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ABSTRACT

Increases in chicken production are mainly due to specialised breeds. However, of increasing importance are the local breeds, known for their ability to adapt to the environment and for their unique products. Conventional poultry products contain lower levels of n-3 fatty acids compared to those obtained from local breeds, therefore the aim of this study was to evaluate the modulation of expression of genes involved in LC-PUFA biosynthesis pathways according to genetic background, diet conditions, and sex. Animals from two local breeds and a commercial line were fed different diets: control and experimental diet (10% linseed supplementation). For each breed and diet group, both males and females were reared. The RNA was extracted from 36 liver samples and was sequenced by RNAseq method. Bioinformatic analysis was carried out to find differentially expressed genes (DEGs) from different comparisons between experimental groups. Results showed low impact of diet on DEGs related to fatty acid biosynthesis, but linseed diet increased percentage of n-3 fatty acids of liver. Sex , particularly the female groups, and genetic background determined the differential expression of genes related to LC-PUFA biosynthesis. Specifically, females of local breeds shared 23 up-regulated genes when compared to their respective commercial line groups. Some of the shared genes had a role in *de novo* triglyceride biosynthesis (*MTTPL* and *GPAM*), and in genes involved in *de novo* FA biosynthesis (*ACACA* and *SCD*) was detected. In conclusion, sex and local genetic background appear to have influence on the expression of genes related to LC-PUFA synthesis.

INTRODUCTION

To date, commercial chicken lines have dominated the poultry market so far, thanks to the fast growth and high production performances. Besides the standard commercial chicken lines, of increasing importance are the local breeds which are able to adapt to the environment where they live (Perini *et al.*, 2021), and for their different quality products compared to conventional ones (Franzoni *et al.*, 2021). Unfortunately, the productivity traits are critical for local breeds, which are instead appreciated for the diversity of their products, which could include economically exploitable traits (Moula *et al.,* 2010). Currently, it is still

difficult to obtain economic feedback from breeders who decide to raise local breeds because of low competitiveness on productive performances.

A potential valorisation strategy is the enrichment in terms of nutritional value of meat and eggs. Enhancing the quantity of n-3 fatty acids in chicken meat and eggs through the diet can have important impacts: i) supplementing the fatty acid intake in the human diet, as it is usually rich in n-6 and lacking in n-3 fatty acids (Mariamenatu & Abdu, 2021); ii) chickens are able to convert precursors of n-3 in long chain (> 20 carbon atoms) polyunsaturated fatty acids (LC-PUFA); iii) finally, eggs and meat enriched with n-3 would have increased market potential compared to standard animal products.

The LC-PUFAs, eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) are the main form of n-3. The fatty acid profiles of poultry meat and eggs can be readily enriched in EPA and DHA through the diet (Cartoni Mancinelli *et al.*, 2022). Although conventional poultry meat contains low levels of EPA and DHA, several factors such as sex, feed, and genetic background may influence their quantity. In particular, genetic background is reported in the literature as a crucial factor: higher concentrations of n-3 LC-PUFA are synthesized by local breeds compared to commercial lines (Cartoni Mancinelli *et al.*, 2021).

The application of a diet rich in precursors of n-3 (α-linolenic acid: 18:3n-3, ALA) can affect the amount of LC-PUFA in meat and eggs, and may change the expression level of genes involved in the process of lipid biosynthesis and elongation. In mammals, these two metabolic pathways, mainly occur in the adipose tissue, while in chicken the majority (90%) takes place in the liver (Nematbakhsh *et al.*, 2021). To elucidate the complete expression profile of liver genes, RNAseq provides a useful tool. This transcriptomic approach can accurately study tissue transcriptomes with high resolution and depth. Increasingly, the nutrigenomic approach is used to investigate the effect of diets on metabolic processes also in poultry (Soglia *et al.*, 2022). A recent study of the liver transcriptome after folic acid supplementation in the diet of broiler chickens found that peroxisome proliferator activated receptor (PPAR) signalling is the pathway most activated by the enriched diet (Zhang *et al.*, 2021).

In recent years, many studies tried to elucidate which genes are principally involved in the biosynthesis process of LC-PUFA. Studies focused on the evaluation of the presence/activity of elongase of very long

chain fatty acid (*ELOVL*) and fatty acid desaturase (*FADS*) enzymes (Cartoni Mancinelli *et al.*, 2022; Lee *et al.*, 2016). Although recently many studies have highlighted some pathways involved in the biosynthesis of LC-PUFAs (e.g. *PPAR* signaling), there remains a lack of scientific research in this area (Mihelic *et al.*, 2020). The aim of this study was to determine the modulation of expression of genes involved in LC-PUFA biosynthesis pathways, according to genetic background (local breed *vs* commercial line), diets, and sex.

MATERIALS AND METHODS

Animal ethics

Birds were raised, handled and processed according to the European legislation for the protection of chickens kept for meat production (European Commission, 2007), the protection of animals at the time of killing (European Commission, 2009) and the protection of animals used for scientific purposes (European Commission, 2010). The experimental protocol was positively evaluated and approved by the Ethical Committee of the University of Perugia (ID: 62700_15/07/2020).

Experimental design

The trial was carried out in the experimental section of the Department of Agricultural, Food and Environmental Sciences (University of Perugia, Italy). Specifically, three different chicken genetic backgrounds were used: Robusta Maculata (RM) and Bionda Piemontese (BP) as local breeds and Ross 308 (ROSS) as a commercial line. Both RM and BP are dual-purpose breeds, in particular BP shows higher aptitude for laying eggs (200 eggs/year *vs* 150 eggs/year of RM) (www.pollitaliani.it). One-day old chicks of each sex were housed in an environmentally-controlled poultry facility and vaccinated against coccidiosis, infectious bronchitis, Marek's Disease, Newcastle Disease and Gumboro. Until 21 days of age, all the chickens received the same starter diet. Then each genetic background was divided into two groups: one fed a standard diet (control group, Ct), the second fed the standard diet supplemented with 10 % extruded linseed (experimental group, L). The two diets were formulated to meet the nutritional recommendations of Ross 308 birds (Table S1) (Aviagen, 2019). Three replicates/group were formed, each representing both

male and female chickens (5 males + 5 females/each replicate). Chickens were raised in different indoor pens at the same stocking density (5 chickens/m^2) and the temperature was set according to the age of the birds (20-32°C; Relative Humidity: 65-72%). Water and feed were provided *ad libitum*. At 81 days of age, live body weight was recorded (g) for all the birds which were later slaughtered in a commercial slaughterhouse. From each replicate, two birds (one for each gender) were selected. Table S2 schematically explains the experimental design used in the present study.

Tissue collection and RNA extraction:

Liver tissue was collected from all the animals. Around 1 g of tissue was collected in a 2 mL cryogenic vial (Corning® Inc., Corning, NY), instantaneously frozen in liquid nitrogen, and stored at -80° C until RNA extraction. The remaining part of liver tissue was collected and stored at -20°C for further analysis of fatty acids (FA) profile.

At this stage, RNA was extracted only from liver tissue. Samples were prepared from 36 animals, with three biological replicates for each experimental group (Table S2). RNA isolation was performed with a NucleoSpin RNA Mini kit for RNA purification (Macherey-Nagel, Germany) as recommended by the manufacturer, starting from 30 mg of tissue. A homogenization step was carried out using an Omni Tissue Homogenizer (TH) - Omni, Inc) in ice. DNA contamination was removed with a DNase enzyme (included in the kit) during the incubation step. RNA concentration was measured with a Qubit 3.0 fluorometer (Life Technologies) and Qubit RNA HS Assay Kit (Life Technologies) according to manufacturer instruction. Total RNA (~1.5 µg/sample) was sent to Genewiz (South Plainfield, NJ, USA) for quality check, library preparation and Illumina sequencing. For testing RNA quality, the Agilent (Santa Clara, CA) 2100 Bioanalyzer Nano Kit was used, and all the samples showed RNA integrity (RIN) numbers ≥6.5 (data not shown). Library preparation was carried out through polyA + selection and paired-end (PE) sequencing was run on an Illumina NovaSeq System that generated 150 bp PE reads.

Bioinformatic analysis

RNAseq data were quality checked by FastQC software (Brown *et al.*, 2017). Paired qualified reads were mapped to the chicken reference genome (GRCg6a, accession number: PRJNA13342) using STAR software (Dobin *et al.,* 2015) with default parameters. The mapping rates for each sample were assessed using Samtools 'flagstat' command (Heng Li *et al.*, 2009). The mapped BAM file was then used in featureCounts software that, assigning raw alignments to annotated genes, generates the raw expression count file used for gene expression quantification (Liao *et al.*, 2014). An overview of genomic distance in the dataset was performed through Principal Component Analysis using the ggplot2 R package. Differential expression (DE) analysis was performed using the DESeq2 R package (Love *et al.*, 2014). The P-value adjustment was made using Benjamini and Hochberg's approach for controlling the False Discovery Rate (FDR). Genes with a P-adjusted value < 0.05 and -2> Log2 fold change (LFC) >2 were used as thresholds for significant DE by DESeq2. Visualization of contrast between different experimental groups was performed by EnhancedVolcano package in R (Blighe *et al.,* 2022). The overlap of significant genes among experimental groups was assessed with Venn diagrams built with the Interactivenn web tool (Heberle *et al.*, 2015). Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity) was used to identify gene ontology pathways, and regulatory networks to which DE genes belong, as well as upstream regulators. Ingenuity Pathway Analysis can transform a set of genes into a number of relevant networks based on comprehensive records maintained in the Ingenuity Pathways Knowledge Base. According to DEG input, IPA software calculates a z-score value for each biological pathway. When a pathway has been categorized with a z-score > 2, it resulted as an activated pathway. Moreover, to confirm the IPA results, gene ontologies (GO) were examined using the Panther (v17.0) database according to the Statistical over-representation test. Fisher's test was used to correct for False Discovery Rate (Figure S1) (Mi *et al.*, 2019). Differential expression (DE) analysis was performed in individual contrasts according to diet, sex and genetic background, separately. In particular we evaluated six diet contrasts, six contrasts for sex and four contrasts for each local breed against the ROSS birds (Table S3).

Fatty acids (FA) profile

The fatty acids profile was determined from the same samples used for the RNAseq. Lipids were extracted from 5 g of liver/sample based on the methods described in Folch *et al.* (1957). The fatty acids were identified in the form of their methyl esters using a Varian Gas Chromatograph (CP-3800) and a DB wax capillary column (25 mm ø, 30 m long). The establishment of each fatty acid was done in relation to the retention time with respect to fatty acid methyl ester standards (FAME, Sigma-Aldrich, Bellefonte, PA). The relative quantity of each fatty acid present in the liver was calculated using heneicosanoic acid (C21:0; Sigma-Aldrich) as the internal standard. Data were expressed as % of total FA. The average amount of each FA was used to calculate the sum of total PUFA of the n-3 and n-6 series. After using the function of the Shapiro test (for normality test), data were analysed with ANOVA and with Tukey's *post-hoc* test in R software. All results are expressed as mean ± SEM, with the level of significance set at $p < 0.05$.

