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Sex-specific effects of a methionine-restricted maternal diet on liver transcript levels and fatty liver production in mule ducks

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ABSTRACT

Sex-specific effects of maternal diet on offspring phenotypes have been reported in farm animals including in poultry. The present study was conducted in mule ducks, investigating the long-term effects of a methionine-restricted maternal diet on the production performance of the offspring of both sexes. Sixty female ducks were divided into two groups and fed either a control diet containing 4.0 g/kg of methionine or a restricted diet containing 2.5 g/kg of methionine. Next, 254 offspring were divided into four subgroups of 60–67 animals, according to maternal diet and sex. Their growth performance was recorded until 87 days of age. Then, plasma parameters were measured on these non-overfed ducks (**NOFDs**) at D87 and 60 of them were sacrificed, representing 15 males and 15 females in each maternal diet group. Carcass traits were recorded, and the liver transcript level of 170 genes mainly involved in energy or one-carbon metabolism was studied. The remaining 194 ducks were overfed during 12.5 days -until 100 days of age- for fatty liver production. Then, zootechnical traits and plasma parameters were measured on these 194 overfed ducks (**OFDs**), and the liver transcript level of the same 170 genes was studied. The results showed that the methionine-restricted maternal diet affected traits in NOFDs but in females only, with lower liver lipid and DM percentages (*P*-value = 0.006 and *P*-value = 0.004, respectively) and a lower plasma cholesterol level (*P*-value = 0.020). In OFDs, after the overfeeding period, fatty liver weight was reduced in both sexes by around 53 g, or almost 10% (*P*-value = 0.016 and 0.017 in females and males, respectively). Only females showed a tendency to lower liver lipid and DM percentages (*P*-value = 0.078 and *P*-value = 0.062, respectively) and their plasmatic aspartate aminotransferase activity was reduced (*P*-value = 0.025). In addition, 18 genes differentially expressed between maternal diet groups were identified in the liver of females only. All were up-regulated in the restricted group and involved in either energy or one-carbon metabolism. These findings showed that the methionine-restricted maternal diet had long-term effects on liver traits, impacting production performance in both sexes. Importantly, these effects were sex-specific, and robust enough to still be observed after the overfeeding period, despite the major reorganisation of metabolic pathways this implied.

Implications

While significant advancements have been made in genetics and husbandry of farmed birds, the influence of maternal nutrition on offspring performance has only recently gained attention. Here, in a duck model, we showed that a methionine-restricted maternal diet impacted hepatic expression of genes of interest in female offspring and decreased fatty liver production in offspring of both sexes. These findings suggest that producers should ensure that maternal diets meet appropriate levels of methionine and other methyl donors to optimise production performance.

Introduction

The effects of maternal diet on offspring phenotypes and performance have been well documented in farm animals (Caton et al., 2020; Khanal and Nielsen, 2017; Chavatte-Palmer et al., 2016; Sinclair et al., 2016), including in poultry where maternal diet determines the nutritional composition of the egg (Abdel-Moneim et al., 2023; Andrieux et al., 2022; Jha et al., 2019;

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Métayer Coustard et al., 2019; Morisson et al., 2017). Indeed, nutrient availability during embryonic development has been shown to interfere with key metabolic pathways and consequently to shape individual phenotypes over the long term, a phenomenon known as nutritional programming. For example, in laying hens, early protein restriction by albumen removal at day 1 of incubation resulted in adult hens laying smaller eggs with a lower proportion of albumen (Willems et al., 2013) and with an altered liver transcriptome (Willems et al., 2016). In poultry again, Hu and co-authors reported that *in ovo* injection of betaine affected hepatic cholesterol metabolism in newly hatched chicks (Hu et al., 2015) and subsequently protected them from hepatic steatosis induced by a high-fat diet (Hu et al., 2017). Furthermore, when betaine was administered to hens instead of being injected into eggs, it induced epigenetic modulations altering the hepatic expression of several genes in their 57-day-old male offspring (Hu et al., 2020; Hou et al., 2018). Indeed, nutrients such as methionine and betaine - which feed the one-carbon metabolism- contribute to the transfer of methyl groups essential to numerous cellular processes such as the epigenetic control of gene expression via DNA methylation (Clare et al., 2019; Van Winkle and Ryznar, 2019; Xu and Sinclair, 2015).

On the other hand, effects of the maternal diet may be sex-specific. As sex-biased expression of genes carried by sex chromosomes begins as soon as the embryonic transcriptome is activated, it leads to sex-specific expression not only in the gonads but also in somatic tissues. Sex chromosome-encoded transcriptional and epigenetic factors initiate sex-specific regulatory cascades and sex-specific pathways, resulting in sex-biased gene expression and sex-specific cellular functions that persist throughout life. Subsequently, the effects of gonadal hormones interact with sex-biased gene effects contributing to sex-specific phenotypes (Arnold, 2019; Deegan and Engel, 2019; Engel, 2018). Thus, sex-specific metabolic programming effects have been reported in birds. For example, in chicken, the female offspring of dams subjected to dietary restriction had more abdominal fat at 6 weeks of age than those of control dams, which was not the case for the male offspring (van der Waaij et al., 2011). Sex-specific effects of embryonic thermal manipulation on the hypothalamus transcriptome of 35-day-old quails submitted to a heat challenge were also reported (Vitorino Carvalho et al., 2021).

We therefore investigated whether reducing methionine (**Met**) content in the diet of female ducks could affect liver metabolism and fatty liver production in both male and female offspring. In mule ducks, which are sterile offspring of female common ducks (*Anas platyrhynchos*) and male Muscovy ducks (*Cairina moschata*) (Marie-Etancelin et al., 2008), fatty liver production results from the development of a reversible hepatic steatosis, obtained by a 12-day period of overfeeding. In our study, the restricted (Group R) and control (Group C) female common ducks were fed diets containing either 2.5 or 4.0 g/kg of Met, respectively. At hatching, Group R newly hatched ducklings (**NHDs**) showed reduced BWs and altered plasma parameters (Bodin et al., 2019). Moreover, in a study targeting the liver transcripts of 170 genes related to energy metabolism, one-carbon metabolism and epigenetic mechanisms, we identified 28 differentially expressed genes (**DEGs**) between the two groups of NHDs. We also showed that a number of the studied genes were differentially expressed according to duckling sex (Sécula et al., 2022a, 2022b). In the new study presented in this article, we evaluated long-term, sex-specific effects of the methionine-restricted maternal diet. To this end, we looked for altered phenotypic traits and for hepatic DEGs in non-overfed ducks (**NOFDs**) and in overfed ducks (**OFDs**), before and after the overfeeding period, respectively.

Material and methods

Experimental design

This study was conducted and is reported in accordance with the ARRIVE guidelines (Percie du Sert et al., 2020). The experimental design and the composition of the experimental diets have already been described (Bodin et al., 2019). Briefly, 60 female common ducks received an adequate level of Met until the age of 10 weeks and were then divided into two groups and fed experimental diets. The Group C received a control growing diet containing 4.0 g/kg of Met while the Group R received a restricted growing diet containing 2.5 g/kg of Met (−37% comparing to the control diet) during the growing period from 10 to 16 weeks of age. They then received reproduction diets containing 4.0 and 2.5 g/kg, respectively, from 16 to 51 weeks of age. Both growing and reproduction diets are presented in Supplementary Table S1. The Met contents of these experimental diets were measured (INVIVO LABS SAS; Chateau Thierry, France), and the results were in accordance with the expected levels (2.4, 2.5, 4.0 and 4.0 g/kg for the restricted growing, restricted reproduction, control growing, and control reproduction diets, respectively, after correction by the DM of the feeds). Egg-laying performance were recorded from onset to 51 weeks of age. The laying peak was reached at 22 weeks of age, and eggs were collected from females aged 30–34 weeks to record egg weight and to determine egg composition. The mule duckling production was performed with the semen of Muscovy drakes aged 41–43 weeks. These Muscovy drakes were not subjected to any dietary treatment and were fed commercial diets (SOAL Haut-Mauco, France). The starting, growing and reproduction diets contained 175, 155 and 175 g/kg of CP, respectively, and 5.0, 3.9 and 4.3 g/kg of Met, respectively. Artificial inseminations were carried out twice a week, and fertilised eggs were collected from females aged 32–36 weeks and stored at 16 °C before incubation. Female reproduction traits and duckling viability have been published (Bodin et al., 2019). Ducklings that were the offspring of Group R and Group C females were subsequently assigned to Group R and Group C, respectively.

Zootechnical measurements and sample collection

Data collected on NHDs at hatching (D1) -such as weight, down colour, plasma parameters and hepatic transcripts of 170 targeted genes- have already been published (Sécula et al., 2022a, 2022b; Bodin et al., 2019). In this new study, we collected new data on NOFDs at D87 and on OFDs at D100 to study the long-term effects of the nutritional programming. The recorded traits are summarised in Fig. 1. The ducklings were reared separately according to their sex and their mother's diet (four subgroups: Group R females, Group R males, Group C females, and Group C males). To this end, 254 ducklings were housed in pens of 30–34 animals (eight pens, two replicates of the four subgroups). They were reared in the same building under the same conditions and fed *ad libitum* until 8 weeks of age. They received a starting diet (PAG1301, SOAL Haut-Mauco, France; Supplementary Table S2) until 4 weeks of age, then a growing diet (PAG1320, SOAL Haut-Mauco, France; Supplementary Table S2) until 8 weeks of age. They were then fed the same growing diet but for only 1 h a day between 8 and 12 weeks of age to prepare them for overfeeding. Feed intake from D1 to D87 was measured per pen, and individual average daily gain between D1 and D87 was calculated and expressed in g/d. Animal growth was monitored by weighing the animals at D1, D29, D57 and D87. For each of Group C and Group R, 15 NOFDs of both sexes were sacrificed (60 animals in total). These mule ducks were selected on the basis of their BW at D87

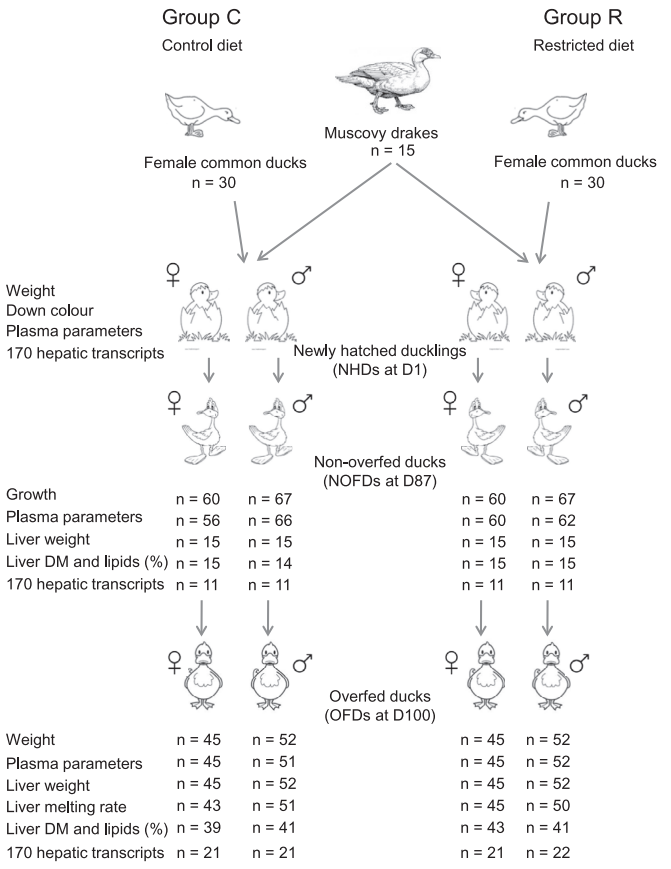


Fig. 1. Experimental design. Sixty female common ducks were divided into two groups at 10 weeks of age. Group R and Group C were fed restricted diets containing 2.5 g/kg Met and control diets containing 4.0 g/kg Met, respectively. The production of mule ducklings was performed with the semen of Muscovy males fed with commercial feed. Then, ducklings of both sexes, 127 in each group, were weighed at D1, D29, D57 and D87. At 87 days of age, before the overfeeding, plasma parameters were studied in all the non-overfed ducks (NOFDs) and some of them were weighted before being sacrificed (15 males and 15 females in each group). Liver weight was measured as well as the liver DM and lipid percentages. In addition, gene expression analyses were performed for 170 targeted genes to look for liver differential gene expression. The remaining animals were overfed twice a day during 12.5 days corresponding to 25 meals, and weighed at D100 before being slaughtered. For all the overfed ducks (OFDs), plasma parameters were studied, liver weight was measured as well as the liver DM and lipid percentages. The liver melting rate was also measured. In addition, gene expression analyses were performed for the same 170 targeted genes to look for liver differential gene expression. The number of animals studied is indicated for each trait.

to be representative of the four subgroups (Group R females, Group C females, Group R males and Group C males). The remaining animals (n = 194) were settled in the overfeeding room, in cages of four animals. They were overfed with corn twice a day for 12.5 days -corresponding to 25 meals- and following the predefined feed intake curve shown in Supplementary Fig. S1. The meals were prepared with 53% Palma 146 (SOAL, Haut-Mauco, France; Supplementary Fig. S1) and 47% water. At the end of the overfeeding period, at D100, cumulative feed intake was calculated as the amount of feed ingested over the 25 meals for each OFD. All OFDs were weighed.

