release 4.1. Functional Specification. In: VSN International Ltd, Hemel Hempstead, HP1 1ES, UK; 2015.
56. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J R Stat Soc. 1995;57:289–300.
57. Murdoch D, Chow E. A Graphical Display of Large Correlation Matrices. Am Stat. 1996;50:178–80.
58. Friendly M. Corrgrams: Exploratory displays for correlation matrices. Am Stat. 2002;56:316–24.
59. Percie du Sert N, et al. The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. BMJ Open Sci. 2020;4:e100115.

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