RESULTS

Sequencing data analysis

Average mapping rate was 85.11% across all samples, 98% of which were seen to be properly paired. Among all the samples, the average quality score for each base was > Q30 and the average GC content in sequenced samples was around 52% (data not shown). After all quality checks, all 36 samples were taken forward for further analysis. In order to shed light on genetic similarity, a principal component analysis (PCA) plot was computed and visualized using R software. This showed genetic distance between the samples according to the comparison of Principal Component 1 and 2 (PC1 and PC2) (Figure 1). Genetic diversity was appreciable, especially between the two main clusters seen in Figure 1 representing male and female birds. The male cluster showed less genetic variance compared to the female one, and the ROSS birds grouped closest to each other within each cluster, because of their standard genetic background. On the contrary, the two local breeds showed more genetic variance within the experimental groups and were clearly separated from the ROSS cluster.

Body weight and fatty acids profile of animals

The effect of diet was evaluated according to the results from live body weight (Figure S2) and the percentage of n-3 and n-6 PUFA, and LC-PUFA (Figure 2). The diet had no statistical impact on the body weight at slaughtering of the two local breeds, either in males or females. Instead, the L diet significatively decreased the body weight in ROSS (male and female).

Figure 2 represent the n-6 and n-3 PUFA proportion in liver, and the sum of LC-PUFA. Figure 2a shows the percentage of LC-PUFA was not statistically affected by the diet. On the contrary, the n-6 PUFA (ranging from 18% to 34% approximately) showed higher level than n-3 PUFA (from 4% to 12). Moreover, the control diet generally increased the n-6 percentage in both sexes of ROSS and in RM_M. On the contrary, the L diet significantly enhanced the n-3 level in all groups, excepted for RM females.

Differentially expressed genes (DEGs)

Each experimental group was compared against one another to check differential gene expression. This was done on the basis of: diet, sex, and genotype (Table S3). In particular, we were interested in comparing local breeds to the broiler (ROSS) genotype. Appendix S1 lists all significantly differentially expressed genes in each comparison. Moreover, all genes related to FA metabolism, and which were found to be differentially expressed, are reported in Table S4.

Diet contrast

For the evaluation of the possible effect of diet on gene expression between sexes and genetic background, the linseed diet group was compared to the control diet group within each sex and within each genetic background. Experimental groups formed by “genetic background” and “sex” were taking in consideration individually. Linseed diet groups have been used as experimental groups, hence the up and down-regulated genes and the GO analyses referring to them. Figure 3 shows the volcano plots resulting from each comparison. Figures 2a-b showed the DEGs detected for the female (BP_F) and male (BP_M) Bionda Piemontese experimental groups respectively. In BP_F, the diet played a limited role in differentiating gene expression level, indeed Figure 3a had only eight differentially expressed genes with a *P*-value < 0.05, of

which some involved in fatty acid metabolism (e.g. *LYG2*, *SCD*), being found down-regulated with the L diet. Other important genes involved in FA synthesis (*ACACB*, *ACACA*, *FASN*) had a significant *P*-value but had lower LFC values (between -2 and 2) (Appendix S1).

In the BP_M group, the diet contrast showed 280 significant genes, and the Gene Ontology study confirmed that the most influenced pathways were cellular lipid metabolic process (GO:0044255) and lipid metabolic process (GO:0006629) (Appendix S2). Regarding the Robusta Maculata breed, volcano plots for DEGs in females (RM_F) and males (RM_M) are shown in Figures 2c-d respectively. Contrary to the BP birds, the RM_F comparison showed more DEGs than the RM_M group, namely 83 and 13 genes. Gene ontology analysis in each group showed that the RM_F group had biological processes which were significantly enriched in the L diet, although not directly related with FA biosynthesis, and males did not show anything significant (i.e. 'de novo' protein folding (GO:0006458)) (Appendix S2). Finally, the last genetic background evaluated for the effect of diet was the commercial ROSS hybrid (Figure 3e-f). In both female and male groups, a limited number of DEGs were identified (72 and 13, respectively). This is similar to the GO biological annotations associated with FA metabolism. None of the six diet contrasts resulted in a significantly activated biological pathway in IPA analysis.

Effect of sex

The effect of sex on gene expression was assessed as follows: the experimental groups compared within the same genetic background and diet groups but differing by sex. Female groups were taken as the experimental group, hence the up and down regulated genes and GO analysis are referred to “F” group in each contrast. Looking at Figure 4a, it is easy to appreciate the numerous DEGs up-regulated in females. For each BP comparison, as shown in Figures 4a-b, the GO annotations on up-regulated genes clarify the sexual dimorphism in the most typical way: cellular response to estrogen stimulus (GO:0071391) and response to estrogens (GO:0043627) (Appendix S3). When comparing the ROSS broiler birds, and examining the linseed diet group (L), significant biological enrichment was found in organo-nitrogen compound biosynthetic process (GO:1901566), for example (Appendix S3). With regard to FA pathways, in BP birds there was

activation of lipid pathways in females, namely lipid transport (GO:0006869) and lipid localization (GO:0010876). This result from Panther was also confirmed using IPA software. Fatty acid metabolism was seen to be significatively enhanced in the two female groups of the BP breed. The significance of fatty acid concentration pathway in RM chickens treated with linseed diet was also indicated (Appendix S3). In particular, the data in Appendix S3 shed light on the genes that were differentially expressed in females within the experimental group. The females expressed some genes strictly related to FA metabolism differentially to males within respective groups. For examples: *ELOVL2* in BP_Ct, *ELOVL2*, *FADS1* and *FADS2* in BP_L, and *SCD* in RM_L.

Genetic background comparison

One of the aims of this study was to assess the impact of breed and genetic makeup on the profile of LC-PUFA expression. In particular, the comparison between local breeds and the commercial line (same sex and diet) was examined.

Figure 5 represents the DEGs from the BP *vs* ROSS comparison. BP breed represented the experimental group, which up and down regulated genes and GO analysis are referred to. Figures 5a-b show females treated with control and linseed diet respectively. According to results from Panther, many pathways related to lipid metabolism were up-regulated in the BP breed, such as fatty acid biosynthetic process (GO:0006633) and long-chain fatty acid metabolic process (GO:0001676) (Appendix S4). In the female control diet group, the up-regulated genes found through IPA were related to FA metabolism: *SCD*, *CYP1A1*, *FASN*, *ACACA*, *THRSP*, *FAR1*, *FADS1* (Appendix S4). Figure 5b showed the results for a genetic background contrast with females fed the linseed diet. The results from GO analysis showed a large number of lipid related pathways, the most important being lipid metabolic process (GO:0006629). Some of the genes related to this, which were more highly expressed in BP relative to the commercial line include *FABP3*, *SCD*, *GPAM*, *LPIN1*, *SREBF2* and *ACACA* (Appendix S4). Meanwhile, Figures 5c-d exhibit the contrast of BP with ROSS birds, in males fed the two different diets. With the control diet, BP males showed higher expression of genes involved in fatty acid metabolic process (GO:0006631) in comparison with ROSS. BP males fed the

linseed diet do not show DEGs significant in FA metabolism (e.g. *FASN*, *ACACA*, *ACACB)* when compared with ROSS (Appendix S4), but amino acid biosynthetic processes are highlighted in the GO analysis.

We next wanted to compare Robusta Maculata (RM) *vs* Ross308 (ROSS) groups. The experimental groups consisted of the RM breed, hence up and down regulated genes and GO analysis are referred to as "RM" breed groups. The volcano plots are presented in Figure 6, of which the first two (Figure 6a-b) represented the contrast in females, with control and experimental diets, respectively. Figure 6a showed 391 genes with a P-value lower than 0.05, of which 234 genes had a value of -2 > LFC >2, and 121 genes in RM with LFC >2. The Panther GO analysis clearly showed that within the up-regulated genes, fatty acid metabolic process (GO:0006631) was clearly enhanced, with similar pathways activated in female linseed diet groups. IPA analysis showed significance of LC-PUFA synthesis in RM birds fed the control diet and synthesis of FA in RM birds fed the linseed diet (Appendix S5). The RM female groups shared DEGs clearly involved in FA pathways, such as *SCD*, *ACACA*, and *THRSP*. On the contrary, in RM males (Figures 6c-d), no pathways related to FA appeared as significantly involved in birds fed either diet. Instead, immune pathways were active in Ct diet birds, while there were no significant pathways in birds fed the supplemented diet (Appendix S5).

Comparison of local vs commercial females

According to the results from the sex comparison previously described, females, for the most part, appeared more specialized for FA production compared to males. Moreover, in the comparison of genetic backgrounds, all the female groups from local breeds showed higher activation of FA-related pathways compared to commercial hybrid birds. In order to understand which DEGs were common to the local breeds, a Venn diagram was generated (Figure 7). Here we took into consideration only the female birds, and only the genes differentially expressed in local breeds (BP and RM) compared to ROSS. Interestingly, 23 genes were commonly up-regulated among local breeds (Appendix S6). A heat map was generated to graphically represent the 23 shared genes and their expression level among all female samples (Table 1) (Figure 8). GeneMania software was used to identify any connection between them, with *GPAM* being

suggested as being directly related to *THRSP* and *SCD*. In fact, we saw *GPAM* as being up-regulated in both BP female groups and in RM females fed the control diet, but not the linseed diet (Figure S3).

DISCUSSION

Many studies have reported how the diet can modulate the expression of genes in different tissues (Sevane *et al.*, 2014; Szalai *et al.*, 2021). Here, we fed animals with a diet rich in the precursor of n-3 LC-PUFA. We designed the experiment assuming that diet plays a key role in changing lipid metabolism in the liver. Indeed, vitamin supplementation (Niu *et al.*, 2009) taurine (He *et al.*, 2019) and zinc oxide nanoparticles (Ramiah *et al.*, 2019) have been used to modulate gene expression of FA related genes, whereas the supplementation of dietary PUFA is widely used to increase LC-PUFA concentration in animal products of different animal species, such as dairy cows, rabbit and turkey (Castellini *et al.*, 2022; Kliem *et al.*, 2019; Szalai *et al.*, 2021). Chickens are usually an ideal target for diet supplementation, especially linseed based supplements (Head *et al.*, 2019; Jing *et al.*, 2013; Sevane *et al.*, 2014; Zhang *et al.*, 2021).

In the present study, the different diets affected the body weight of commercial birds. This result is explained by the fact that during the experimental trial, the ROSS chickens showed problems of adaptation to the linseed diet, consuming around 7% less quantity of the L diet with respect to the Ct diet, both in males and in females. In other studies was already reported how extruded linseed diet could lead to lower body weight gain and lower feed intake in broiler (Avazkhanloo *et al.,* 2020; Anjum *et al.*, 2013). This outcome indicated once again how the local breeds can easily adapt themselves to different environmental factor (e.g. alternative diet sources), while commercial lines encounter difficulties. In the present study, n-3, n-6 and LC-PUFA of liver were examined. LC-PUFA, which are directly related to the desaturation and elongation ability, was not affected by diet, conversely to n-6 and n-3 levels. The percentage of n-6 was generally higher in the Ct diet because the Ct diet is higher in linoleic acid (LA), which is the precursors of n-6 PUFA. On the other hand, the L diet positively affected the n-3 PUFA, and indeed the n-3 is significantly higher in the L diet than in the corresponding Ct groups (Figure 2). Both these results are commonly found in the literature (Hang *et al.*, 2018; Head *et al.*, 2019; Meineri *et al.*, 2018). Head *et al.* (2019) described the

modulation of FA profile in a given tissue through the diet, and also provided insights into low effect of the diet on the gene expression. The fact that the trend of LC-PUFA in the different experimental groups was similar confirms this assertion. Thus, it seems that the entity of LC-PUFA produced by liver is mainly due to the level of dietary precursors. On the same time, the type of precursor furnished α-linolenic (ALA, n-3) or linolenic (LA, n-6) determined an alternative accumulation of the same PUFA series in the liver. Indeed, we found that the dietary supplementation of linseed affected expression of genes involved in FA metabolism in only one group (BP_M group – Appendix S2). The most highly expressed genes in the linseed diet group included *PPARGC1A,* and *LPIN1*. *PPARGC1A* is a co-activator of PPARγ which is a transcription factor that participates in induction and stimulation of fat-specific genes and fatty acid bio-synthesis (Wang *et al.*, 2017). *LPIN1* is involved in synthesis and transport of triacylglycerol, a major constituent of chicken lipids (Desert *et al.*, 2018), and also found to be over expressed in a chicken line selected for intramuscular fat content together with *PPARγ* (Liu *et al.*, 2020). The remaining contrasts (n=5) for diet did not show enriched pathways, and for this reason, we can assert that diet is the factor with lowest impact on gene expression of fatty acid metabolism. Although no GO enrichment was discovered regarding FA metabolism in the other 5 diet contrasts, there are some interesting genes down-regulated with the L diet belonging to the BP_F and RM_F groups. Indeed, in BP_F the L diet showed down-regulation of *LYG2*, *ACACA*, *FASN*, *SCD* and in RM_F, down-regulation of *PLIN2* and *GPAM*, all important genes in FA metabolism and discussed later in this section. This is also in agreement with Head et al. (2019) who ascertained that the effect of linseed on the expression of 14 genes involved in FA metabolism in chicken liver was low. We therefore saw differing modulation of FA metabolism in liver between genetic backgrounds and sexes through diet.