The sixty selected NOFDs and all OFDs were conventionally slaughtered by electronarcosis and bleeding, between 12 and 14 h after the last meal. Blood samples were collected and centrifuged for 15 min at 1 700 g and 4 °C to separate the plasma that were stored at –20 °C. These ducks were weighed after bleeding and removal of feathers, legs and wing tips. Their livers were removed and weighed. Liver samples were collected and immediately immersed in liquid nitrogen before being stored at –80 °C to further evaluate liver DM and lipid percentages and for further RNA analyses. For OFDs only, another liver sample (60 g) was collected to further assess the liver melting rate. The ratio between liver and feed intake during the overfeeding period was calculated individually as the liver weight measured in OFD minus the average liver weight in NOFDs inside the subgroup of interest (either Group R females, Group C females, Group R males or Group C males), divided by the individual cumulative feed intake. It is expressed in g of liver / kg of feed as already used by Bonnefont and co-authors (Bonnefont et al., 2019).

Plasma parameters

For all the plasma samples collected in NOFDs before overfeeding, and in OFDs after overfeeding, six plasma parameters were analysed with an ABX Pentra 400 clinical chemistry analyzer from Horiba Medical (Grabels, FR) at the ANEXPLO platform (Toulouse, France: https://anexplo.genotoul.fr/) with assay reagents from HORIBA ABX SAS for individual plasma glucose (Ref No. A11A01668), cholesterol (Ref No. A11A01634), and triglyceride (Ref No. A11A01640) levels, as well as for individual plasma alkaline phosphatase (**ALP**) (Ref No. A11A01626), alanine aminotransferase (**ALT**) (Ref No. A11A01627) and aspartate aminotransferase (**AST**) (Ref No. A11A01629) activities. For free fatty acid quantitative determination, the assay reagents were from SOBIODA SAS (Wako Chemicals GmbH: NEFA C; Code No. 999-75406 and NEFA C Standard; Code No. 270-76499).

Liver parameters

The liver samples collected in NOFDs and OFDs and stored at –80 °C were ground into powder using a Retsch grinder at 30 Hz for 45 s in liquid nitrogen. A portion of the liver powder was dried in an oven at 105 °C for 24 h for DM content determination. Another portion of the liver powder was used to assess lipid content by extracting all lipids by homogenisation in chloroform and methanol 2:1 (v/v) and measuring them using the method of Folch et al. (Folch et al., 1957). The lipid content was measured in duplicates and averaged. The fatty liver melting rate was also evaluated for OFDs. For this, individual samples of liver (60 g) were placed in individual tin cans, cooked in an autoclave at 85 °C for 60 min, and stored overnight at 4 °C. The melted fat was removed, and the melting rate was then calculated as the percentage of loss from the initial liver weight.

Quantitative PCR and gene expression analysis

Liver samples were also used for gene expression analysis. Animals were selected to be representative of their subgroup, on the BW at slaughter. In total, 44 samples were chosen in NOFDs (11 by subgroup) and 85 samples in OFDs (21 or 22 by subgroup, as the liver weight variability was strongly higher after overfeeding). Thus, 80–100 mg of tissue powder was processed for RNA extraction and reverse transcription as previously reported (Sécula et al., 2022b). Primer design and quantitative PCR validation have already been described for the first 100 targeted genes (Sécula et al., 2022b) and for the 70 other ones (Sécula et al., 2022a) as well as the identification of potential reference genes (Supplementary Table S3). Gene expression was quantified using 96.96 Dynamic Array Integrated Fluidic Circuits and the Fluidigm BioMark HD system. The entire experiment was conducted on 168 liver samples (39 samples of NHD livers for the two studies already published, and 129 samples for the current study) and a total of 170 genes, either targeting one-carbon metabolism and epigenetic mechanisms (70 genes)

or playing a role in energy metabolism (100 genes) (Supplementary Table S3). As the technology used did not allow for all samples and genes to be analysed on the same chip, care was taken to randomise the samples on two chips and the genes in each specific target amplification, thus making a total of four chips. For each of the four arrays, a 14-cycle specific target amplification was performed on the cDNA samples, a calibrator sample (either a pool of the 44 cDNA samples for NOFDs or a pool of the 85 cDNA samples for OFDs), a pool of the 168 cDNA samples in fivefold dilutions (to determine PCR amplification efficiency), a duck genomic DNA control, an internal control (human genomic DNA) and a negative control (Tris-EDTA). Gene expression analysis was conducted in NOFDs and OFDs as already reported twice (Sécula et al., 2022a, 2022b). At the end of these analyses, after eliminating outliers and genes with more than 25% missing data, 145 genes remained for NOFDs and 143 genes for OFDs.

Statistical analyses of results

The statistical unit is the animal for all analyses, as we measured some individual data: weights, liver RNA expressions... Phenotypic traits and differential gene expression were analysed separately for NOFDs and OFDs. For phenotypic traits, data were analysed directly without normalisation, except for plasma parameters where the values were log-transformed to obtain normal distributions. For the analysis of differential gene expression, for each gene, the few missing values were imputed within each group of the same sex and maternal diet using the imputPCA function with three principal components from the missMDA package of R software (Josse and Husson, 2016). These normalised and imputed relative expressions were then transformed using the function qqnorm() (Becker et al., 1988) to make the data follow a centred reduced normal distribution as already reported (Sécula et al., 2022a, 2022b). The same statistical model was then applied on all data.

ANOVAs were conducted using a linear mixed model fitted with ASReml software (Gilmour et al., 2015). This model included maternal diet, duckling sex and the interaction between them as fixed effects, and the duckling associated with its relationship matrix as a random effect. We also investigated the sex-specific impact of the Met-restricted maternal diet by comparing Group R females vs Group C females and Group R males vs Group C males. ANOVAs were therefore conducted using a linear mixed model including the maternal diet as fixed effect and the duckling associated with its relationship matrix as a random effect. For gene expression, the probabilities associated with Wald tests for fixed effect were corrected for multiple tests using the Benjamini-Hochberg correction and were called as ***P*-value (BH)**, as the results are used as a list of genes differentially expressed. For phenotypic traits and plasmatic parameters, significant traits were selected with a *P*-value < 0.05. For gene expression, significant DEGs were selected with a *P*-value (BH) < 0.05.

Correlation matrices were built for traits showing sex-specific effects of the Met-restricted maternal diet, separately for Group C and Group R females, and for Group C and Group R males. They were plotted with the package corrplot of R (version 4.3.0) using the functions rcorr and corrplot (Friendly, 2002; Murdoch and Chow, 1996), and only the correlations with a *P*-value < 0.05 were reported on the plots. Then, partial least squares (**PLS**) were performed in regression mode between the traits and the genes that had a significant maternal diet effect in females. The PLS networks were drawn separately for the Group R and the Group C females. They were plotted with the package MixOmics (6.24.0) using the functions pls and network (Rohart et al., 2017).

Results

Effects of maternal diet and offspring sex on phenotypic traits and liver transcripts in non-overfed ducks

Feed intake from D1 to D87 was measured per pen, and the individual average daily feed intake between D1 and D87 was calculated. It was 167.32 and 162.10 g/d in Group R and Group C, respectively, and 156.36 and 173.98 g/d in females and males, respectively. These results suggested that maternal diet had no impact on the offspring's food intake up to D87. However, sex had an impact on food intake, with an average individual food intake lower in females by around 18 g/d - or around 10% - compared with the average individual food intake in males. Growth measurements were made up to D87, and plasma parameters were recorded at D87 on the 244 NOFDs. Then, a subset of 15 animals by group of sex and maternal diet (n = 60 in total), representative of each subgroup was slaughtered and other phenotypic traits such as weight and chemical composition of the livers were recorded. The results are shown in Table 1. The statistical results of the expression of the 145 studied genes in the liver of NOFDs are presented in Supplementary Table S4, and only the statistical results of the DEGs are shown in Table 2.

No significant differences were observed between the two diet groups (Diet *P*-value > 0.05), neither for BWs at D29, D57 and D87 (BW_D29, BW_D57, BW_D87_NOFD, respectively), nor for average daily gain between D1 and D87 (ADG_D1_D87), nor for plasma parameters in NOFDs at D87 (log_Plasma_ALP_NOFD, log_Plasma_Chol_NOFD, log_Plasma_Gluc_NOFD, log_Plasma_Trigly_NOFD and log_Plasma_FFA_NOFD; Table 1), nor for the liver weight and the proportion of liver weight to bled animal weight (LiverW_NOFD and LiverW/AnimBledW_NOFD) in NOFDs at D87. However, a lower percentage of lipids and DM was observed in the liver when comparing Group R to group C (for lipids: Liver_Lip_NOFD: 3.99 ± 0.144 and 4.38 ± 0.144, in Group R and Group C, respectively; Diet *P*-value = 0.002 and for DM: Liver_DM_NOFD; 27.21 ± 0.193 and 27.75 ± 0.195, in Group R and Group C, respectively; Diet *P*-value = 0.024). This might suggest some differences in liver metabolism, but none of the 145 studied genes were differentially expressed between the two groups of maternal diet (Diet *P*-value (BH) > 0.1; Supplementary Table S4).

As expected, a large number of phenotypic traits differed or tended to differ between the two sexes of mule ducks at D87 (Sex *P*-value < 0.05 or Sex *P*-value < 0.1; Table 1). All growth parameters differed between the sexes. Thus, while females had a higher hatching BW (BW_D1_NHD, Sex *P*-value = 0.027), they subsequently showed lower BWs during their growth (BW_D29, BW_D57 and BW_D87_NOFD, Sex *P*-value < 0.001) and lower average daily gain from D1 to D87 (ADG_D1_D87; Sex *P*-value < 0.001) than males. These lower BWs are to be linked to the lower feed intake observed in females.

Plasma parameters also differed or tended to differ between the sexes, with the exception of free fatty acid content. Females had a higher percentage of lipids in the liver (Liver_Lip_NOFD; Sex *P*-value < 0.001), and they also tended to have a higher proportion of liver (LiverW/AnimBledW_NOFD; Sex *P*-value = 0.069). Moreover, significant interactions between the maternal diet effect and the sex of the offspring effect were detected for the BW at D29 (BW_D29; Sex*Diet *P*-value = 0.012) and for the percentage of DM in the liver (Liver_DM_NOFD; Sex*Diet *P*-value = 0.031) whereas this interaction tented to be significant for the percentage of lipids in the liver (Liver_Lip_NOFD; Sex*Diet *P*-value = 0.090). Finally, of the 145 genes studied, 27 showed differential expression in the liver between sexes (Sex *P*-value (BH) < 0.05) and eight other ones showed a tendency to be differentially expressed (0.05 < Sex

Table 1
Phenotypic traits measured in mule ducks from D1 to D87 and at D100. The first part of the table describes the traits measured from D1 to D87, before overfeeding. The second part of the table describes the traits measured in overfed ducks (OFDs) at D100. Means and SEs are given for the two maternal diet groups (Group R and Group C) and for both sexes.

		Maternal diet group[1,2,3]				Sex[4]						
		Group R		Group C		Female		Male		*P*-value[5]		
Traits	n	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Diet	Sex	Sex*Diet
Before overfeeding												
BW_D1_NHD (g)	254	32.84[a]	1.139	35.45[b]	1.137	34.47[c]	1.123	33.82[d]	1.121	<0.001	0.027	0.647
BW_D29 (g)	254	1 220.7	41.09	1 235.0	41.03	1 136.2[c]	40.51	1 319.6[d]	40.45	0.385	<0.001	0.012
BW_D57 (g)	254	2 672.5	68.76	2 633.2	68.62	2 523.8[c]	67.81	2 781.8[d]	67.65	0.196	<0.001	0.153
BW_D87_NOFD (g)	254	3 352.5	84.22	3 373.5	84.03	3 239.3[c]	83.10	3 486.7[d]	82.82	0.621	<0.001	0.339
ADG_D1_D87 (g/d)	254	38.60	0.958	38.82	0.956	37.27[c]	0.946	40.15[d]	0.942	0.653	<0.001	0.346
Log Plasma_ALP_NOFD	244	0.04	0.048	0.01	0.048	−0.01[c]	0.048	0.06[d]	0.047	0.388	0.002	0.641
Log Plasma_Chol_NOFD	244	−0.01	0.031	0.02	0.031	−0.05[c]	0.031	0.06[d]	0.031	0.201	<0.001	0.176
Log Plasma_Gluc_NOFD	244	0.00	0.011	0.02	0.011	0.00	0.011	0.02	0.011	0.258	0.062*	0.581
Log Plasma_Trigly_NOFD	244	0.01	0.062	−0.03	0.062	0.05[c]	0.062	−0.07[d]	0.061	0.366	0.006	0.184
Log Plasma_FFA_NOFD	244	0.03	0.068	0.03	0.068	0.00	0.069	0.05	0.067	0.937	0.459	0.274
AnimBledW_NOFD (g)	60	2 564.8	44.68	2 572.4	45.03	2 487.3[c]	45.01	2 649.9[d]	44.27	0.904	0.002	0.233
LiverW_NOFD (g)	60	70.50	2.937	67.57	2.917	68.99	2.914	69.08	2.898	0.347	0.979	0.982
Liver_DM_NOFD (%)	59	27.21[a]	0.193	27.75[b]	0.195	27.57	0.192	27.39	0.195	0.024	0.468	0.031
Liver_Lip_NOFD (%)	59	3.99[a]	0.144	4.38[b]	0.144	4.47[c]	0.142	3.90[d]	0.142	0.002	<0.001	0.090
LiverW/AnimBledW_NOFD (%)	60	2.75	0.082	2.63	0.082	2.78	0.082	2.60	0.081	0.266	0.069*	0.611
After overfeeding												
BW_D100_OFD (g)	194	5 051.8	95.92	5 159.6	95.58	4 883.4[c]	94.95	5 328.0[d]	94.06	0.052*	<0.001	0.927
ADG_NOFD_OFD (g/d)	194	128.14[a]	3.349	134.45[b]	3.338	123.79[c]	3.334	138.81[d]	3.276	0.007	<0.001	0.986
FeedIntake_OFD (g)	194	9 515.8	73.29	9 496.5	72.98	9 372.4	73.12	9 639.8	71.58	NA	NA	NA
Log Plasma_ALP_OFD	193	−0.03	0.089	−0.12	0.088	−0.16[c]	0.088	0.01[d]	0.087	0.197	0.020	0.937
Plasma_ALT_OFD	193	2.45	0.094	2.56	0.093	2.53	0.094	2.49	0.091	0.129	0.526	0.875
Log Plasma_AST_OFD	193	1.02	0.149	1.15	0.149	1.15	0.148	1.03	0.147	0.134	0.104	0.991
Log Plasma_Chol_OFD	193	0.82	0.052	0.83	0.052	0.81	0.052	0.83	0.051	0.731	0.525	0.297
Log Plasma_Gluc_OFD	193	0.57	0.061	0.63	0.061	0.76[c]	0.061	0.44[d]	0.060	0.244	<0.001	0.962
Log Plasma_Trigly_OFD	193	0.99	0.068	0.95	0.068	1.11[c]	0.068	0.83[d]	0.066	0.722	<0.001	0.010
Log Plasma_FFA_OFD	193	0.32	0.061	0.31	0.061	0.17[c]	0.063	0.45[d]	0.059	0.943	0.001	0.928
AnimBledW_OFD (g)	194	4 373.4	87.97	4 441.8	87.80	4 217.8[c]	87.10	4 597.5[d]	86.36	0.174	<0.001	0.890
AATW_OFD (g)	194	147.09	3.604	148.23	3.599	141.85[c]	3.63	153.47[d]	3.52	0.664	<0.001	0.127
LiverW_OFD (g)	194	490.81[a]	31.855	544.83[b]	31.770	510.64	31.484	525.00	31.289	0.001	0.274	0.833
AATW/AnimBledW_OFD (%)	194	3.37	0.092	3.32	0.092	3.36	0.092	3.33	0.090	0.569	0.705	0.053
LiverW/AnimBledW_OFD (%)	194	11.21[a]	0.598	12.23[b]	0.597	12.05[c]	0.592	11.38[d]	0.587	0.002	0.018	0.666
Liver_DM_OFD (%)	164	67.40	0.644	68.32	0.646	68.03	0.639	67.69	0.638	0.085*	0.535	0.567
Liver_Lip_OFD (%)	164	55.03	0.859	56.06	0.861	55.88	0.851	55.22	0.850	0.133	0.333	0.439
Liver_MeltingRate_OFD (%)	189	30.55[a]	4.660	35.62[b]	4.652	39.86[c]	4.604	26.31[d]	4.581	0.031	<0.001	0.618

Abbreviations: BW_D1_NHD = BW of newly hatched ducklings at D1; BW_D29 = BW at D29; BW_D57 = BW at D57; BW_D87_NOFD = BW of non-overfed ducks at D87; ADG_D1_D87 = average daily gain between D1 and D87; log_Plasma_ALP_NOFD = individual plasma alkaline phosphatase activity in non-overfed ducks converted into logarithms; log_Plasma_Chol_NOFD, log_Plasma_Gluc_NOFD, log_Plasma_Trigly_NOFD and log_Plasma_FFA_NOFD = individual plasma cholesterol, glucose, triglyceride and free fatty acid levels in non-overfed ducks, converted into logarithms; AnimBledW_NOFD = animal weight after bleeding and removal of feathers, legs and wing tips, of non-overfed ducks; LiverW_NOFD = liver weight of non-overfed ducks; Liver_DM_NOFD = percentage of liver DM in non-overfed ducks; Liver_Lip_NOFD = percentage of liver lipids in non-overfed ducks; LiverW/AnimBledW_NOFD = ratio of liver weight to bled animal weight in non-overfed ducks; BW_D100_OFD = BW of overfed ducks at D100; ADG_NOFD_OFD = average daily gain during overfeeding; FeedIntake_OFD = individual feed intake during overfeeding; log Plasma_ALP_OFD, log Plasma_ALT_OFD, and log Plasma_AST_OFD = individual plasma alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase activities in overfed ducks, converted into logarithms; log Plasma_Chol_OFD, log Plasma_Gluc_OFD, log Plasma_Trigly_OFD, log Plasma_FFA_OFD = individual plasma cholesterol, glucose, triglyceride and free fatty acid levels in overfed ducks, converted into logarithms; AnimBledW_OFD = animal weight (without blood, feathers, legs and wing tips) in overfed ducks; AATW_OFD = abdominal adipose tissue weight in overfed ducks; LiverW_OFD = fatty liver weight in overfed ducks; AATW/AnimBledW_OFD = ratio of abdominal adipose tissue weight to bled animal weight in overfed ducks; LiverW/AnimBledW_OFD = ratio of fatty liver weight to bled animal weight in overfed ducks; Liver_DM_OFD, Liver_Lip_OFD = percentage of fatty liver DM and lipids in overfed ducks; Liver_MeltingRate_OFD = fatty liver melting rate in overfed ducks.

[1] Group R mule ducks from dams fed the methionine-restricted diet.
[2] Group C mule ducks from dams fed the control diet.
[3] Values within a row with different superscripts (a or b) differ significantly at $P < 0.05$ for the maternal diet effect.
[4] Values within a row with different superscripts (c or d) differ significantly at $P < 0.05$ for the sex effect.
[5] Stars (*) indicate *P*-values comprised between 0.05 and 0.1.

P-value (BH) < 0.1) (Table 2). They are either involved in energy metabolism, one-carbon metabolism or epigenetic mechanisms.

Effects of maternal diet and offspring sex on phenotypic traits and liver transcripts in overfed ducks

Mule ducks were then overfed twice a day for 12.5 days. Feed intake during the overfeeding period was measured for each animal, and total intake was calculated (FeedIntake_OFD). At the end of the overfeeding period, the BW was measured (BW_D100_OFD) and average daily gain during the overfeeding period was assessed (ADG_NOFD_OFD). Then, phenotypic traits and plasma parameters were recorded. All the results are given in Table 1. The statistical results of the expression of the 143 genes in the liver of OFDs are presented in Supplementary Table S5, and only the statistical results of the DEGs are shown in Table 3.

The results showed that the BW before overfeeding was not affected by the maternal diet (BW_D87_NOFD, Diet *P*-value > 0.1; Table 1), and that food intake during the overfeeding period (FeedIntake_OFD) was not affected either, although it was not possible to establish a *P*-value because the values are mainly identical between all animals. However, the BW after overfeeding

Table 2
Hepatic gene expressions in non-overfed ducks (NOFDs). No gene was differentially expressed neither for the Diet effect nor for the Sex*Diet interaction. The genes listed in this table are the ones which were either significantly differentially expressed or tented to be differentially expressed, according to the sex of the mule ducks. For each gene, LS-Means and SEs are given for the two maternal diet groups and for both sexes. The Benjamini-Hochberg (BH) corrected *P*-values for the diet effect, the sex effect, and their interaction are given.

	Maternal diet group[1,2]				Sex						
	Group R		Group C		Female		Male		*P*-value (BH)[3]		
Gene	LS-Mean	SE	LS-Mean	SE	LS-Mean	SE	LS-Mean	SE	Diet	Sex	Sex*Diet
ABCA1	−0.23	0.143	0.23	0.143	−0.69	0.143	0.69	0.143	0.410	<0.001	0.837
ACSL1	−0.22	0.199	0.23	0.200	−0.45	0.200	0.46	0.199	0.564	0.004	0.890
ACSL5	−0.04	0.170	0.04	0.170	−0.61	0.170	0.61	0.170	0.944	<0.001	0.890
ALDOB	0.09	0.162	−0.09	0.162	−0.66	0.162	0.66	0.162	0.846	<0.001	0.963
ARHGEF28	0.10	0.196	−0.10	0.196	−0.40	0.196	0.40	0.196	0.853	0.021	0.890
BHMT	0.18	0.278	−0.22	0.280	−0.64	0.279	0.60	0.276	0.564	<0.001	0.963
BHMT2	0.12	0.304	−0.14	0.305	−0.61	0.304	0.59	0.301	0.723	<0.001	0.963
CBS	−0.20	0.222	0.21	0.222	−0.34	0.222	0.35	0.221	0.642	0.065*	0.890
CD36	−0.17	0.217	0.18	0.217	−0.39	0.217	0.39	0.216	0.703	0.031	0.963
CTSL2	0.05	0.146	−0.05	0.146	−0.73	0.146	0.73	0.146	0.872	<0.001	0.942
CYP2E1	0.16	0.197	−0.16	0.198	−0.40	0.198	0.40	0.197	0.723	0.020	0.890
DHFR	0.17	0.180	−0.17	0.180	−0.79	0.180	0.78	0.179	0.543	<0.001	0.963
ELAVL1	0.14	0.299	−0.04	0.301	0.51	0.300	−0.40	0.297	0.853	0.004	0.963
ERRFI1	−0.30	0.394	0.33	0.395	0.09	0.393	−0.05	0.390	0.867	0.091*	0.963
ESR1	0.03	0.183	−0.03	0.183	−0.48	0.183	0.48	0.183	0.944	0.002	0.837
FAS	−0.07	0.341	0.12	0.342	0.56	0.340	−0.51	0.337	0.846	<0.001	0.946
GFPT2	−0.01	0.329	−0.11	0.331	0.49	0.329	−0.62	0.326	0.898	<0.001	0.890
HMGCR	0.07	0.188	−0.05	0.189	−0.73	0.189	0.76	0.187	0.853	<0.001	0.963
IL6ST	0.17	0.162	−0.16	0.162	−0.71	0.162	0.73	0.161	0.571	<0.001	0.963
LDHA	0.01	0.191	0.00	0.192	0.58	0.192	−0.58	0.191	0.991	<0.001	0.963
MEF2C	−0.02	0.160	0.02	0.160	−0.68	0.160	0.68	0.160	0.944	<0.001	0.963
MTHFD1L	−0.20	0.271	0.16	0.273	−0.46	0.272	0.42	0.270	0.700	0.008	0.963
MTHFD2	−0.03	0.192	0.03	0.192	0.49	0.192	−0.49	0.192	0.944	0.003	0.963
MTR	−0.13	0.205	0.13	0.205	−0.33	0.205	0.33	0.205	0.830	0.091*	0.963
MTRR	−0.18	0.194	0.18	0.194	0.34	0.194	−0.34	0.194	0.703	0.065*	0.837
MTTP	−0.02	0.379	−0.02	0.381	−0.52	0.379	0.48	0.376	0.996	<0.001	0.963
NR1H4	−0.12	0.203	0.12	0.203	0.36	0.203	−0.36	0.203	0.846	0.056*	0.963
PRKAA1	0.14	0.132	−0.14	0.132	−0.76	0.132	0.76	0.132	0.642	<0.001	0.837
PRKAB1	0.10	0.363	−0.09	0.364	−0.33	0.363	0.33	0.360	0.853	0.054*	0.963
PRKAG2	−0.07	0.218	0.09	0.219	−0.50	0.219	0.52	0.218	0.853	<0.001	0.837
RAN	0.23	0.276	−0.18	0.277	0.35	0.277	−0.30	0.275	0.642	0.086*	0.963
SHMT1	−0.19	0.227	0.18	0.228	−0.37	0.228	0.36	0.227	0.700	0.034	0.837
TET1	0.03	0.287	−0.10	0.288	0.34	0.288	−0.40	0.285	0.880	0.044	0.963
TPI1	0.03	0.207	−0.03	0.207	−0.33	0.207	0.33	0.207	0.944	0.091*	0.963
VLDLR	0.10	0.195	−0.10	0.196	0.63	0.196	−0.64	0.195	0.844	<0.001	0.963