This study also evaluated the effect of sexual dimorphism and its impact on FA metabolism. A clear separation between male and female birds is appreciable in Figure 1, underlying a divergent level of expression in genes between the two groups. The background of sex effect on FA metabolism was investigated by Poureslami *et al.* (2010b) where they described the poor effect of sex on final concentration of MUFA and PUFA of the n-3 and n-6 series (Poureslami *et al.,* 2010a). Elsewhere, Lopez-Ferrer *et al.* used dietary linseed oil, finding that abdominal fat percentage in male chickens was significantly lower than in

the female chickens (López-Ferrer *et al.*, 2001). This was corroborated in human where the differential ability to activate PPARα together with the well-documented effect of sex hormones on lipid metabolism may highlight sex as a significant factor in plasma FA levels (Thifault *et al.*, 2013). Differences between male and female chickens have never been thoroughly described and hence our interest in including sex effect in our analysis. BP_F, in both diet groups, showed a greater enrichment for estrogen pathways because it was the most egg-layer type chicken in the dataset. Indeed, estrogen in laying hens stimulates the liver to synthesize fatty acids for egg enrichment (Hanlon *et al.*, 2022). As reported by Ayres *et al.* (2013), differential expression of genes related to the female W chromosome are essential for sex determination in chickens. Some of them (*NIPBL* and *UBAP2*) have also been found to be central to sex determination in Japanese quail (Caetano-Anolles *et al.*, 2015). Regarding lipid metabolism, some genes were found to be more highly expressed in females with respect to males. This is the case for *ELOVL2* (in both BP contrasts), *FADS1*, *FADS2* (in BP_L) and *SCD* (in RM_L, and in both ROSS groups) that code for enzymes directly involved in desaturation and elongation of FA in the diet, such as ALA (18:3n-3), resulting in LC-PUFA (Head *et al.,* 2019). Interestingly, the results can be appreciated in the comparison of females with males in the BP breed. Besides the finding of elongases and desaturase-related genes being more highly expressed in females, other genes directly involved in FA metabolism were also identified. For instance, *APOB* plays a vital role in the assembly and secretion of triacylglycerol-rich lipoprotein in the liver of egg-laying chickens (Ma *et al.,* 2017), in line with the BP breed having a major aptitude in laying, especially when compared to ROSS (meat type) and RM birds (dual-purpose). Moreover, in the BP_L group, the female highly expressed genes of the fatty acid-binding protein (*FABP*) family, particularly *FABP3* and *FABP1*, which are considered biomarkers for intramuscular fat content. Bongiorno *et al.* (2022) reported that the saturated fatty acid (SFA) of breast meat was mainly influenced by gender. On the other hand, in the RM breed the higher female expression of *THRSP* strongly suggests a control of lipogenic targets (Resnyk *et al.*, 2017). Activated estrogen pathways could explain differences in genes related to FA in females. In 2017 Zhang *et al.* found three miRNAs involved in down-regulation of genes related to FA metabolism. More specifically, estrogen eliminates the suppressive effect of miRNAs on the target gene *ELOVL5*. Interestingly, estrogen suppresses

the host *SLIT2* gene, thus decreasing the expression of intronic miR-218-5p to promote hepatic synthesis of long-chain polyunsaturated fatty acids in the liver (Zhang *et al.,* 2017). Although *ELOVL5* was not found to be directly overexpressed in females in the present study, many genes functionally related to it (i.e. *ELOVL2*, *SCD*, *FADS1*, *FADS2*) were identified. It is possible that estrogen pathways could regulate FA metabolism in general through the down-regulation of miR-218-5p, which regulates the genes related to FA metabolism, thus enhancing FA biosynthesis. Interestingly, it is the same miRNA (miR-218-5p, regulated by estrogen) that has a role in *FADS1* regulation in liver (Hong Li *et al.*, 2016), and *ELOVL2* regulation in chicken muscle (Zhang *et al.,* 2018). A more comprehensive evaluation of estrogen modulation via miRNA of FA metabolism should be investigated in further studies.

In the present study, we observed that the genetic background was the variable having the largest effect on differential gene expression. Initially, we expected differing expression of genes for FA metabolism between the local breeds and in particular when compared to ROSS broilers. In the BP breed, a huge effect was identified in all four experimental groups when compared against ROSS birds. According to gene expression rates, the two female groups for each diet were the most divergent. These results suggested not only that the females had higher expression of genes involved in FA metabolism, but also that the BP female exhibited higher expression of genes responsible for FA metabolism, when compared to the ROSS females. In particular, the female control diet group showed an enrichment for genes involved in the long-chain fatty acid metabolic process (GO:0001676) supported by both GO analysis and the related genes *ELOVL2*, *SCD*, *FADS1*, *FADS2*, *THRSP*, *FABP3* and *LPIN1*.

With males belonging to the local breeds, an effect was only observed within the BP breed, suggesting the importance of FA processing in this breed. RM females also showed interesting results with regards the FA process. On the contrary, the comparison between RM_M and ROSS_M did not show significant differences in genes involved in the FA processes. This lower effect in males of local breeds could be due to the estrogen stimulus that was strongly activated in females from local breeds and results in the difference in expression of genes related to LC-PUFA. Comparing all the results from contrasts of BP and RM female groups against the ROSS birds highlights 23 genes that are shared between the female local breeds (Figure

7 and Figure 8, Appendix S6). Some of these are still uncharacterized (*LOC101747680*, *LOC107053670*, *LOC426220*, *LOC101749589*, *LOC112532382*, *LOC107050519*, *LOC107053691*, *LOC112532439*), but three of them are already reported in the literature with regards FA metabolism related functions. The *LOC101747680* gene codes for a C-like protein and it is located on chromosome 11, and *LOC107053670* is a non-coding RNA situated on chromosome 6, each with roles which are still unclear. *LOC426220* is located on the W sex chromosome, and is an avidin-related protein 6-like, and with all other avidin-like molecules localized on chromosome Z, suggesting a sex-specific regulation. Moreover, two studies have found this gene strongly correlated with egg yolk, vitelline membrane, and white coloured eggs (Gloux *et al.*, 2019; Niskanen *et al.*, 2005). Regarding eggs and fertility, another three genes were found differentially expressed in local breed females: *WDFC8*, *CTSEAL*, and *ZP1* (Table 1). *WDFC8* is clearly related to "WAP four-disulphide core domain proteins". Members of this family are involved in various aspects of mucosal immunity (Wilkinson *et al.,* 2011). *WDFC8* was also found highly overexpressed in the liver of laying hens (Gloux *et al.*, 2019) and has been shown to protect egg yolk precursors from proteolytic activities/inactivation in the plasma, on their way from the liver to the growing oocytes (Marie Bourin *et al.*, 2012). Moreover, *WFDC8* has been found as a unique protein in egg yolk of chicken (Farinazzo *et al.*, 2009). The avian-specific *CTSEAL* gene was found to be overexpressed in liver of laying hens and the translated protein shares large sequence similarity with cathepsin D, suggesting *CTSEAL* as an accessory of cathepsin D in the processing of egg yolk precursors (Bourin *et al.*, 2012; Gloux *et al.*, 2019). *ZP1* gene was also up-regulated in the liver of hens, where the protein is synthesized and then transported to the ovary to be inserted in the perivitelline membrane surrounding the oocyte (Gloux *et al.*, 2019). Here, it plays a fundamental role in the first interactions between spermatozoa and the oocyte (Bausek *et al.*, 2004). These three genes were differentially expressed in liver of BP and RM birds, with an explanation being that these are multi-purpose breeds and are thus more suitable chickens for egg production than the ROSS broilers. Table 1 and Figure 8 also show other genes not known to be involved in a particular pathway or showing a functional relationship with others. An example is chitinase (*CHIA*), which is a gene coding for a major protease-resistant glycosidase with a physiological role as a digestive enzyme that breaks down chitin-

containing organisms in the chicken gastrointestinal tract (Tabata *et al.*, 2018). Another example is Doublecortin (*DCX*) – a member of a family of microtubule-associated proteins that are required for neuronal migration during cortical development (Vermillion *et al.*, 2014). Tumor Necrosis Factor Receptor Superfamily, Member 14 (*TNFRSF14*) was also found differentially expressed. It mediates apoptosis signalling, and can allow cancer cells to escape the immune process (Guo *et al.*, 2020).

The remainder of genes found differentially expressed in females of local breeds (compared to ROSS), are involved in FA metabolism. The *ABHD12B* and *ABHD5* genes are part of the α/β hydrolase domain-containing family appointed to mobilization of lipids. In particular, *ABHD12B* has been highlighted by Li et al (2020) as a hydrolyser of very long chain lysophosphatidylserine lipids in human cells (Li *et al.*, 2021). *ABHD5* the most well-characterized gene of its family codes for a protein cofactor of the ATGL enzyme and stimulates triacylglycerol hydrolase activity. Ouyang et al (2016) reported that overexpression of *ABDH5* markedly decreased the triglyceride content of preadipocytes in chicken (Ouyang *et al.*, 2016). This mechanism is modulated via the two above-mentioned genes and might explain the question of why native breed hens raised in free-range systems have less fat but higher polyunsaturated fatty acids in their meat muscles (Sokołowicz *et al.*, 2016). We discovered other genes differentially expressed in local female breeds belonging to the cytochrome P450 family were also highlighted: *CYP2C45* and *RP11-400G3.5*. Cytochrome P-450 2C45 (*CYP2C45*) was the most highly expressed cytochrome P-450 isoform in chicken liver and is a unique isoform in avian species (Watanabe *et al.*, 2013). Moreover, Zhao *et al.* (2019) have shown that *CYP2C45* was overexpressed in liver of overfed geese, hence promoting hepatic steatosis. Eventually, this resulted in the up-regulation of some genes involved in FA metabolism (i.e. *PK* and *ALOX5*) acting via *PPAR* pathways (Zhao *et al.*, 2019). *RP11-400G3.5* is reported as a *CYP2C21*-like pseudogene and is the closest gene to *CYP2C45* on chromosome 6 (Watanabe *et al.*, 2013). In fact, both of these genes showed similar function, especially in arachidonic and linolenic acid metabolism in KEGG pathways (data not shown). Furthermore, in local breeds we found significantly more expression in two genes having a role in de novo triglyceride biosynthesis (*MTTPL* and *GPAM*), two genes involved in *de novo* FA biosynthesis (*ACACA* and

SCD), confirmedly involved in desaturation of FA in comparison to ROSS birds. All those genes have been seen to be involved in FA or lipid metabolism (Figure S3).