P-value (BH) = Benjamini-Hochberg (BH) corrected *P*-values.
[1] Group R mule ducks from dams fed the methionine-restricted diet.
[2] Group C mule ducks from dams fed the control diet.
[3] Stars (*) indicate *P*-values (BH) comprised between 0.05 and 0.1.

(BW_D100_OFD) in Group R tended to be around 100 g lower than in Group C (Diet *P*-value = 0.052), and the average daily gain during the 12.5 days of overfeeding (ADG_NOFD_OFD) was also lower in Group R than in Group C (Diet *P*-value = 0.007). Moreover, the fatty liver weight was more than 50 g lower (LiverW_OFD, Diet *P*-value = 0.001) and the ratio of liver weight to bled animal weight was significantly reduced too in Group R ducks compared to Group C ducks (LiverW/AnimBledW_OFD; Diet *P*-value = 0.002). However, the reduced availability of Met in the maternal diet improved fatty liver quality, as the fatty liver melting rate (Liver_MeltingRate_OFD), was five points lower in Group R ducks compared to Group C ducks (Diet *P*-value = 0.031) even if the percentage of lipids in the liver did not differ between the two groups (Liver_Lip_OFD; Diet *P*-value = 0.133). Hepatic metabolism was therefore modified in ducks whose mothers were fed a Met-restricted diet. In addition, six DEGs were identified between the two maternal diet groups, all up-regulated in Group R when compared to Group C. They were *ACADM*, *ACOX1*, *ADK*, *APOB*, *IL6ST*, and *MTHFR* (Diet *P*-value (BH) < 0.05; Table 3).

Once again, and as expected, many phenotypic traits differed between the two sexes of mule ducks after overfeeding (Table 1). The feed intake seemed lower during the overfeeding period in females than in males (FeedIntake_OFD, 9 372 g in females vs 9 640 g in males; no statistics because the values are mainly identical between animals), and the average daily gain was also lower in females than in males (ADG_NOFD_OFD, 123.8 g in females vs 138.8 g in males; Sex *P*-value < 0.001). In plasma, glucose and triglyceride levels were higher in females than in males (log_Plasma_Gluc_OFD and log_Plasma_Trigly_OFD; Sex *P*-value < 0.001 for both), while free fatty acid level and ALP activity were lower (log_Plasma_FFA_OFD and log_Plasma_ALP_OFD; Sex *P*-value = 0.001 and Sex *P*-value = 0.020, respectively). Fatty liver weight did not differ between the two sexes (LiverW_OFD, Sex *P*-value > 0.1); thus, the ratio of liver weight to bled animal weight was higher in females when compared to males (LiverW/AnimBledW_OFD, Sex *P*-value = 0.018). However, the fatty livers produced by females were of poorer quality, with a much higher melting rate than in males (39.86 vs 26.31%, with a Sex *P*-value < 0.001). Of all the genes studied in the liver, 50 showed differential expression between the two sexes and 15 others showed a tendency to be differentially expressed (Sex *P*-value (BH) < 0.05 and 0.05 < Sex *P*-value (BH) < 0.1, respectively; Table 3). Of the 50 genes significantly differentially expressed in OFDs, 18 were already differentially expressed in NOFDs (*ABCA1*, *ACSL1*, *ALDOB*, *ARHGEF28*, *BHMT*, *CD36*, *CTSL2*, *CYP2E1*, *DHFR*, *ELAVL1*, *HMGCR*, *IL6ST*, *MEF2C*, *MTHFD2*, *MTTP*, *PRKAA1*, *TET1* and *VLDLR*) and, of these, eight were

Table 3
Hepatic gene expressions in overfed ducks (OFDs). For each gene, LS-Means and SEs are presented for the two maternal diet groups (Group R and Group C) and for both sexes. The Benjamini-Hochberg (BH) corrected *P*-values of the diet effect, the sex effect and their interaction are given.

	Maternal diet group[1,2,3]				Sex[4]						
	Group R		Group C		Female		Male		*P*-value (BH)[5]		
Gene	LS-Mean	SE	LS-Mean	SE	LS-Mean	SE	LS-Mean	SE	Diet	Sex	Sex*Diet
AACS	−0.11	0.143	0.12	0.145	0.31[c]	0.145	−0.31[d]	0.143	0.599	0.011	0.245
ABCA1	0.10	0.177	−0.11	0.178	−0.60[c]	0.177	0.59[d]	0.175	0.559	<0.001	0.994
ACADM	0.32[a]	0.153	−0.32[b]	0.155	−0.01	0.155	0.01	0.153	0.047	0.973	0.059*
ACADS	0.24	0.340	−0.26	0.342	−0.21	0.337	0.19	0.336	0.237	0.063*	0.548
ACAT1	0.23	0.129	−0.24	0.130	−0.39[c]	0.130	0.39[d]	0.129	0.182	<0.001	0.059*
ACOX1	0.43[a]	0.137	−0.45[b]	0.139	−0.13	0.139	0.11	0.137	0.001	0.340	0.621
ACSL1	0.19	0.161	−0.20	0.163	−0.29[c]	0.163	0.28[d]	0.161	0.307	0.017	0.158
ACSL5	0.20	0.145	−0.21	0.147	−0.23	0.147	0.22	0.145	0.293	0.068*	0.271
ADK	0.32[a]	0.187	−0.32[b]	0.188	−0.18	0.187	0.18	0.185	0.047	0.142	0.561
AHCY	0.22	0.316	−0.23	0.317	−0.23[c]	0.314	0.22[d]	0.312	0.253	0.024	0.245
ALDOB	0.20	0.167	−0.20	0.168	−0.52[c]	0.168	0.52[d]	0.166	0.253	<0.001	0.140
APOB	0.30[a]	0.136	−0.32[b]	0.137	−0.35[c]	0.137	0.34[d]	0.136	0.047	0.002	0.383
ARHGEF28	−0.04	0.135	0.03	0.137	−0.49[c]	0.137	0.48[d]	0.135	0.893	<0.001	0.867
BCL2	0.01	0.231	−0.02	0.232	0.26[c]	0.230	−0.27[d]	0.228	0.909	0.022	0.257
BHMT	−0.06	0.313	0.07	0.314	−0.23[c]	0.311	0.24[d]	0.309	0.809	0.045	0.482
BHMT2	−0.01	0.213	0.03	0.214	−0.20	0.213	0.22	0.211	0.907	0.097*	0.674
CD36	0.12	0.140	−0.12	0.142	−0.28[c]	0.142	0.27[d]	0.140	0.567	0.022	0.073*
CHDH	0.17	0.331	−0.14	0.332	−0.18	0.328	0.21	0.327	0.500	0.086*	0.705
CPT1A	0.13	0.164	−0.15	0.166	−0.32[c]	0.165	0.30[d]	0.163	0.512	0.009	0.191
CTSL2	0.09	0.136	−0.11	0.137	−0.61[c]	0.137	0.59[d]	0.135	0.590	<0.001	0.160
CYP2E1	0.16	0.136	−0.17	0.138	−0.38[c]	0.138	0.37[d]	0.136	0.384	0.001	0.156
DGAT2	−0.07	0.206	0.07	0.207	0.23	0.206	−0.23	0.204	0.809	0.060*	0.315
DHCR24	0.19	0.298	−0.25	0.299	−0.28[c]	0.296	0.21[d]	0.294	0.263	0.025	0.747
DHFR	0.02	0.153	−0.05	0.154	−0.80[c]	0.153	0.77[d]	0.151	0.841	<0.001	0.158
DICER1	−0.02	0.261	0.08	0.262	0.40[c]	0.260	−0.34[d]	0.258	0.838	0.001	0.909
DNMT3A	−0.10	0.232	0.11	0.233	0.27[c]	0.232	−0.26[d]	0.229	0.639	0.027	0.561
EED	0.04	0.146	−0.03	0.148	0.31[c]	0.148	−0.31[d]	0.146	0.893	0.014	0.548
EHHADH	0.22	0.222	−0.22	0.223	−0.28[c]	0.222	0.28[d]	0.220	0.253	0.014	0.174
ELAVL1	0.24	0.198	−0.22	0.200	0.32[c]	0.199	−0.30[d]	0.197	0.237	0.009	0.290
ELOVL6	−0.02	0.224	0.02	0.225	−0.41[c]	0.223	0.41[d]	0.221	0.907	<0.001	0.245
ERRFI1	0.13	0.176	−0.13	0.177	−0.30[c]	0.177	0.29[d]	0.175	0.559	0.015	0.621
FASN	0.20	0.217	−0.21	0.219	−0.24[c]	0.217	0.23[d]	0.215	0.307	0.049	0.548
FTCD	0.16	0.177	−0.17	0.178	−0.24	0.178	0.23	0.175	0.432	0.056*	0.965
GLP1R	0.04	0.277	−0.02	0.278	0.34[c]	0.276	−0.31[d]	0.274	0.907	0.004	0.857
HDAC1	−0.08	0.336	−0.03	0.338	0.19[c]	0.333	−0.30[d]	0.332	0.907	0.021	0.621
HMGCR	0.03	0.116	−0.05	0.118	−0.65[c]	0.118	0.63[d]	0.116	0.841	<0.001	0.263
HNF4A	0.02	0.237	−0.02	0.238	0.24	0.236	−0.24	0.234	0.907	0.056*	0.396
HSBP1	−0.03	0.178	0.04	0.180	0.38[c]	0.179	−0.38[d]	0.177	0.893	0.001	0.989
IL6ST	0.27[a]	0.136	−0.29[b]	0.137	−0.60[c]	0.136	0.58[d]	0.135	0.033	<0.001	0.073*
INSIG2	0.21	0.207	−0.20	0.208	−0.22	0.207	0.24	0.204	0.293	0.056*	0.652
IYD	0.09	0.145	−0.10	0.147	−0.22	0.147	0.21	0.145	0.678	0.089*	0.158
MEF2C	0.14	0.119	−0.15	0.121	−0.58[c]	0.121	0.57[d]	0.119	0.381	<0.001	0.130
MMP2	−0.04	0.303	0.02	0.304	0.34[c]	0.301	−0.36[d]	0.299	0.907	0.001	0.876
MSRA	0.14	0.354	−0.25	0.355	−0.46[c]	0.350	0.35[d]	0.349	0.307	<0.001	0.073*
MTHFD2	−0.04	0.192	0.02	0.193	0.34[c]	0.192	−0.36[d]	0.190	0.907	0.002	0.174
MTHFR	0.29[a]	0.295	−0.35[b]	0.296	−0.02	0.293	−0.03	0.291	0.047	0.997	0.273
MTRR	0.10	0.156	−0.09	0.158	0.22	0.158	−0.21	0.156	0.678	0.097*	0.747
MTTP	−0.04	0.166	0.04	0.168	−0.42[c]	0.167	0.41[d]	0.165	0.858	<0.001	0.160
NR4A3	−0.20	0.165	0.21	0.166	0.24	0.166	−0.22	0.164	0.293	0.063*	0.916
PARP1	0.15	0.222	−0.17	0.223	0.32[c]	0.222	−0.34[d]	0.219	0.432	0.004	0.832
PCK1	0.11	0.203	−0.13	0.204	−0.42[c]	0.203	0.40[d]	0.201	0.559	<0.001	0.174
PPARG	0.00	0.154	0.00	0.156	0.31[c]	0.156	−0.31[d]	0.154	0.982	0.012	0.315
PPARGC1A	0.02	0.229	−0.03	0.230	−0.24[c]	0.228	0.24[d]	0.226	0.907	0.046	0.174
PRKAA1	0.07	0.101	−0.08	0.102	−0.74[c]	0.102	0.73[d]	0.101	0.622	<0.001	0.235
PRKAB1	0.22	0.212	−0.21	0.213	−0.42[c]	0.212	0.43[d]	0.210	0.237	<0.001	0.156
RAN	0.14	0.302	−0.20	0.303	0.16	0.300	−0.21	0.298	0.384	0.097*	0.621
RBBP4	0.18	0.213	−0.15	0.214	0.46[c]	0.212	−0.43[d]	0.210	0.381	<0.001	0.621
SDHA	0.03	0.256	−0.09	0.257	−0.24	0.255	0.17	0.253	0.837	0.086*	0.453
SLC6A6	0.15	0.139	−0.16	0.141	−0.37[c]	0.141	0.37[d]	0.139	0.446	0.001	0.273
SOD1	0.27	0.167	−0.27	0.168	−0.31[c]	0.168	0.31[d]	0.166	0.129	0.007	0.450
TET1	−0.08	0.148	0.08	0.149	0.29[c]	0.149	−0.29[d]	0.148	0.761	0.021	0.978
TET2	0.09	0.147	−0.09	0.148	0.28[c]	0.148	−0.28[d]	0.147	0.678	0.025	0.438
TNFSF10	0.14	0.139	−0.15	0.141	−0.33[c]	0.141	0.33[d]	0.139	0.461	0.004	0.158
TYMS	−0.05	0.292	0.04	0.294	0.24[c]	0.290	−0.26[d]	0.289	0.873	0.024	0.720
UGDH	0.06	0.147	−0.06	0.149	0.29[c]	0.149	−0.29[d]	0.147	0.823	0.021	0.621

(*continued on next page*)

Table 3 (*continued*)

	Maternal diet group[1,2,3]				Sex[4]						
	Group R		Group C		Female		Male		*P*-value (BH)[5]		
Gene	LS-Mean	SE	LS-Mean	SE	LS-Mean	SE	LS-Mean	SE	Diet	Sex	Sex*Diet
UHRF1	−0.10	0.310	0.01	0.311	0.24[c]	0.308	−0.32[d]	0.306	0.838	0.008	0.476
VLDLR	0.08	0.167	−0.07	0.168	0.61[c]	0.167	−0.60[d]	0.165	0.678	<0.001	0.849
WNT11	0.05	0.217	−0.06	0.218	0.22	0.217	−0.23	0.215	0.838	0.071*	0.482
XPO5	0.08	0.257	−0.14	0.258	0.20	0.256	−0.26	0.254	0.616	0.056*	0.383

P-value (BH) = Benjamini-Hochberg (BH) corrected *P*-values.
[1] Group R mule ducks from dams fed the methionine-restricted diet.
[2] Group C mule ducks from dams fed the control diet.
[3] Values within a row with different superscripts (a or b) differ significantly at *P*-value (BH) < 0.05 for the maternal diet effect.
[4] Values within a row with different superscripts (c or d) differ significantly at *P*-value (BH) < 0.05 for the sex effect.
[5] Stars (*) indicate *P*-values (BH) comprised between 0.05 and 0.1.

already differentially expressed in NHDs (*ABCA1*, *BHMT*, *DHFR*, *ELAVL1*, *HMGCR*, *MEF2C*, *PRKAA1* and *VLDLR*) (Sécula et al., 2022a, 2022b).

Sex-specific effects of maternal diet on phenotypic traits and liver transcripts

The measurements and analyses described above made it possible to observe both the effects of the Met-restricted maternal diet and the effects of the offspring sex on phenotypic traits and hepatic gene expressions. We then sought to further explore the sex-specific response to the maternal Met restriction. To this end, we investigated the effects of the Met-restricted maternal diet on phenotypic traits and liver transcripts for each sex individually. The results are given in Supplementary Table S6 for the phenotypic traits, in Supplementary Table S7 for the liver gene expressions in NOFDs, and in Supplementary Table S8 for the liver gene expressions in OFDs. Only measurements showing sex-specific differences induced by the Met-restricted maternal diet are presented in Table 4 for the phenotypic traits and in Table 5 for the liver transcripts. Examples of the sex-specific effects of maternal diet on the traits studied in NHDs, NOFDs and OFDs are shown in Fig. 2.

The Met-restricted maternal diet had a very significant impact on hatching weight with a reduction of around 3 g for both sexes, (BW_D1_NDH, Diet *P*-value < 0.001; Table 4 and Fig. 2A). However, the striking feature at hatching was the difference in down colour induced by the maternal Met restriction in male ducklings only (L_Back_NHD, a_Back_NHD and b_Belly_NHD, Diet *P*-value < 0.05; Table 4 and Fig. 2B and 2C). Then, the Met-restricted maternal diet had no major effect on growth until D87, as indicated by the daily weight gain measured between D1 and D87 (ADG_D1_D87), which was equivalent for Group C and Group R within sex (Supplementary Table S6). In NOFDs, none of the plasma parameters were affected by the maternal diet (Supplementary Table S6), and none of the 145 studied genes were differentially expressed between the diet groups in male offspring (Supplementary Table S7). However, Group R females showed reduced plasma cholesterol level (log_Plasma_Chol_NOFD, Diet *P*-value = 0.020) and altered liver metabolism compared to Group C females, with lower liver DM and liver lipid percentages (Liver_DM_NOFD, *P*-value = 0.004 and Liver_Lip_NOFD, *P*-value = 0.006; Table 4 and Fig. 2D, 2E and 2F). Thus, the effects of the Met-restricted maternal diet previously described in NOFDs (Table 1) for these two last traits were essentially due to those described in females by these new analyses, as the Met-restricted maternal diet did not affect these traits in males. However, the study of liver transcripts in NOFDs revealed only two genes that tended to be differentially expressed between the diet groups in females, among the 145 studied genes. These were the *ABCA1* and *PPARG* genes (Diet *P*-value < 0.1; Table 5 and Supplementary Table S7).

During the overfeeding period, the average daily weight gain of Group R females was very significantly reduced compared with Group C females, with a difference of over 8 g per day (ADG_NOFD_OFD; *P*-value = 0.008; Table 4). In OFD males, the average daily weight gain in Group R was less reduced, with a difference of 5.5 g per day (ADG_NOFD_OFD; *P*-value = 0.041; Table 4). Fatty liver weight (LiverW_OFD) was reduced in Group R offspring in a comparable way in both sexes, by around 53 g, and with the same level of significance (Diet *P*-value = 0.016 and 0.017 in females and males, respectively; Table 4 and Fig. 2G). As a result, the ratio of fatty liver weight to animal weight was significantly reduced in Group R ducks compared to Group C ducks, in both sexes (LiverW/AnimBledW_OFD; *P*-value = 0.008 and *P*-value = 0.019 in females and males, respectively, Table 4). The ratio between liver and feed intake during overfeeding was reduced in Group R compared to Group C in both sexes (Liver_CFI_Ratio_OFD: 43.8 vs 49.7 g/kg in females from Group R and Group C, respectively, *P*-value = 0.001 and 44.6 vs 50.4 g/kg in males from Group R and Group C, respectively, *P*-value = 0.013, Table 4). However, this reduction in fatty liver weight tended to improve fatty liver quality in females only, where the melting rate showed a downward trend, from 43% in Group C females to 37% in Group R females (Liver_MeltingRate_OFD, *P*-value = 0.053; Table 4 and Fig. 2H). Moreover, in accordance with the results observed in NOFD females, Group R females showed a tendency to lower DM and lipid percentages in livers compared to Group C females (Liver_DM_OFD; *P*-value = 0.062 and Liver_Lip_OFD; *P*-value = 0.078, Table 4). Finally, whereas previous results (Table 1) showed a significant effect of the offspring sex but no effect of the Met-restricted maternal diet on plasma parameters, this new intra-sex study showed that AST activity was reduced in Group R when compared to Group C in females only (log_Plasma_AST_OFD; *P*-value = 0.025; Table 4). Overall, these results showed more marked effects of the Met-restricted maternal diet on liver metabolism in females than in males. This difference in response to maternal diet between the sexes was confirmed by differences in hepatic gene expressions. Indeed, while no differences in expression were observed in males for the 143 genes studied (Supplementary Table S8), differences in expression were observed in females for 35 genes that were either differentially expressed (Diet *P*-value (BH) < 0.05 for 18 of them), or tended to be differentially expressed (Diet *P*-value (BH) between 0.05 and 0.1 for 17 of them; Table 5). They were studied in the context of energy metabolism or one-carbon metabolism and were all up-regulated in Group R females when compared to Group C females, except *SHMT1* that was down-regulated.

Table 4
Significant phenotypic traits for the maternal diet within sex. The first part of the table describes the traits measured in newly hatched ducklings (NHDs) at D1 or later, at D29. The second part of the table describes the traits measured in non-overfed ducks (NOFDs). The third part of the table describes the traits measured in overfed ducks (OFDs). For each phenotypic trait, means and SEs are given for the two maternal diet groups (Group R and Group C) within sex.

		FEMALES[1,2,3]					MALES[1,2,4]				
		Group R		Group C			Group R		Group C		
Traits	n	Mean	SE	Mean	SE	*P*-value[5]	Mean	SE	Mean	SE	*P*-value
BW_D1_NHD (g)	254	32.75^{a}	1.284	35.72^{b}	1.287	<0.001	32.71^{c}	1.244	35.27^{d}	1.241	<0.001
BW_D29 (g)	254	1 144.1	38.81	1 137.1	38.92	0.718	1 300.4^{c}	41.00	1 343.9^{d}	40.88	0.032
L_Back_NHD	254	72.90	0.715	72.47	0.715	0.517	72.03^{c}	0.409	70.71^{d}	0.409	0.022
a_Back_NHD	254	−0.14	0.487	0.23	0.489	0.356	0.42^{c}	0.247	1.41^{d}	0.247	0.005
a_Belly_NHD	254	−2.62	0.285	−1.94	0.286	0.066*	−2.73	0.346	−2.41	0.344	0.295
b_Belly_NHD	254	39.93	0.900	40.03	0.905	0.866	40.93^{c}	0.873	39.42^{d}	0.870	0.004
log_Plasma_Chol_NOFD	244	−0.08^{a}	0.023	−0.03^{b}	0.024	0.020	0.06	0.041	0.07	0.040	0.529
Liver_DM_NOFD (%)	60	27.03^{a}	0.268	28.10^{b}	0.268	0.004	27.38	0.217	27.39	0.225	0.989
Liver_Lip_NOFD (%)	59	4.17^{a}	0.196	4.76^{b}	0.197	0.006	3.82	0.146	3.98	0.147	0.154
BW_D100_OFD (g)	194	4 807.6	101.43	4 935.6	101.72	0.062*	5 298.6	126.62	5 388.0	125.87	0.219
ADG_NOFD_OFD (g/d)	194	119.51^{a}	4.283	128.14^{b}	4.325	0.008	136.84^{c}	3.245	142.33^{d}	3.216	0.041
Liver_CFI_Ratio_OFD (g/kg)	194	43.84^{a}	3.174	49.72^{b}	3.184	0.001	44.65^{c}	4.364	50.45^{d}	4.348	0.013
LiverW_OFD (g)	194	482.57^{a}	26.854	535.10^{b}	26.942	0.016	501.09^{c}	41.924	554.06^{d}	41.771	0.017
LiverW/AnimBledW_OFD (%)	194	11.59^{a}	0.493	12.68^{b}	0.499	0.008	10.91^{c}	0.801	11.94^{d}	0.798	0.019
Liver_DM_OFD (%)	164	67.59	0.452	68.81	0.474	0.062*	67.82	0.667	68.35	0.660	0.429
Liver_Lip_OFD (%)	164	55.18	0.798	56.72	0.822	0.078*	55.54	0.952	55.94	0.942	0.639
Liver_MeltingRate_OFD (%)	189	36.81	4.890	42.86	4.924	0.053*	24.77	4.838	28.23	4.811	0.213
log_Plasma_AST_OFD	193	0.99^{a}	0.100	1.22^{b}	0.098	0.025	0.96	0.205	1.05	0.206	0.436

Abbreviations: BW_D1_NHD = BW of newly hatched ducklings at D1; BW_D29 = BW at D29; L_Back_NHD and a_Back_NHD = CIE-LAB lightness (L*) and redness (a*) values measured on the back of the newly hatched ducklings; a_Belly_NHD and b_Belly_NHD = CIE-LAB redness (a*), and yellowness (b*) values measured on the belly of the newly hatched ducklings; log_Plasma_Chol_NOFD = individual plasma cholesterol level in non-overfed ducks, converted into logarithms; Liver_DM_NOFD = DM percentage in liver DM from non-overfed ducks; Liver_Lip_NOFD = lipid percentage in liver from non-overfed ducks; BW_D100_OFD = BW of overfed ducks; ADG_NOFD_OFD = average daily gain during overfeeding; Liver_CFI_Ratio_OFD = ratio of liver weight gain to feed intake during overfeeding; LiverW_OFD = fatty liver weight in overfed ducks; LiverW/AnimBledW_OFD = ratio of fatty liver weight to bled animal weight in overfed ducks; Liver_DM_OFD and Liver_Lip_OFD = DM and lipid percentages in fatty liver from overfed ducks; Liver_MeltingRate_OFD = fatty liver melting rate in overfed ducks; log Plasma_AST_OFD = individual plasma aspartate aminotransferase activity in overfed ducks, converted into logarithms.