In Table 1 we report the microsomal triglyceride transfer protein-like (*MTTPL*) gene located on chromosome 6, which is an orthologue of *MTTP* on chromosome 4. *MTTP* is responsible for the assembly and subsequent secretion of very low density lipoproteins from hepatocytes and controls the incorporation of triglycerides into apolipoprotein B (Liu *et al.*, 2016). Moreover, *MTTPL* was found overexpressed in liver of chickens fed with a diet rich in LC-PUFA precursor (Liu *et al.*, 2019; Tesseraud *et al.*, 2014). In the present study, *MTTPL* was differentially expressed in the liver of local breeds, underlining their capacity for FA metabolism. This was not the first time that genetic background has been shown to be a factor in liver expression of *MTTPL*: in Hérault 2010 it is differentially expressed in Muscovy duck with respect to Pekin duck fed *ad libitum* (Hérault *et al.*, 2010). Another gene involved in triglyceride metabolism is Glycerol-3-phosphate acyltransferase (*GPAM*), not shown in Table 1, but found differentially expressed in 3 of the 4 comparisons depicted in Figure 7 (Appendix S1). In fact, *GPAM* was found significantly up-regulated in RM_F_Ct, BP_F_Ct and BP_F_L groups, and plays a central role in *de novo* lipogenesis, particularly of triglycerides (Figure 8) (Claire D'Andre *et al.*, 2013). It was observed that *THRSP* (alias *SPOT14*) was discovered to have a reported role in *de novo* lipogenesis. Indeed, the *THRSP* expression level in liver is correlated with its ability to synthesize lipids (Desert *et al.*, 2018). Furthermore, *SPOT14* has been shown to be a direct target of the key lipogenic *SREBF1* transcription factor (Wu *et al.*, 2013) with its expression levels being under control of estrogens in chicken (Ren *et al.*, 2017). However, the biochemical mechanism linking *SPOT14* to *de novo* lipogenesis remains unclear. Another gene regulated by *SREBF1* is acetyl CoA carboxylase (*ACACA*) also identified in this study, it is critically important for the synthesis of long chain fatty acids (Resnyk *et al.*, 2017). The *ACACA* gene encodes for an enzyme which catalyses the conversion of acetyl-CoA to malonyl- CoA, the substrate of the *de novo* lipogenesis (Nematbakhsh *et al.*, 2021). We also identified stearoyl-CoA desaturase (*SCD*) that plays an important role in biosynthesis of LC-PUFA via the PPAR signalling pathway. Along with *ACACA*, *SCD* could be regulated by *SREBF1*, with both genes being involved in FA *de novo* biosynthesis (Resnyk *et al.*, 2017). *SCD,* thanks to its Delta-9 desaturase activity, can

convert palmitic acid (C16:0) and stearic acid (C18:0) to palmitoleic (C16:1 n-7) and oleic acid (C18:1n-9), respectively. *SCD* is recognized as a gene responsible for FA metabolism and having a significant role in intramuscular fat deposition. The third gene regulated by *SREBF1* is not presented in Table 1, but was significant in the comparison between BP_F_Ct and ROSS birds. This is the *FASN* gene and it is involved in *de novo* FA biosynthesis along with *ACACA* and *SCD* (Nematbakhsh *et al.*, 2021).

In conclusion, we provide a comparison of differential gene expression in two native dual-purpose slow-growing chickens, namely RM and BP, compared to commercial ROSS, fed a diet high in n-3 PUFA and assert that high levels of PUFA precursors in the diet do not result in significant changes in expression of genes involved in FA metabolism, whichever the genetic background or sex studied. The diet had an effect on phenotype as body weight, especially in ROSS birds which were significantly lighter in the L diet. On the contrary, it is clear that sex is an important factor in FA processes. Indeed, females differentially express elongases and desaturase genes (*ELOVL2*, *FADS1*, *FADS2,* and *SCD*) with a central role in LC-PUFA production, particularly in the BP breed. The BP breed is also suggested to be the genetic background with better capacity for modulation of expression of genes involved in FA metabolism. The two local breeds compared with ROSS commercial birds, have shown a significantly higher modulation of gene expression in liver, which underlines a greater aptitude of local breeds in FA metabolism and final LC-PUFA production. Finally, this study has shed light on the capability of local chicken breeds to modulate the expression of some of the genes involved in FA metabolism, as well as the gene expression in females compared to males. Furthermore, the local breeds had the ability to adapt easily to a different diet without change in final weight, enriching the tissue (liver in this case) with n-3, hence having the potential for the establishment of high-quality products.

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DATA AVAILABILITY STATEMENT

RNAseq raw data are available in NCBI (BioProject ID PRJNA865899).

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TABLES

Table 1: Differentially expressed genes shared by BP and RM female groups when compared to ROSS birds. Genes are related to Figure 7 and 8 and Appendix S6.

Gene ID	Gene name	Functions	Reference
ABHD12B	*Abhydrolase Domain-Containing Protein 12B*	Hydrolyzation of the very long chain lysophosphatidylserine lipids	(Z. Li *et al.*, 2021)
ABHD5	*Abhydrolase Domain Containing 5*	Crucial gene for fat mobilization and functions as a protein cofactor of ATGL	(Ouyang *et al.*, 2016)
ACACA	*Acetyl-CoA Carboxylase Alpha*	Catalysing carboxylation of acetyl-CoA to malonyl-CoA, which is the rate-limiting step in fatty-acid synthesis	(Pirany *et al.*, 2020)
CHIA	*Chitinase*	Chinase activity	(Tabata *et al.*, 2017)
CTSEAL	*Cathepsin E-A-like protein*	Process egg yolk proteins	(M. Bourin *et al.*, 2012)
CYP2C45	*Cytochrome P450 Family 2 Subfamily C Member 45*	Promotes hepatic steatosis by inducing glycolysis-related genes and PPAR pathway	(Zhao *et al.*, 2019)
RP11-400G3.5	*cytochrome P450 2C21-like (CYP2C21L)*	Unknown	(Watanabe *et al.*, 2013)
DCX	*Doublecortin*	Member of a family of microtubule-associated proteins that are required for neuronal migration during cortical development	(Vermillion *et al.*, 2014)
LOC101747680	*C-factor-like*	-	

LOC101749589	-	-	-
LOC107050519	-	-	-
LOC107053670	-	-	-
LOC107053691	-	-	-
LOC112532382	-	-	-
LOC112532439	-	-	-
LOC426220	*Avidin-related protein 6-like*	Correlated with egg yolk	(Gloux *et al.*, 2019)
MTTPL	*Microsomal triglyceride transfer protein-like*	Secretion and lipid metabolism	(Gloux *et al.*, 2019)
SCD	*Stearoyl-CoA desaturase*	Desaturation of palmitic and stearic acid to palmitoleic acid (C16:1) and oleic acid (C18:1), respectively	(Nematbakhsh *et al.*, 2021)
THRSP	*Thyroid hormone responsive*	Transcription factor involved in control of lipogenic enzymes	(Cui *et al.*, 2018)
TMEM30CP	*Transmemebrane protein 30C*	Unknown	-
TNFRSF14	*Tumor necrosis factor receptor superfamily, member 14*	Mediated apoptosis, leading the cancer cells to escape during the immune process	(Guo *et al.*, 2020)
WFDC8	*WAP four-disulphide core domain protein 8*	Correlated with egg yolk	(Gloux *et al.*, 2019)
ZP1	*Zona pellucida sperm-binding protein 1*	Female fertility	(Bausek *et al.*, 2004)

FIGURES

Figure 1. Principal component analysis (PCA) of all samples. Colours define different genetic background (BP = Bionda Piemontese; RM = Robusta Maculata; ROSS = Ross 308); shapes represent different diet (Ct = Control diet; L = Linseed diet). The two clusters represent the sex: males (M) in the smallest cluster on bottom right, and females (F) in top left.

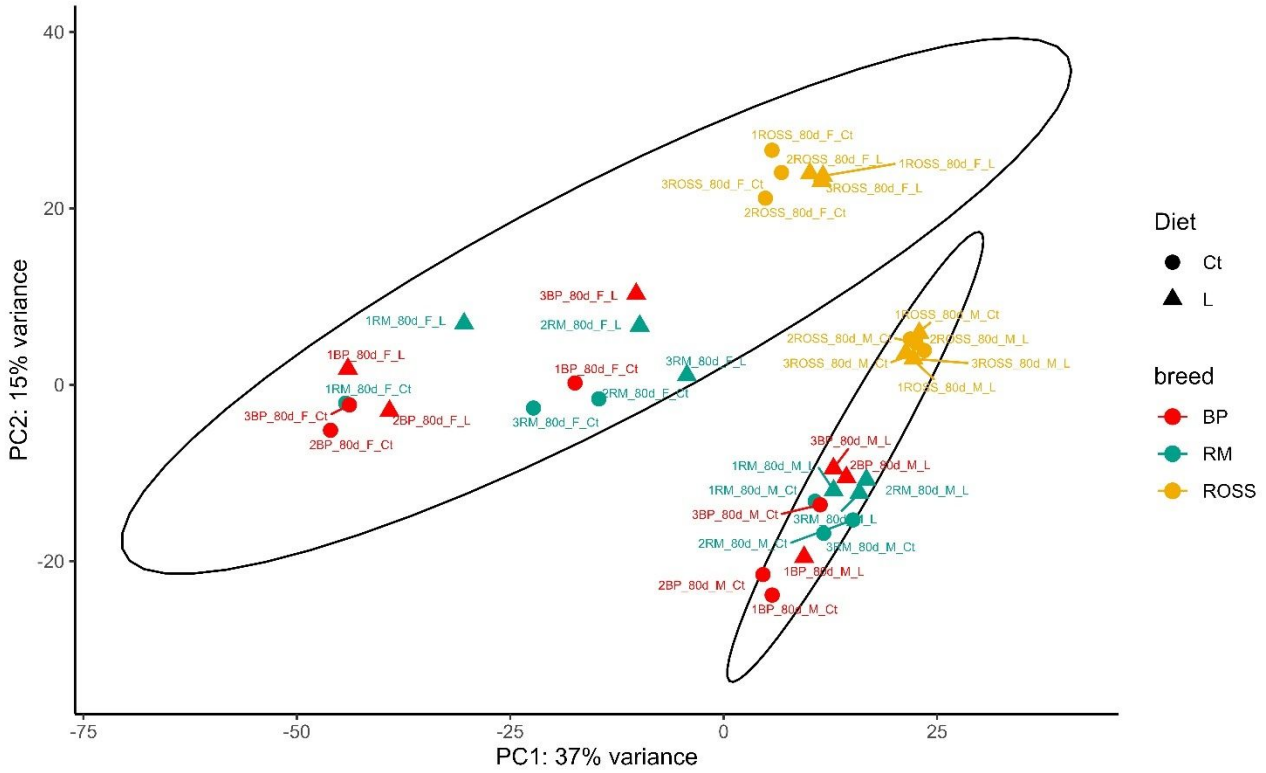

PC2: 15% variance
PC1: 37% variance
40
20
0
-20
-75
-50
-25
0
25
1ROSS_80d_F_Ct
2ROSS_80d_F_L
1ROSS_80d_F_L
3ROSS_80d_F_Ct
3ROSS_80d_F_L
2ROSS_80d_F_Ct
3BP_80d_F_L
1RM_80d_F_L
2RM_80d_F_L
1BP_80d_F_L
1BP_80d_F_Ct
3RM_80d_F_L
1RM_80d_F_Ct
2RM_80d_F_Ct
3BP_80d_F_Ct
2BP_80d_F_L
3RM_80d_F_Ct
2BP_80d_F_Ct
1ROSS_80d_M_Ct
2ROSS_80d_M_Ct
2ROSS_80d_M_L
3ROSS_80d_M_Ct
3ROSS_80d_M_L
1ROSS_80d_M_L
3BP_80d_M_L
1RM_80d_M_L
2BP_80d_M_L
1RM_80d_M_Ct
2RM_80d_M_L
3BP_80d_M_Ct
3RM_80d_M_L
2RM_80d_M_Ct
3RM_80d_M_Ct
1BP_80d_M_L
2BP_80d_M_Ct
1BP_80d_M_Ct
Diet
Ct
L
breed
BP
RM
ROSS

Figure 2. Effect of diet on the percentage of total n-6 (a), total n-3 (b) and total PUFA (c) in relation to total FA in liver tissue. All the comparisons were assessed by experimental groups (same genetic background and sex, but different diet). *P < 0.05, ***P < 0.001 (post hoc Tukey's test). The data are presented as mean ± standard error.