[1] Group R mule ducks from dams fed the methionine-restricted diet.
[2] Group C mule ducks from dams fed the control diet.
[3] Values within a row with different superscripts (a or b) differ significantly at $P < 0.05$ in females.
[4] Values within a row with different superscripts (c or d) differ significantly at $P < 0.05$ in males.
[5] Stars (*) indicate *P*-values comprised between 0.05 and 0.1.

Then, correlations between the phenotypic traits listed in Table 4, which showed a sex-specific response to maternal diet, were studied. Correlation matrices between the significant traits are shown in Fig. 3 for females and in Fig. 4 for males. The same matrices with the correlation values are shown in Supplementary Fig. S2 for the females and in Supplementary Fig. S3 for the males. The BW at hatching (BW_D1_NHD) was positively correlated with the BW after overfeeding (BW_D100_OFD, 0.49) and the fatty liver weight (LiverW_OFD, 0.42) in Group C females (FC) (Fig. 3 and Supplementary Fig. S2) and with the BW after overfeeding (BW_D100_OFD, 0.39) and the fatty liver melting rate (Liver_MeltingRate_OFD, 0.42) in Group R males (MR) (Fig. 4 and Supplementary Fig. S3). Two down colour traits measured in NHDs showed correlations with traits measured in OFDs. The belly a* value in NHDs was positively correlated with the BW in OFDs (BW_D100_OFD, 0.29) and with the fatty liver weight (LiverW_OFD, 0.38) in Group R males whereas the belly b* value was negatively correlated with DM and lipid percentages of fatty liver (Liver_DM_OFD; −0.44 and Liver_Lip_OFD; −0.44) in Group C females. It may be pointed out that the plasma cholesterol level measured in NOFDs before the overfeeding period showed no correlations with traits measured in OFDs after the overfeeding period, neither in females nor in males (Fig. 3 and Fig. 4). As expected, the fatty liver weight (LiverW_OFD) was positively correlated with the melting rate (Liver_MeltingRate_OFD) in both females and males of both diet groups and also with the plasma AST activity (Plasma_AST_OFD) in Group C females and in Group R and C males.

Finally, the correlations between the significant traits for the maternal diet (Table 4) and the liver DEGs between the diet groups (Table 5) were also studied in Group R and Group C female OFDs. The networks are shown in Fig. 5. As no gene was significant for the maternal diet effect in Group R and Group C OFD males (Table 5), no network was drawn. The results showed that the relationships between liver DEGs and phenotypic traits differed between the two groups of females (Group R vs Group C). The networks of Group R females were less dense than those of Group C females. They also differed in the genes and traits involved. However, it should be noted that, although the level of expression of the *ADK* gene differed between the two groups of females, its level of expression remained correlated with both the fatty liver weight and the ratio between this fatty liver weight and the weight of the bled animal in both groups of females (LiverW_OFD; −0.57 in both groups and LiverW_AnimalBledW_OFD; −0.57 in Group R females and −0.65 in Group C females). The level of expression of the *MEF2C* gene also appeared in the networks of both groups of females, but was correlated with the fatty liver melting rate in Group R females (Liver_MeltingRate_OFD; −0.54), whereas it was correlated with the ratio between the fatty liver weight and the weight of the bled animal in Group C females (LiverW_AnimalBledW_OFD; −0.56). The level of expression of the *SIRT1* gene was correlated with DM and lipid percentages of the fatty liver in Group R females (Liver_Lip_OFD; 0.63 and Liver_DM_OFD; 0.62), whereas it was positively correlated with duckling back colour data in Group C females (L_Back_NHD: −0.60 and a_Back_NHD: 0.60). Surprisingly, inside this Group C females, the duckling belly and back colours did not belong to the same networks. For the belly, the a* value (a_Belly_NHD) correlated with the expression level of the *ADK* (0.57) and *CTH* (0.54) genes and the b* value (b_Belly_NHD) with the expression level of the *ADK*

Table 5
Differentially expressed genes for the maternal diet within each sex, in non-overfed ducks (NOFDs) and overfed ducks (OFDs). For each gene, LS-Means and SEs are given for the two maternal diet groups intra sex. The Benjamini-Hochberg (BH) corrected *P*-values of the diet effect within sex are given.

	FEMALES[1,2,3]					MALES[1,2]				
	Group R		Group C		*P*-value	Group R		Group C		*P*-value
Genes	LS-Mean	SE	LS-Mean	SE	(BH)[4]	LS-Mean	SE	LS-Mean	SE	(BH)
Non-Overfed Ducks (D87)										
ABCA1	−0.57	0.249	0.57	0.249	0.096*	0.00	0.307	0.00	0.307	0.996
PPARG	0.57	0.250	−0.57	0.250	0.096*	0.13	0.304	−0.13	0.304	0.878
Overfed Ducks (D100)										
ACADM	0.64[a]	0.251	−0.61[b]	0.249	<0.001	0.02	0.215	−0.02	0.220	0.971
ACADS	0.34	0.399	−0.31	0.395	0.068*	0.15	0.212	−0.16	0.217	0.842
ACAT1	0.53[a]	0.186	−0.53[b]	0.186	0.002	−0.15	0.295	0.15	0.297	0.842
ACOX1	0.44[a]	0.197	−0.44[b]	0.197	0.020	0.34	0.315	−0.37	0.317	0.313
ACSL1	0.47[a]	0.194	−0.47[b]	0.194	0.013	−0.08	0.409	0.06	0.410	0.864
ACSL5	0.41	0.365	−0.34	0.361	0.053*	0.01	0.215	−0.01	0.220	0.971
ADK	0.41	0.273	−0.36	0.271	0.052*	0.27	0.276	−0.31	0.278	0.832
AHCY	0.39	0.365	−0.30	0.362	0.058*	−0.01	0.390	−0.10	0.391	0.914
ALDOB	0.49[a]	0.191	−0.49[b]	0.191	0.007	−0.08	0.388	0.01	0.389	0.922
APOB	0.49[a]	0.322	−0.46[b]	0.319	0.008	0.14	0.414	−0.18	0.415	0.842
BMF	0.33	0.239	−0.35	0.238	0.084*	−0.16	0.212	0.17	0.217	0.842
CD36	0.44[a]	0.197	−0.44[b]	0.197	0.020	−0.19	0.211	0.20	0.216	0.842
CPT1A	0.38	0.204	−0.38	0.204	0.058*	−0.10	0.405	0.07	0.406	0.842
CTH	0.45[a]	0.300	−0.39[b]	0.297	0.023	0.06	0.215	−0.066	0.220	0.889
CTSL2	0.35	0.243	−0.38	0.241	0.063*	−0.15	0.213	0.15	0.218	0.842
CYP2E1	0.47[a]	0.364	−0.40[b]	0.361	0.020	−0.18	0.386	0.16	0.387	0.842
DHFR	0.31	0.407	−0.32	0.403	0.090*	−0.24	0.287	0.25	0.290	0.841
EHHADH	0.43[a]	0.198	−0.43[b]	0.198	0.020	0.01	0.319	−0.03	0.321	0.971
ENO1	0.34	0.207	−0.34	0.207	0.090*	−0.13	0.281	0.13	0.284	0.842
ESR1	0.36	0.205	−0.36	0.205	0.063*	−0.03	0.275	0.03	0.278	0.949
GART	0.45[a]	0.261	−0.40[b]	0.259	0.020	−0.13	0.213	0.14	0.218	0.842
IL6ST	0.58[a]	0.213	−0.58[b]	0.211	<0.001	−0.02	0.389	−0.07	0.390	0.957
IYD	0.51[a]	0.383	−0.33[b]	0.380	0.020	−0.15	0.212	0.16	0.217	0.842
MEF2C	0.42[a]	0.332	−0.35[b]	0.329	0.038	−0.15	0.322	0.13	0.324	0.842
MSRA	0.37[a]	0.392	−0.46[b]	0.389	0.020	−0.09	0.315	0.09	0.317	0.842
MTHFR	0.41[a]	0.372	−0.54[b]	0.368	0.004	0.17	0.368	−0.14	0.369	0.842
PCK1	0.36	0.205	−0.36	0.205	0.063*	−0.09	0.369	0.05	0.370	0.864
PRKAA1	0.34	0.320	−0.37	0.317	0.063*	−0.13	0.360	0.096	0.362	0.842
PRKAB1	0.59[a]	0.361	−0.43[b]	0.358	0.002	−0.02	0.258	0.00	0.261	0.983
PRKAG2	0.35	0.274	−0.39	0.272	0.058*	−0.02	0.288	0.00	0.290	0.971
SHMT1	−0.37	0.204	0.37	0.204	0.058*	0.08	0.279	−0.07	0.282	0.864
SIRT1	0.32[a]	0.378	−0.42[b]	0.375	0.033	0.01	0.263	−0.03	0.267	0.971
SLC6A6	0.36	0.205	−0.36	0.205	0.063*	−0.03	0.277	0.02	0.280	0.971
SOD1	0.37	0.240	−0.35	0.239	0.062*	0.19	0.211	−0.20	0.216	0.842
TNFSF10	0.33	0.303	−0.40	0.300	0.056*	−0.12	0.214	0.12	0.219	0.842

P-value (BH) = Benjamini-Hochberg (BH) corrected *P*-values.
[1] Group R mule ducks from dams fed the methionine-restricted diet.
[2] Group C mule ducks from dams fed the control diet.
[3] Values within a row with different superscripts (a or b) differ significantly at $P < 0.05$ in females.
[4] Stars (*) indicate *P*-values comprised between 0.05 and 0.1.

(0.59), *CTH* (0.56) and *AHCY* (0.54) genes. For the back, the a* value correlated with the expression level of *ACAT1* (0.53), *PRKAA1* (0.75), *CTSL2* (0.59) and *SIRT1* (0.60) genes and the L* value correlated with the expression level of *SIRT1* (−0.60), *TNFSF10* (−0.57) and *CTSL2* (−0.61) genes (Fig. 5).

Discussion

We had already investigated the effects of the Met-restricted maternal diet on phenotypic traits in newly hatched ducks, showing effects on their energy metabolism (Bodin et al., 2019) and reporting a number of DEGs in their liver (Sécula et al., 2022a, 2022b). In this new study, we wanted to test the long-term effects of the Met-restricted maternal diet on the non-overfed ducks, at D87, and on the performance of ducks in the context of fatty liver production, at D100, after a period of overfeeding. Indeed, the period of overfeeding, which consists of giving a large quantity of a corn-based diet twice a day, leads to a high hepatic *de novo* lipogenesis activity resulting in a reversible hepatic steatosis with the accumulation of lipids mainly triglycerides in the liver. Thus, this strong reshuffling of metabolic pathways could have erased any effects of the Met-restricted maternal diet observed prior to overfeeding.

Maternal methionine restriction impacted fatty liver production

Apart from hatching, maternal Met restriction did not affect the offspring's BW until D87, i.e., 12 weeks of age. This contrasts with the results of Liu and co-authors, who showed that maternal Met restriction (0.27 vs 0.37%) reduced the BW not only at hatching but also at 7 weeks of age in chickens (Liu et al., 2020). At 12 weeks of age, the reduced percentage of lipids in the liver indicated a lower fattening of the offspring from Met-restricted dams. However, no significant differences in plasma parameters or gene expression in the liver were observed. Thus, the long-term effects of maternal restriction were quite subtle, and might be expected to disappear after the overfeeding period. Nevertheless, the average daily gain during the 12.5 days of overfeeding was lower in the Group R than in the Group C, leading to a lower BW at the end of the overfeeding period, partly due to a reduced fatty liver

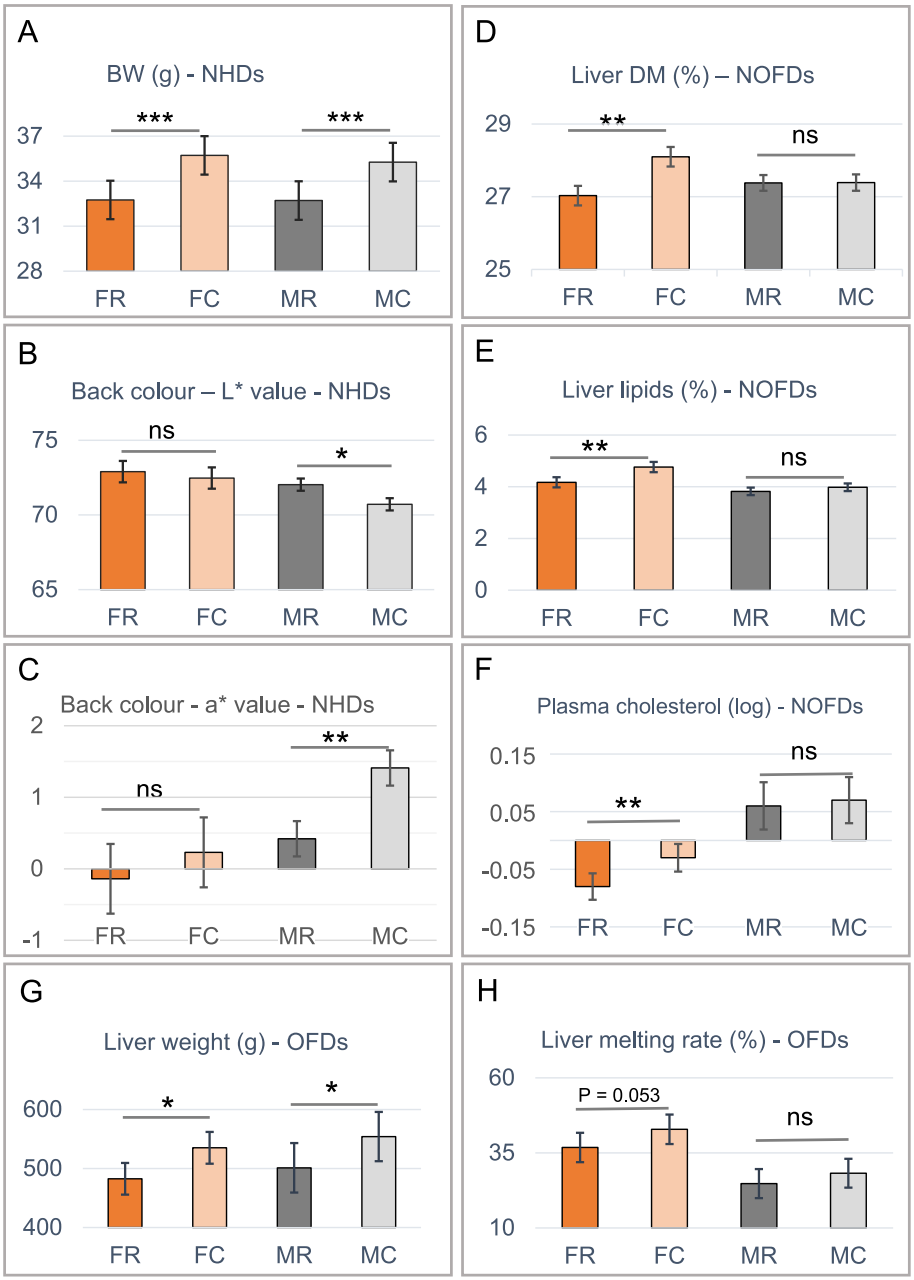
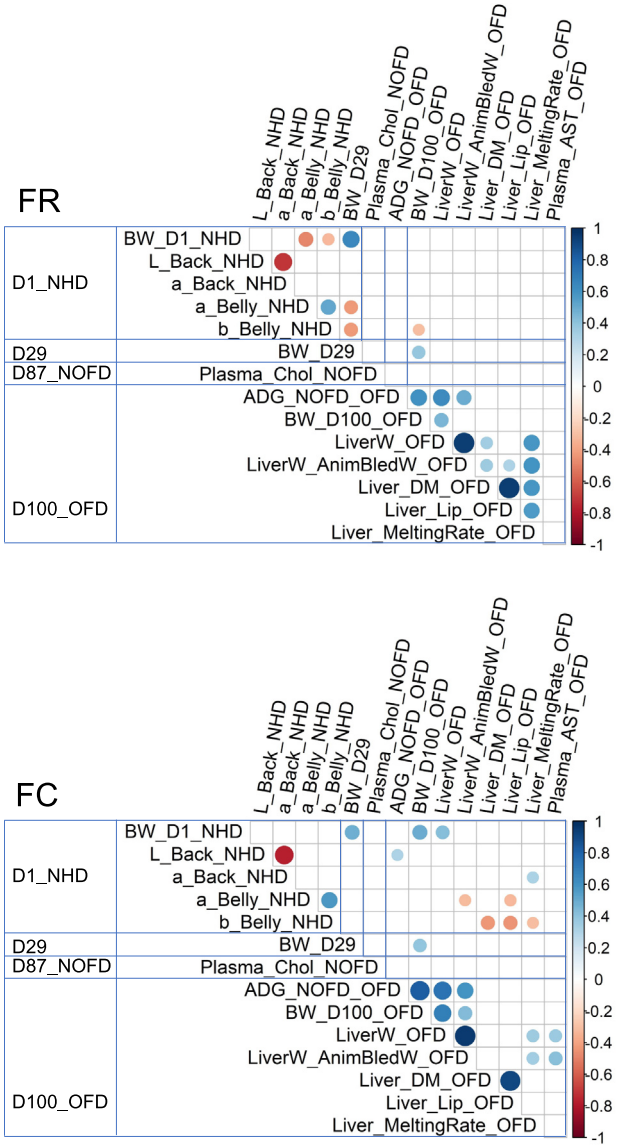


Fig. 2. Examples of maternal diet effects on phenotypic traits within each sex, in newly hatched ducklings (NHDs), non-overfed ducks (NOFDs) and overfed ducks (OFDs). Data are presented as Means ± SEs, as in Table 4. For each trait, females (FR and FC) are shown in orange, while males (MR and MC) are shown in grey. The offspring from dams fed the methionine-restricted diet (Group R: females FR and males MR) are presented in dark colours while the offspring from dams fed the control diet (Group C: females FC and males MC) are in light ones. The significance of difference is given by the diet *P*-value inside sex for each trait (***: $P < 0.001$; **: $P < 0.01$ and *: $P < 0.05$, ns: not significant). A: BW of newly hatched duckling (NHDs); B: CIE-LAB lightness value (L*) for down colour on the back of newly hatched ducklings (NHDs); C: CIE-LAB redness value (a*) for down colour on the back of newly hatched ducklings (NHDs); D: DM percentage in the liver from non-overfed ducks (NOFDs); E: lipid percentage in the liver from non-overfed ducks (NOFDs); F: plasma cholesterol level in non-overfed ducks (NOFDs), converted into logarithms; G: fatty liver weight in overfed ducks (OFDs); H: fatty liver melting rate in overfed ducks (OFDs).

weight (by more than 50 g, i.e., almost 10%). A reduction in the liver melting rate was observed in the Group R, in line with a lower liver weight, that can be explained by the positive genetic correlation (0.80 ± 0.07) between these two traits (Marie-Etancelin et al., 2011). However, while the fatty liver weight was affected by maternal diet, the hepatic lipid level, the proportion of abdominal fat to the weight of the bled animal and plasma parameters did not differ between the two diet groups after the overfeeding period. Nevertheless, the reduction in fatty liver weight indicated that liver metabolism was altered in ducks whose mothers were fed a reduced Met diet, and six DEGs were identified between the two diet groups after the overfeeding period (*ACADM*, *ACOX1*, *ADK*, *APOB*, *IL6ST* and *MTHFR*). These six genes were up-regulated in the restricted group and involved in either energy or one-carbon metabolism. *ACADM* and *ACOX1* genes both encode enzymes involved in the fatty acid beta-oxidation pathway. *APOB* gene encodes an apolipoprotein of low-density lipoproteins (LDL) and *IL6ST* encodes gp130, a signal transducer for interleukin 6 (IL6), which is involved in the GP130-STAT3 signalling pathway in humans with non-alcoholic fatty liver disease (NAFLD) (Min



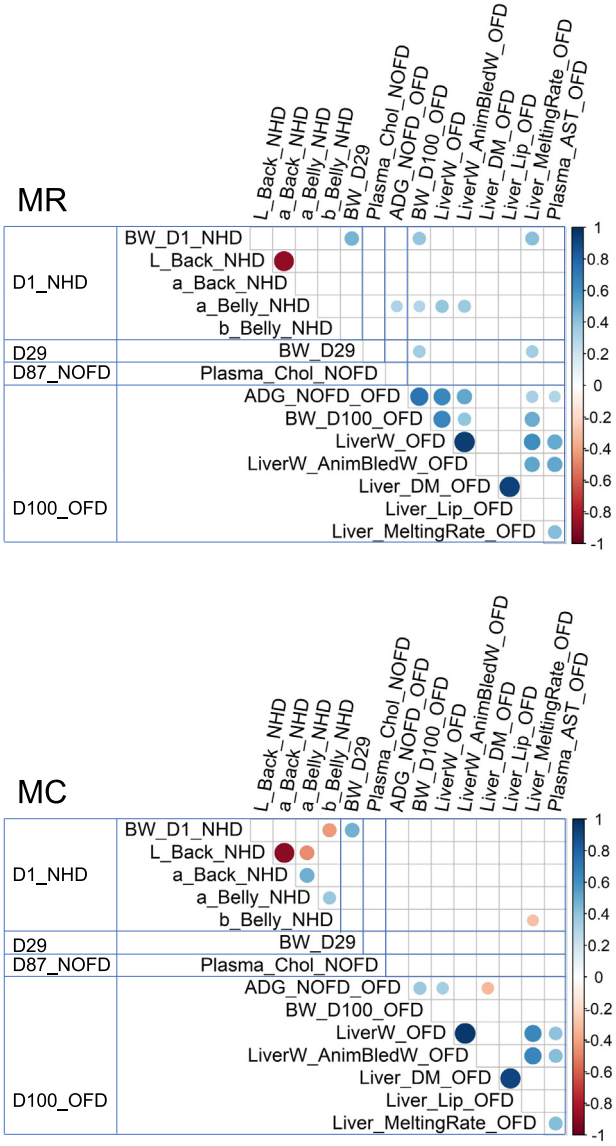


Fig. 3. Correlation matrices between traits showing sex-specific effects of the Met-restricted maternal diet in female mule ducks. The correlation matrices were plotted for the female offspring from dams fed the methionine-restricted diet (Group R females: FR; n = 45) and for the female offspring from dams fed the control diet (Group C females: FC; n = 45). The colour scale indicates the strength of the correlation; blue for a positive correlation and red for a negative one. The raw data were used, and only the significant correlations (differing from zero with a *P*-value < 0.05) were plotted. Phenotypic traits are BW of newly hatched ducklings (BW_D1_NHD), CIE-LAB lightness (L*) and redness (a*) values for the down colour on the duckling back (L_Back_NHD and a_Back_NHD); CIE-LAB redness (a*) and yellowness (b*) values for the down colour on the duckling belly (a_Belly_NHD and b_Belly_NHD) at hatching, BW at D29 (BW_D29), plasma cholesterol level in non-overfed ducks (Plasma_Chol_NOFD), average daily gain during overfeeding (ADG_NOFD_OFD), BW of overfed ducks (BW_D100_OFD), and parameters measured in overfed ducks: fatty liver weight (LiverW_OFD), ratio of fatty liver weight to bled animal weight (LiverW_AnimBledW_OFD), DM percentage of fatty liver (Liver_DM_OFD), lipid percentage of fatty liver (Liver_Lip), fatty liver melting rate (Liver_MeltingRate_OFD) and plasma activity of aspartate aminotransferase (Plasma_AST_OFD).