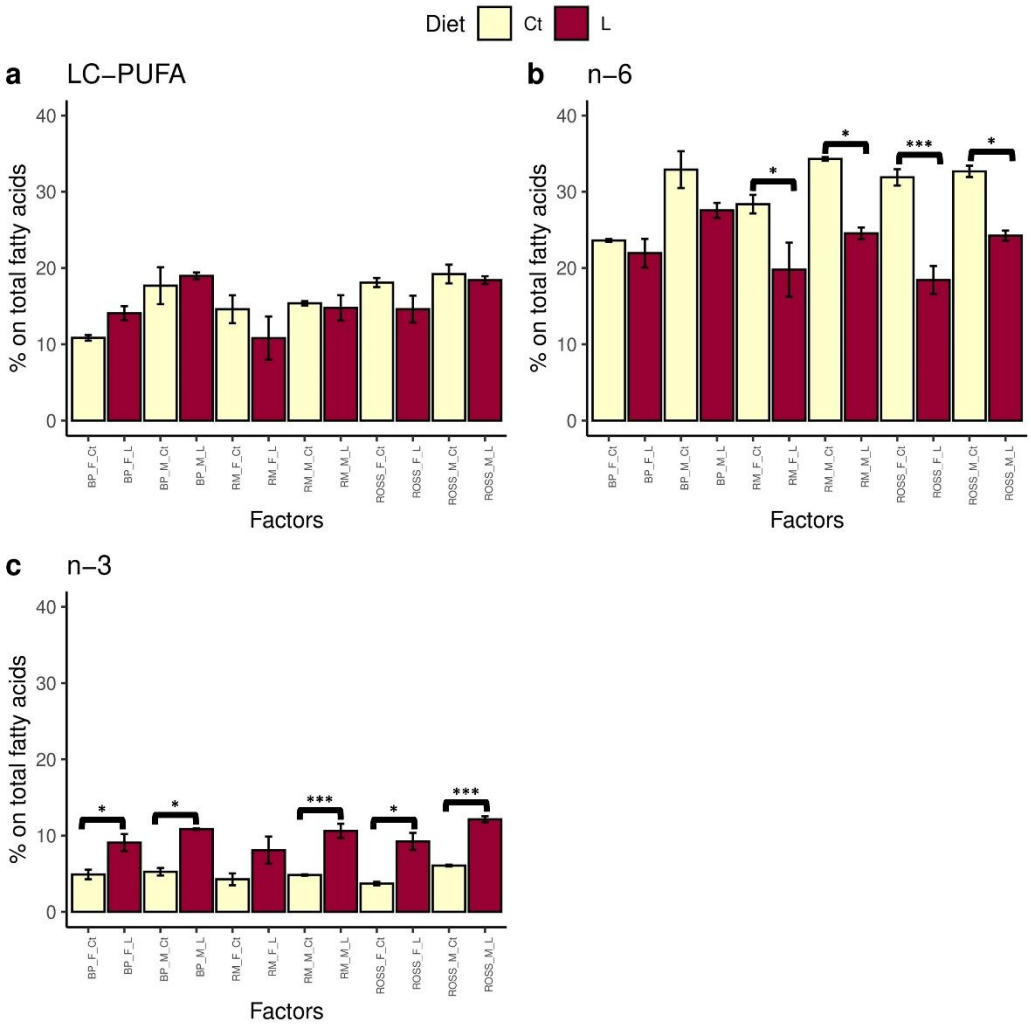


Figure 3. Volcano plot of differentially expressed genes (DEGs) between the Linseed diet (L) and Control diet (Ct) groups. Note: The red dots indicate genes with a P-adjusted value < 0.05 and -2> Log2 fold change >2. The red dots on the right quadrant of the figures are up-regulated in Linseed diet, the ones on the left are down-regulated. The blue dots represent the genes that reached the P-adjusted value < 0.05 but Log2 fold change value is higher than -2 and lower than 2. The green dots represent the genes whose difference in expression level between the Ct and L groups did not reach significance (padj > 0.05).

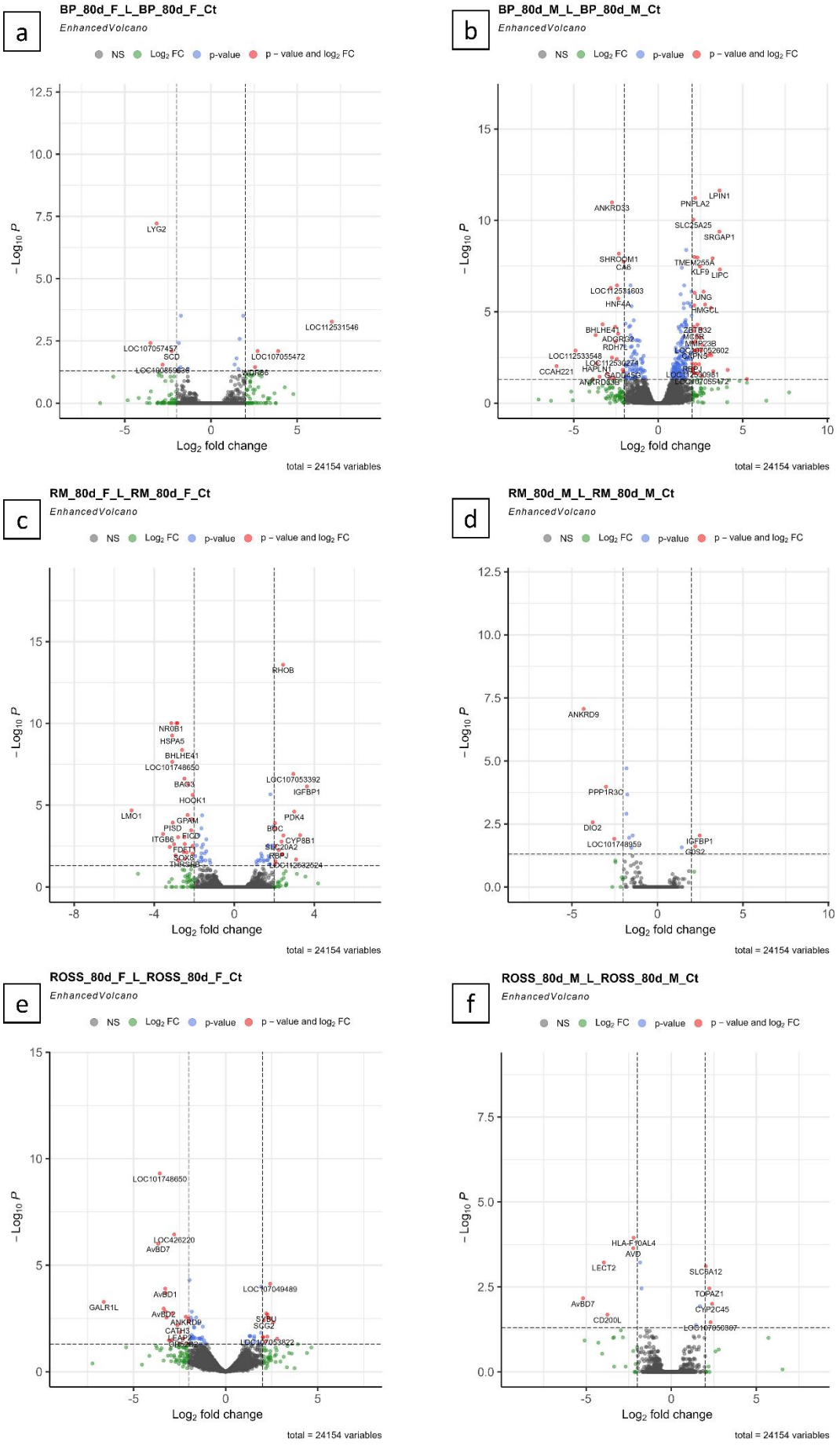

a
BP_80d_F_L_BP_80d_F_Ct
EnhancedVolcano
NS Log2 FC p-value p – value and log2 FC
LYG2
LOC112531546
LOC107057457
SCD
LOC107055472
LOC100859626
MDH2
− Log10 P
Log2 fold change
total = 24154 variables
b
BP_80d_M_L_BP_80d_M_Ct
EnhancedVolcano
NS Log2 FC p-value p – value and log2 FC
LPIN1
ANKRD33
PNPLA2
SLC25A25
SRGAP1
SHROOM1
CA6
TMEM255A
KLF9
LIPC
LOC112531603
HNF4A
LING
HMGCL
BHLHE41
ADGRB3
RDH7L
LOC112533548
CCAH221
HAPLN1
LOC112534224
SADL1
ANKRD33B
LOC107055472
− Log10 P
Log2 fold change
total = 24154 variables
c
RM_80d_F_L_RM_80d_F_Ct
EnhancedVolcano
NS Log2 FC p-value p – value and log2 FC
RHOB
NR0B1
HSPA5
BHLHE41
LOC101748650
BAG3
LOC107053392
IGFBP1
HOOK1
LMO1
GPAM
PISD
ITGB6
FICD
FDCT
SOX5
THNSL2
PDK4
BDC
CYP8B1
SLC20A2
RBPJ
LOC112532524
− Log10 P
Log2 fold change
total = 24154 variables
d
RM_80d_M_L_RM_80d_M_Ct
EnhancedVolcano
NS Log2 FC p-value p – value and log2 FC
ANKRD9
PPP1R3C
DIO2
LOC101748959
IGFBP1
GDS2
− Log10 P
Log2 fold change
total = 24154 variables
e
ROSS_80d_F_L_ROSS_80d_F_Ct
EnhancedVolcano
NS Log2 FC p-value p – value and log2 FC
LOC101748650
LOC428220
AvBD7
AvBD1
LOC107049489
GALR1L
AvBD2
ANKRD9
CATHS
LEAP2
SYBU
SGG2
LOC417053822
− Log10 P
Log2 fold change
total = 24154 variables
f
ROSS_80d_M_L_ROSS_80d_M_Ct
EnhancedVolcano
NS Log2 FC p-value p – value and log2 FC
HLA-F10AL4
AVD
LECT2
SLC5A12
TOPAZ1
AvBD7
CYP2C45
CD200L
LOC107050387
− Log10 P
Log2 fold change
total = 24154 variables

Figure 4. Volcano plot of differentially expressed genes (DEGs) between the Female (F) and Male (M) groups. Note: The red dots indicate genes with a P-adjusted value < 0.05 and -2> Log2 fold change >2. The red dots on the right quadrant of the figures are up-regulated for female, the ones on the left are down-regulated. The blue dots represent the genes that reached the P-adjusted value < 0.05 but Log2 fold change value is higher than -2 and lower than 2. The green dots represent the genes whose difference in expression level between the F and M groups did not reach significance (padj > 0.05).

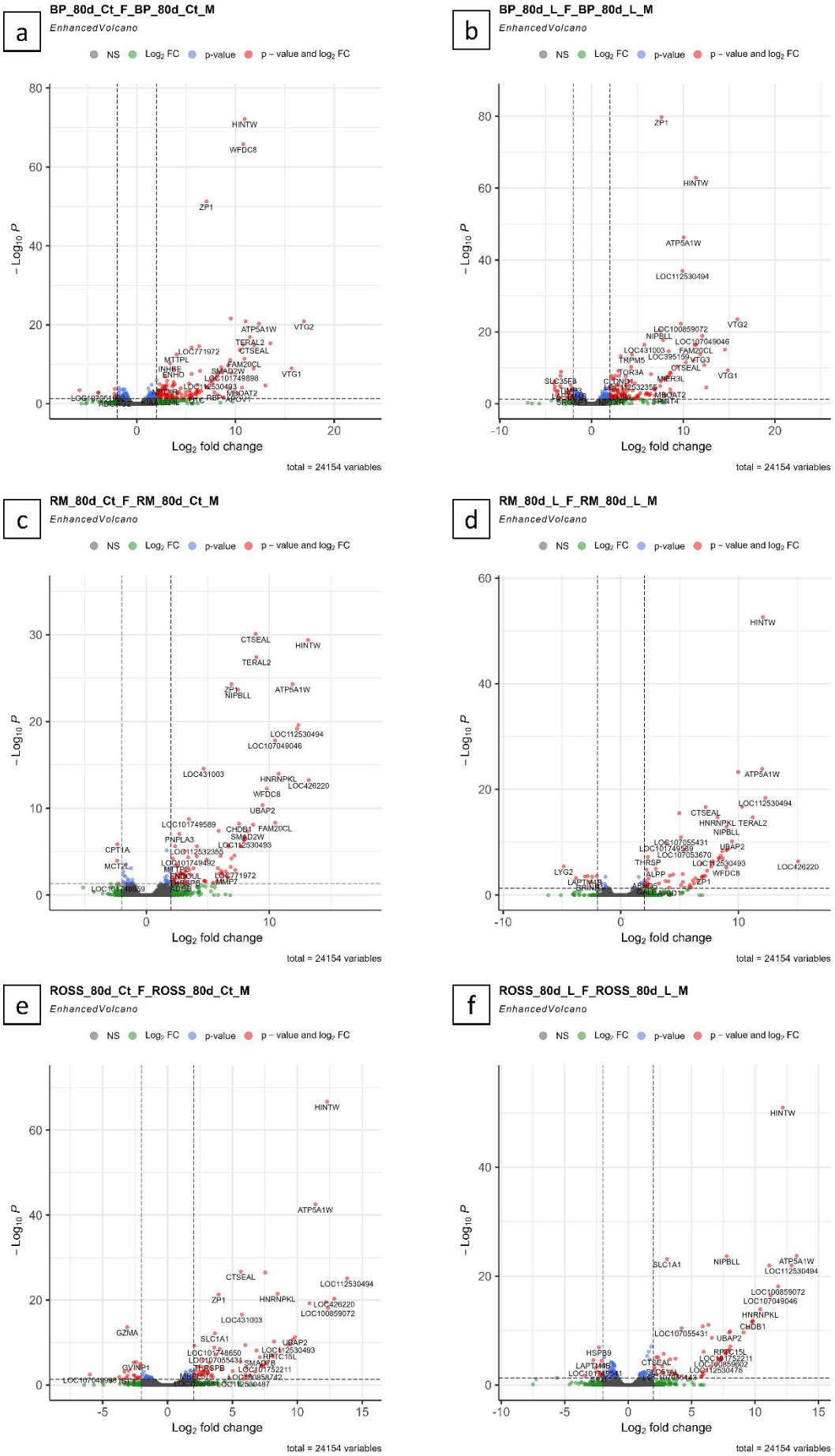

a
BP_80d_Ct_F_BP_80d_Ct_M
EnhancedVolcano
NS Log2 FC p-value p – value and log2 FC
– Log10 P
Log2 fold change
total = 24154 variables
b
BP_80d_L_F_BP_80d_L_M
EnhancedVolcano
NS Log2 FC p-value p – value and log2 FC
– Log10 P
Log2 fold change
total = 24154 variables
c
RM_80d_Ct_F_RM_80d_Ct_M
EnhancedVolcano
NS Log2 FC p-value p – value and log2 FC
– Log10 P
Log2 fold change
total = 24154 variables
d
RM_80d_L_F_RM_80d_L_M
EnhancedVolcano
NS Log2 FC p-value p – value and log2 FC
– Log10 P
Log2 fold change
total = 24154 variables
e
ROSS_80d_Ct_F_ROSS_80d_Ct_M
EnhancedVolcano
NS Log2 FC p-value p – value and log2 FC
– Log10 P
Log2 fold change
total = 24154 variables
f
ROSS_80d_L_F_ROSS_80d_L_M
EnhancedVolcano
NS Log2 FC p-value p – value and log2 FC
– Log10 P
Log2 fold change
total = 24154 variables

Figure 5. Volcano plot of differentially expressed genes (DEGs) between the Bionda Piemontese (BP) and Ross308 (ROSS) groups. Note: The red dots indicate genes with a P-adjusted value < 0.05 and -2> Log2 fold change >2. The red dots on the right quadrant of the figures are up-regulated in the Bionda Piemontese breed, the ones on the left are down-regulated. The blue dots represent the genes that reached the P-adjusted value < 0.05 but Log2 fold change value is higher than -2 and lower than 2. The green dots represent the genes whose difference in expression level between the BP and ROSS groups did not reach significance (padj > 0.05).

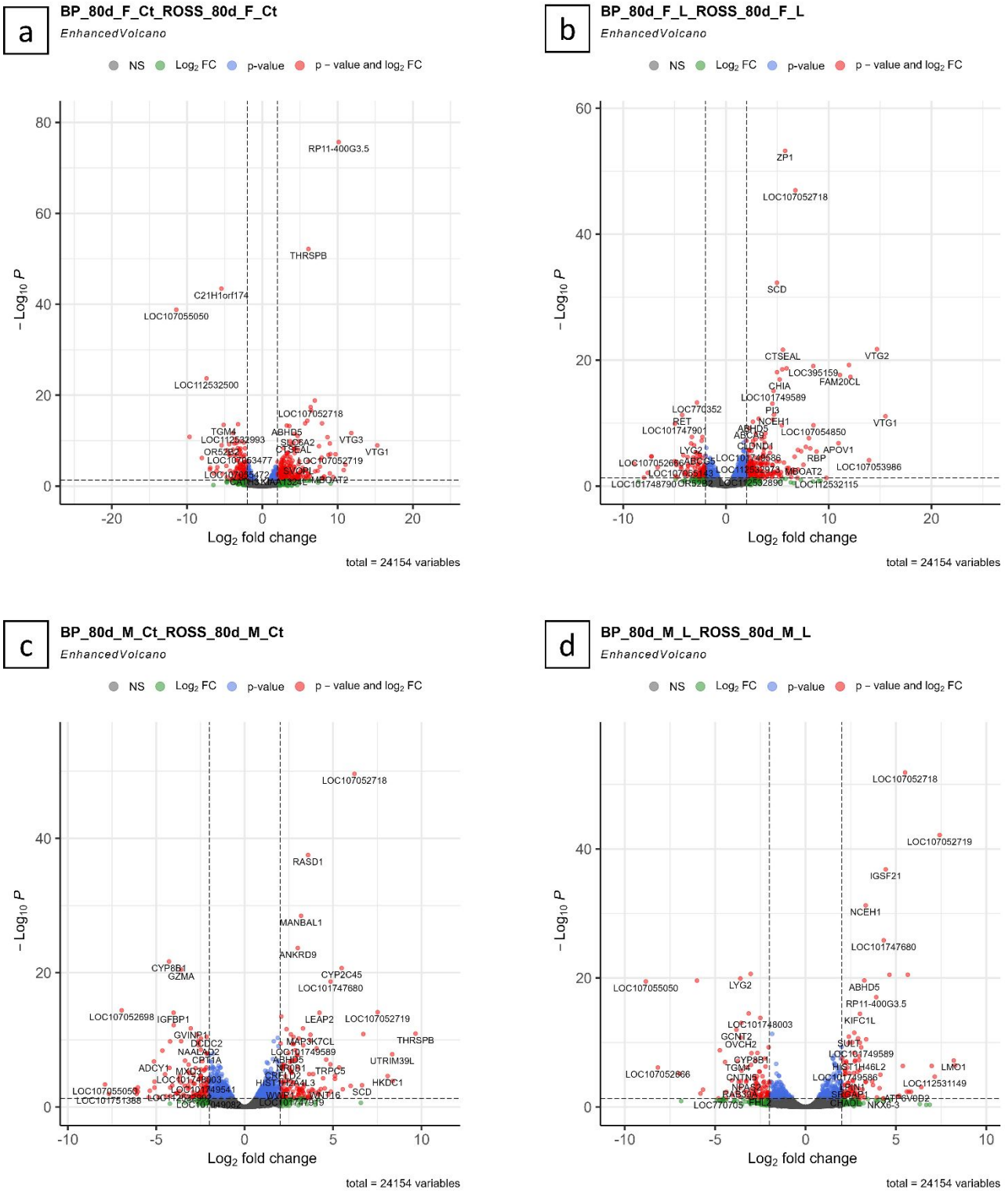


Figure 6. Volcano plot of differentially expressed genes (DEGs) between the Robusta Maculata (RM) and Ross308 (ROSS) groups. Note: The red dots indicate genes with a P-adjusted value < 0.05 and -2> Log2 fold change >2. The red dots on the right quadrant of the figures are up-regulated in the Robusta Maculata breed, the ones on the left are down-regulated. The blue dots represent the genes that reached the P-adjusted value < 0.05 but Log2 fold change value is higher than -2 and lower than 2. The green dots represent the genes whose difference in expression level between the RM and ROSS groups did not reach significance (padj > 0.05).