Fig. 4. Correlation matrices between traits showing sex-specific effects of the Met-restricted maternal diet in male mule ducks. The correlation matrices were plotted for the male offspring from dams fed the methionine-restricted diet (Group R males: MR; n = 52) and for the male offspring from dams fed the control diet (Group C males: MC; n = 52). The colour scale indicates the strength of the correlation; blue for a positive correlation and red for a negative one. The raw data were used, and only the significant correlations (differing from zero with a *P*-value < 0.05) were plotted. Phenotypic traits are BW of newly hatched ducklings (BW_D1_NHD), CIE-LAB lightness (L*) and redness (a*) values for the down colour on the duckling back (L_Back_NHD and a_Back_NHD); CIE-LAB redness (a*) and yellowness (b*) values for the down colour on the duckling belly (a_Belly_NHD and b_Belly_NHD) at hatching, BW at D29 (BW_D29), plasma cholesterol level in non-overfed ducks (Plasma_Chol_NOFD), average daily gain during overfeeding (ADG_NOFD_OFD), BW of overfed ducks (BW_D100_OFD), and parameters measured in overfed ducks: fatty liver weight (LiverW_OFD), ratio of fatty liver weight to bled animal weight (LiverW_AnimBledW_OFD), DM percentage of fatty liver (Liver_DM_OFD), lipid percentage of fatty liver (Liver_Lip), fatty liver melting rate (Liver_MeltingRate_OFD) and plasma activity of aspartate aminotransferase (Plasma_AST_OFD).

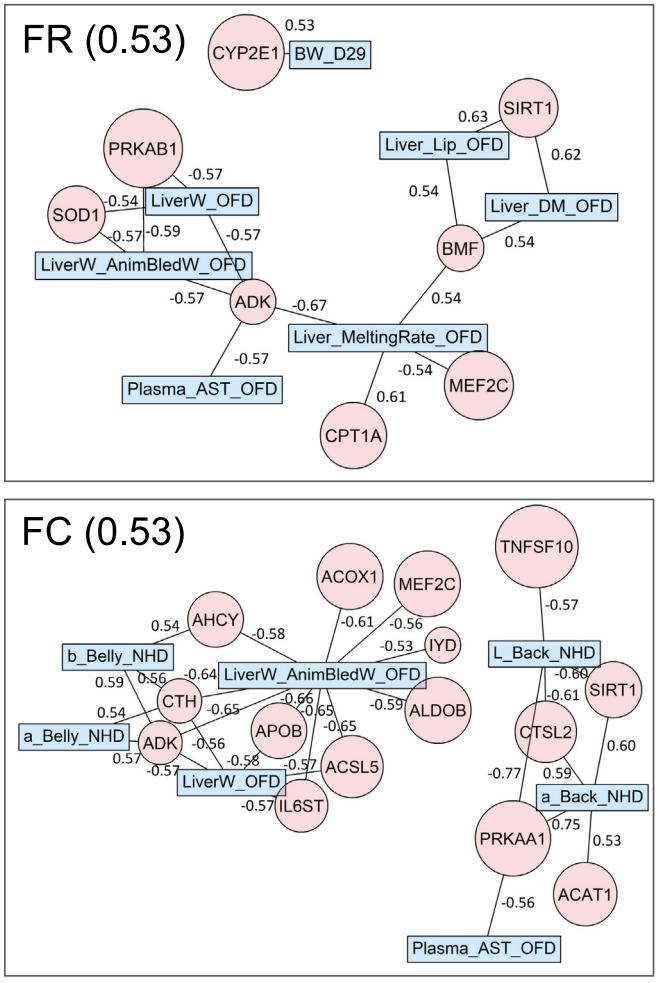


Fig. 5. Network of partial least squares (PLS) regression between phenotypic traits affected by maternal diet and liver gene expressions of differentially expressed genes in female mule ducks after overfeeding. A threshold of 0.53 was applied, and only higher correlations were represented in the networks. FR: The network was drawn with the data of female offspring from dams fed the methionine-restricted diet (Group R; n = 21). FC: The network was drawn with the data of female offspring from dams fed the control diet (Group C; n = 21). Abbreviations: L_Back_NHD and a_Back_NHD = CIE-LAB lightness (L*) and redness (a*) values for the down colour on the back of the newly hatched ducks; a_Belly_NHD and b_Belly_NHD = CIE-LAB redness (a*) and yellowness (b*) values for the down colour on the belly of the newly hatched ducks; BW_D29 = BW at D29; LiverW_OFD = fatty liver weight in overfed ducks; LiverW_AnimBledW_OFD = ratio of fatty liver weight to bled animal weight in overfed ducks; Liver_DM_OFD and Liver_Lip_OFD = DM and lipid percentages in fatty liver from overfed ducks; Liver_MeltingRate_OFD = fatty liver melting rate in overfed ducks; Plasma_AST_OFD = plasma aspartate aminotransferase activity in overfed ducks.

et al., 2015). *MTHFR* and *ADK* are both involved in one-carbon metabolism which is known to be linked to energy metabolism and steatosis (Cai et al., 2017; Christensen et al., 2010).

Offspring sex impacted growth and fatty liver production

The experimental design enabled us to study not only the effect of maternal diet but also the effect of the offspring sex on growth, plasma parameters, and fatty liver production. Sexual dimorphism of the BW is well known in Muscovy duck but is far less marked in mule ducks which is the offspring of a male Muscovy duck and a female common duck. However, and although Baéza and co-authors reported differences in BW only at 2 and 5 weeks of age in mule ducks (Baeza et al., 2000), we observed weight differences between the two sexes throughout growth, from hatching to 12 weeks of age. Thus, while males weighed less than females at hatching, they were heavier at 29 days of age, and remained so until they entered the overfeeding period at 87 days of age, with a weight difference of almost 250 g (or 7.6% of BW), in line with a higher average individual feed intake of the order of 10%.

At 87 days of age, before overfeeding, females tended to have a slightly higher proportion of liver weight and showed a higher liver lipid level. This difference in fattening between the two sexes was accompanied by differences observed for plasma parameters: females had lower glucose and cholesterol levels than males, but a higher triglyceride level. After overfeeding, females maintained a lower weight than males, and their average daily gain during overfeeding was lower than that of males, which is consistent with their lower consumption. Surprisingly, females had a fatty liver weight equivalent to that of males (511 ± 31.5 g and 525 ± 31.3 g, respectively), leading to a higher ratio of fatty liver to bled animal weight (12,1 ± 0.6% vs 11.4 ± 0.6%). The melting rate of female fatty liver was much higher than that of males (39.9 ± 4.6% vs 26.3 ± 4.6%, respectively) that highlights a degradation of fatty liver quality in females, but neither liver lipid nor liver DM contents differed between the sexes. This differs from the results of Marie-Etancelin and co-authors, whose study showed a lower fatty liver weight in females than in males (465 g and 517 g respectively), reduced by 10.6%, and with a melting rate of over 46.9% in females vs 32,0% in males (Marie-Etancelin et al., 2015). These differences in results may be due to the fact that the mule ducks' parental lines were not the same between the two studies. Thus, in the present study, for the same weight of fatty liver and the same liver lipid content, the melting rate was higher in females, underlining a lower fatty liver quality. These results are in accordance with the French production of foie gras that excludes females for high–quality products. Moreover, this highlights different hepatic metabolisms between the two sexes, reflected by a large number of differential transcripts.

Maternal methionine restriction showed sex-specific effects on liver transcript levels and on fatty liver traits

When looking for sex-specific responses to the Met-restricted maternal diet, results showed a variety of situations depending on the traits studied. Some traits were affected by maternal diet in only one of the sexes, while others were affected equally in both sexes. At hatching, the impact of maternal diet on the BW was equivalent in both sexes, with a weight reduction of around 3 g. However, maternal Met restriction induced a difference in down colour in male ducklings only. At D87, before the overfeeding period, maternal diet effects on lipid metabolism were observed in females only, with a lower percentage of liver lipids and a lower plasma cholesterol level, accompanied by a trend towards lower transcript levels for the *ABCA1* and *PPARG* genes, both implicated in energy metabolism. This is consistent with van der Waaij and co-authors' work in chicken, which also showed effects of maternal dietary restriction in female offspring only (van der Waaij et al., 2011).

After the overfeeding period, to distinguish the effect of maternal diet from the effect of feed intake on liver weight gain during overfeeding, we calculated the ratio between liver weight gain and feed intake. In this way, we showed that for the same amount of feed intake, liver weight gain was reduced in Group R offspring when compared to Group C offspring, regardless of sex. Moreover, a positive correlation between liver weight and melting rate was observed in males and females, whatever the maternal diet. This positive correlation had already been reported in male mule ducks (Bonnefont et al., 2019; Marie-Etancelin et al., 2011). In addition, the effects of the Met-restricted maternal diet on liver metabolism

were more marked in females than in males. Group R females showed a tendency to lower liver lipid and DM percentages, and their plasma AST activity was reduced compared to Group C females. AST is known as being a player of the physiology of the liver, and its activity is used as a marker of liver damage in mammals (Schuster et al., 2024; Sookoian and Pirola, 2015; Sookoian, 2012) as well as in ducks (Ming et al., 2017; Chen et al., 2000).

Altogether, these results highlighted differences in liver responses to maternal diet between the sexes and this was confirmed by differences in hepatic gene expression. While we had identified six DEGs between the two diet groups of OFDs in our first analysis, intra-sex analysis showed that the expression differences were attributable to females only. It was therefore not possible to look for correlations between DEGs and altered phenotypic traits in males. In females, the correlation networks differed between the two groups. In group C females, 10 DEGs correlated with fatty liver weight and/or percentage of fatty liver in the carcass whereas in group R females, seven DEGs correlated with liver characteristics affected by maternal deficiency, such as fatty liver weight, melting rate, liver lipid level or liver DM level. In both groups, DEGs correlating with altered phenotypic traits were involved in energy metabolism or one-carbon metabolism, highlighting the links between these two metabolisms as already reported (Clare et al., 2019; Cai et al., 2017; Chango and Pogribny, 2015). Our results are also consistent with previous studies reporting the impacts of maternal diet methyl donor levels on offspring liver metabolism in farmed mammals (Safain et al., 2024; Elolimy et al., 2019; Cai et al., 2014a, 2014b) and farmed birds (Hu et al., 2015, 2017, 2020; Hou et al., 2018).

In conclusion, the nutritional programming inscribed during embryonic development had long-term effects on liver traits and was sufficiently robust to persist after the overfeeding period, despite the major reorganisation of metabolic pathways that it implies. Our study also highlighted the sex-specific effect of nutritional programming. Whereas few studies investigated both males and females in mammalian and avian models, we found that phenotypic traits and liver metabolism depend on the offspring's sex, highlighting the importance of specifying sex in all nutritional programming studies. In the context of fatty liver production, our results showed that reducing methionine levels in the maternal diet led to a 10% decrease in fatty liver weight, in offspring of both sexes. Producers should, therefore, ensure that the maternal diet meets the appropriate levels of methionine and other methyl donors to support optimal production performance. Additionally, since we observed a reduction in fatty liver weight with decreased methionine intake, it would be worth exploring whether increasing methionine levels in the maternal diet could lead to an increase in fatty liver weight.

Supplementary material

Supplementary Material for this article (https://doi.org/10.1016/j.animal.2025.101539) can be found at the foot of the online page, in the Appendix section.

Ethics approval

Experimental procedures and animal care were conducted in compliance with the European Communities Council Directive 2010/63/EU. The experiment was conducted at AVIPOLE - Palmiped Farming Systems Facility – INRAE (Benquet, 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 (authorisation APAFIS#1847-2015092213418825v2).

Data and model availability statement

None of the data were deposited in an official repository. The datasets used and analysed during the current study are available from the corresponding author.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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Declaration of interest

None.

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