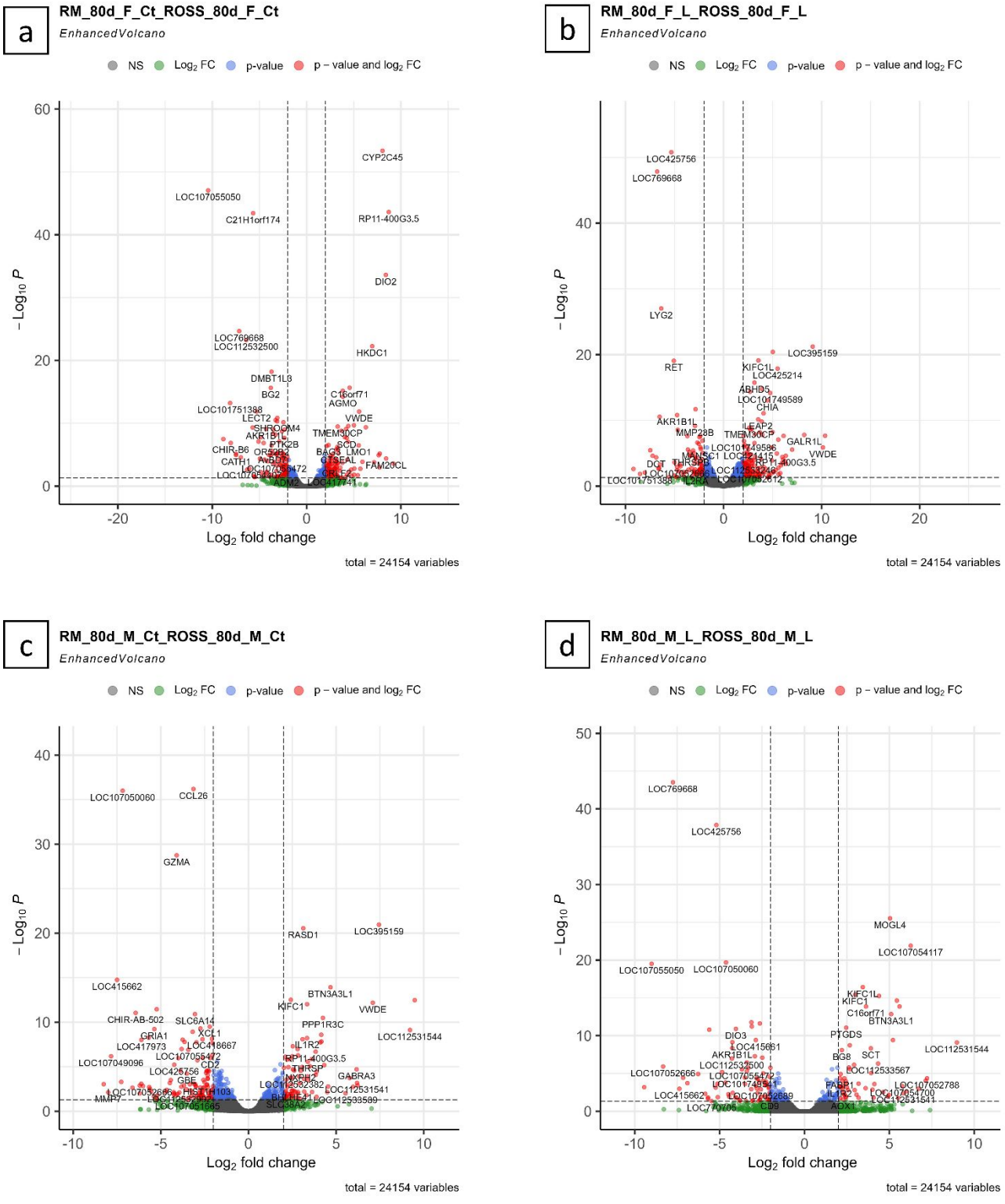


Figure 7. Venn diagram of up-regulated genes in Bionda Piemontese and Robusta Maculata birds compared to Ross308 (ROSS) chickens in female experimental groups.

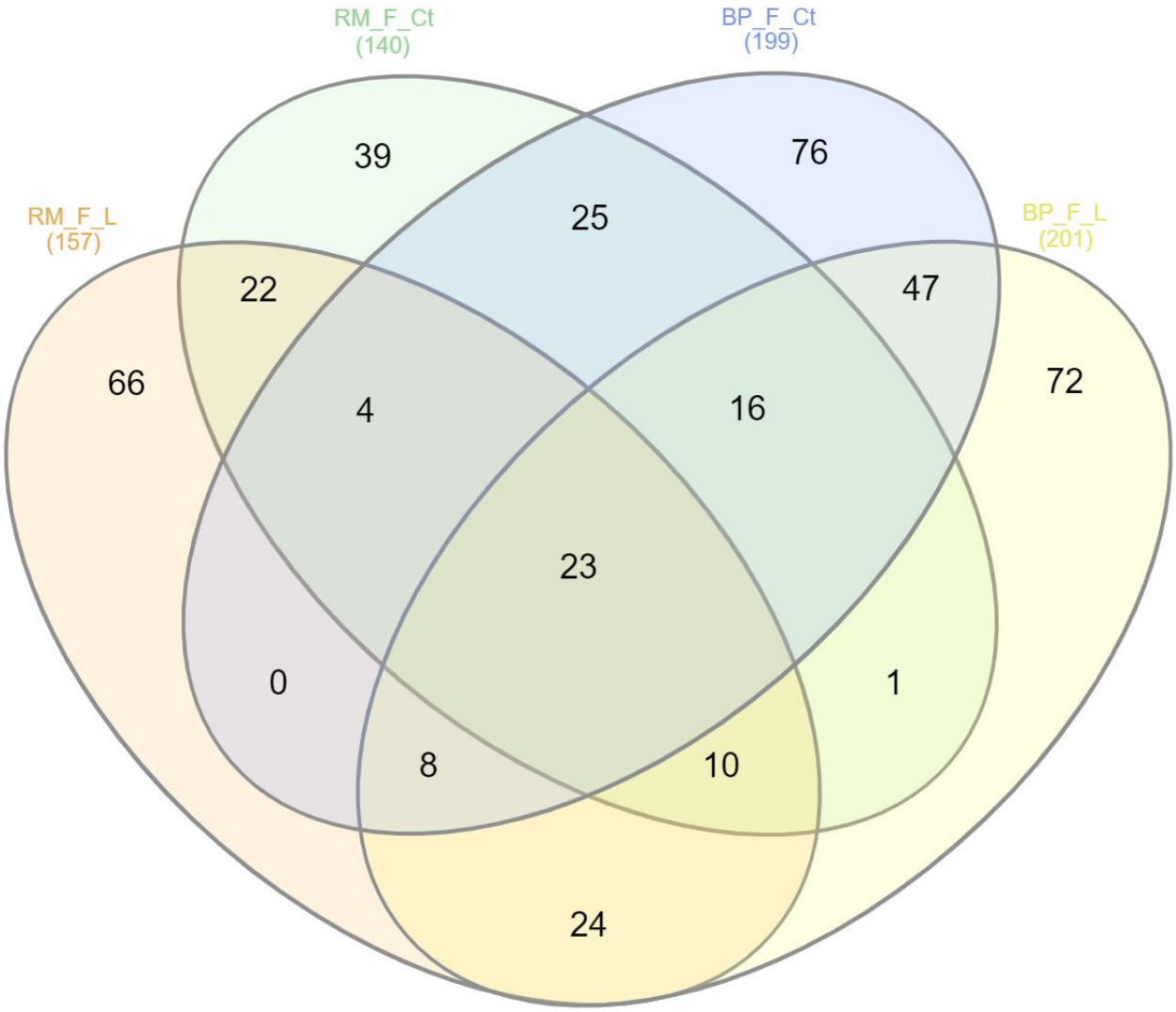
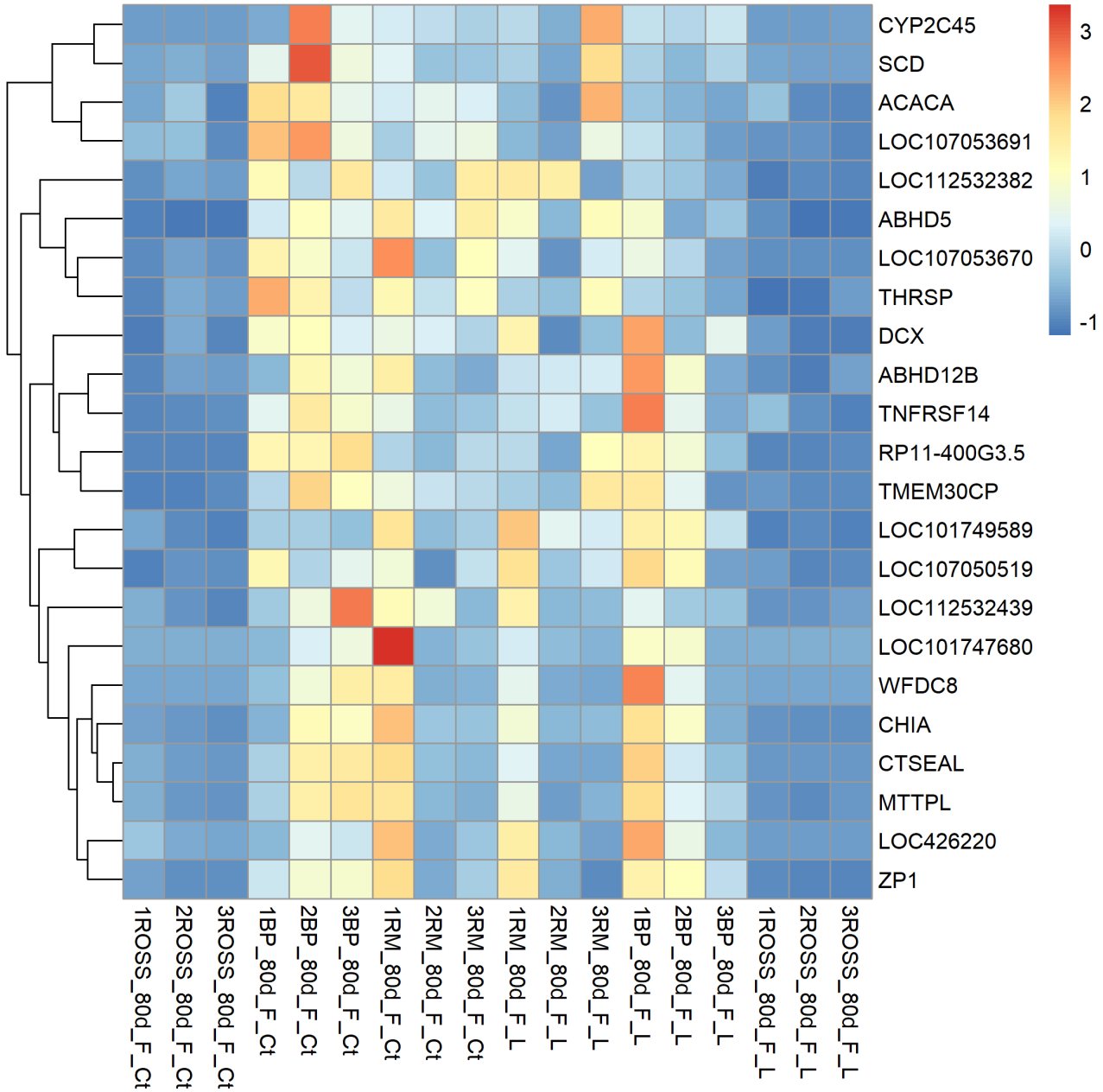


Figure 8. Heatmap showing the K-means clustering of transformed expression values for the 23 genes reported in common between female experimental groups in Figure 7. Red represents higher expression and blue represents lower expression.



SUPPLEMENTARY MATERIALS:

Table S1. Formulation and chemical analysis of chicken diet

Table S2. Outline of experimental design. Birds reared for each experimental group. In brackets the liver samples used for the RNA extraction.

Table S3. Summary of contrasts made in differential expression analysis divided by Diet, Sex, Genetic Background. In bold the groups used as experimental, with up and down regulated genes referred to them.

Table S4. Representation of all genes involved in fatty acids metabolism found as differentially expressed in all experimental groups and divided by the belonging contrast

Figure S1. Step by step bioinformatic procedures adopted in the present study.

Figure S2. Effect of diet (Ct = control, L = linseed) on Live Body Weight in g. ***P < 0.001 (post hoc Tukey's test).

Figure S3. GeneMANIA report of 23 genes (dashed circles) reported in common between female experimental groups in Figure 7. Non-dashed circles represent genes commonly co-expressed with the 23 belonging to our dataset. Legends explain biological function in which the genes are involved.

Appendix S1. DEGs of all the comparison. Six Diet comparisons (up and down regulated genes refer to the Linseed group), six Sex comparisons (up and down regulated genes refer to the female group), four BP *vs* ROSS comparisons (up and down regulated genes are referred to the BP group) and four RM *vs* ROSS comparisons (up and down regulated genes refer to the RM group),
In each comparison is reported just genes with a P-value > 0.05. All sheets report Gene ID; baseMean as the average of the normalized count values, dividing by size factors, taken over all samples; log2FoldChange indicates how much the gene or transcript's expression seems to have changed between the experimental and control groups. This value is reported on a logarithmic scale to base 2; lfcSE as the standard error estimate for the log2 fold change estimate; stat as the value of the test statistic for the gene or transcript; P-value of the test for the gene or transcript; and padj as Adjusted P-value for multiple testing for the gene or transcript.

Appendix S2. Gene Ontology for DEGs in Diet contrasts with significant values in Panther. Gene Ontologies refer to up-regulated genes in the Linseed diet group. The considered contrast for each gene ontology is reported in the title of each sheet. The GO tables are formed by *Gallus gallus - REFLIST* column that shows how many genes are involved in a given pathway. *Name of the contrast column* shows number of genes differentially expressed in the contrast belonging to a given pathway. *Over/under represented* column represents whether the pathway is enriched or down-regulated. *Fold enrichment* column explain how many times the pathway is enriched or down-regulated. *Raw p-value* and *FDR* (False discovery Rate) columns represents the statistical output of the Statistical overrepresentation test in Panther.

Appendix S3. Gene Ontology for DEGs in Sex contrasts with significant values in Panther and in IPA software. Gene Ontologies refer to up-regulated genes in the female sex group. The considered contrast for each gene ontology is reported in the title of each sheet. The GO tables are formed by *Gallus gallus - REFLIST* column that shows how many genes are involved in a given pathway. *Name of the contrast column* shows number of genes differentially expressed in the contrast belonging to a given pathway. *Over/under represented* column represents whether the pathway is enriched or down-regulated. *Fold enrichment* column explains how many times the pathway is enriched or down-regulated. *Raw p-value* and *FDR* (False discovery Rate) columns represents the statistical output of the Statistical overrepresentation test in Panther. IPA analysis used the Sex comparison for each group. Only pathways with a z-score > 2 (activated)

are reported. Titles of each sheet explain the contrast under study and the pathways detected as activated. Each IPA sheet reports ID and full name of genes involved in a given pathway, prediction of the behaviour of a given gene when the pathway is activated, and log2 Fold Change value for every gene in that contrast.

Appendix S4. Gene Ontology for DEGs in Genetic background contrasts (Bionda Piemontese *vs* Ross308) with significant values in Panther and IPA analyses. Gene Ontologies refer to up-regulated genes in the BP breed group. The considered contrast for each gene ontology is reported in the title of each sheet. The GO tables are formed by *Gallus gallus - REFLIST* column that shows how many genes are involved in a given pathway. *Name of the contrast column* showed number of genes differentially expressed in the contrast belonging to a given pathway. *Over/under represented* column represents whether the pathway is enriched or down-regulated. *Fold enrichment* column explain how many times the pathway is enriched or down-regulated. *Raw p-value* and *FDR* (False discovery Rate) columns represents the statistical output of the Statistical overrepresentation test in Panther. IPA analysis used the Breed comparison for each group. Only pathways with a z-score > 2 (activated) are reported.. Titles of each sheet explain the contrast under study and the pathways detected as activated. Each IPA sheet reports ID and full name of genes involved in a given pathway, prediction of the behaviour of a given gene when the pathway is activated, and log2 Fold Change value for every gene in that contrast.

Appendix S5. Gene Ontology for DEGs in Genetic background contrasts (Robusta Maculata *vs* Ross308) with significant values in Panther and in IPA analyses. Gene Ontologies refer to up-regulated genes in the BP breed group. The considered contrast for each gene ontology is reported in the title of each sheet. The GO tables are formed by *Gallus gallus - REFLIST* column that shows how many genes are involved in a given pathway. *Name of the contrast column* shows number of genes differentially expressed in the contrast belonging to a given pathway. *Over/under represented* column represents whether the pathway is enriched or down-regulated. *Fold enrichment* column explain how many times the pathway is enriched or down-regulated. *Raw p-value* and *FDR* (False discovery Rate) columns represents the statistical output of the Statistical overrepresentation test in Panther. IPA analysis used the Breed comparison for each group. Only pathways with a z-score > 2 (activated) are reported. Titles of each sheet explain the contrast under study and the pathways detected as activated. Each IPA sheet reports ID and full name of genes involved in a given pathway, prediction of the behaviour of a given gene when the pathway is activated, and log2 Fold Change value for every gene in that contrast.

Appendix S6. Gene lists of Venn diagram groups reported in Figure 7.

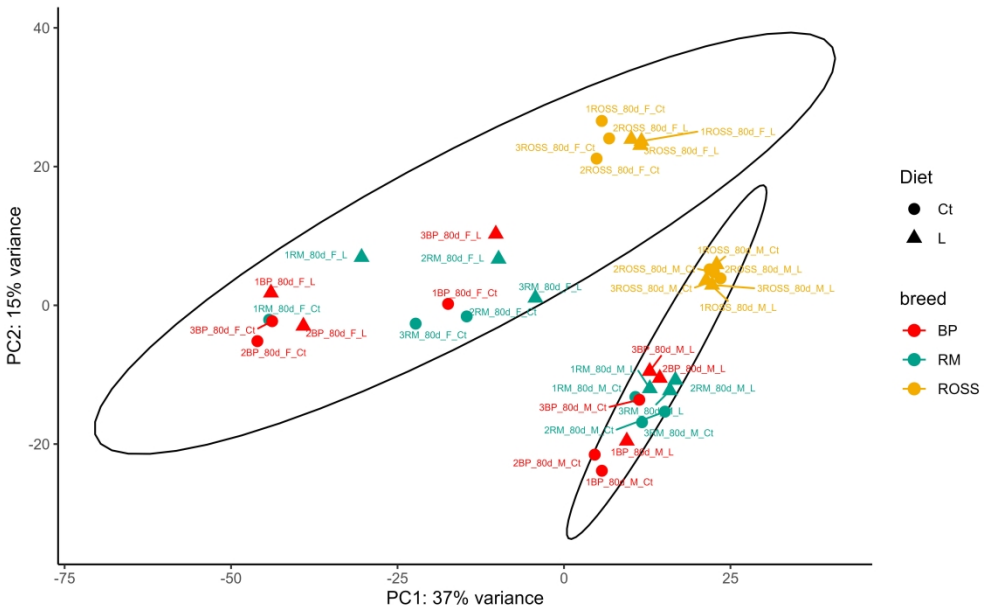


Figure 1. Principal component analysis (PCA) of all samples. Colours define different genetic background (BP = Bionda Piemontese; RM = Robusta Maculata; ROSS = Ross 308); shapes represent different diet (Ct = Control diet; L = Linseed diet). The two clusters represent the sex: males (M) in the smallest cluster on bottom right, and females (F) in top left.

2822x1746mm (72 x 72 DPI)

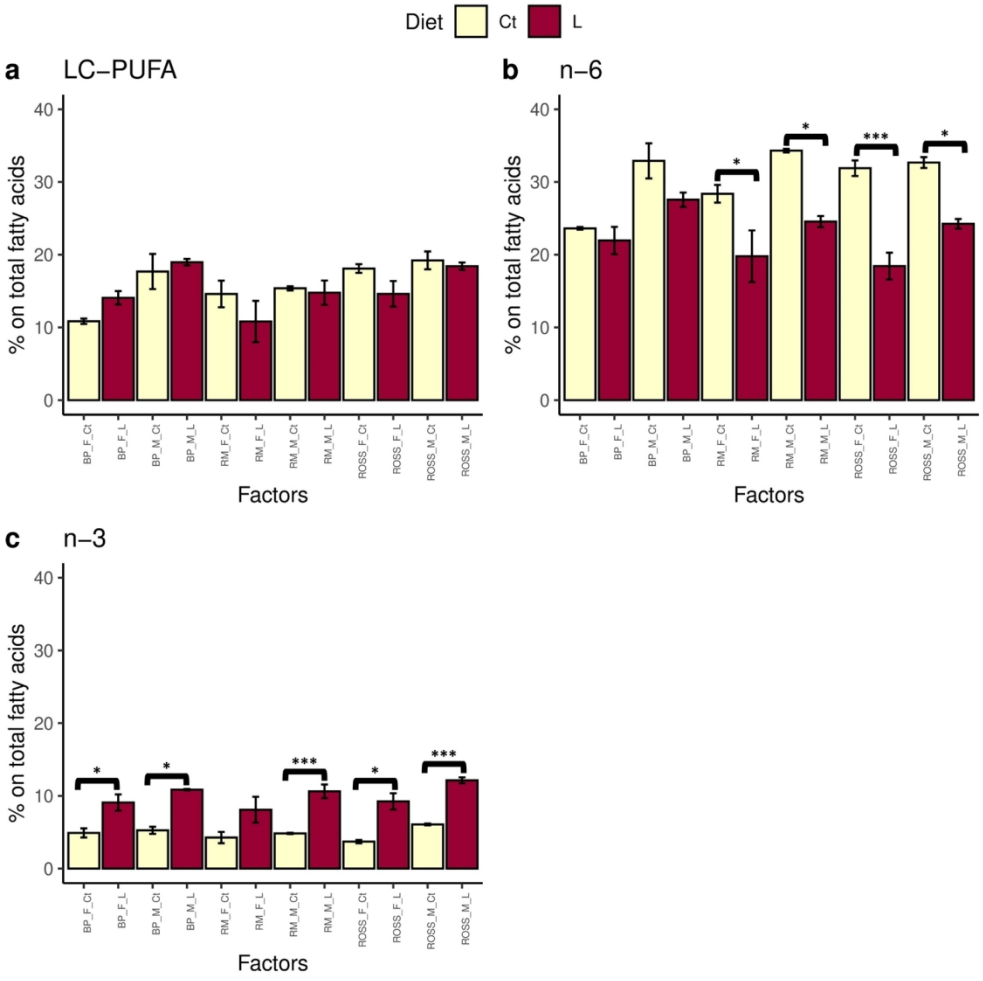


Figure 2. Effect of diet on the percentage of total n-6 (a), total n-3 (b) and total PUFA (c) in relation to total FA in liver tissue. All the comparisons were assessed by experimental groups (same genetic background and sex, but different diet). *P < 0.05, ***P < 0.001 (post hoc Tukey's test). The data are presented as mean ± standard error.

138x141mm (300 x 300 DPI)

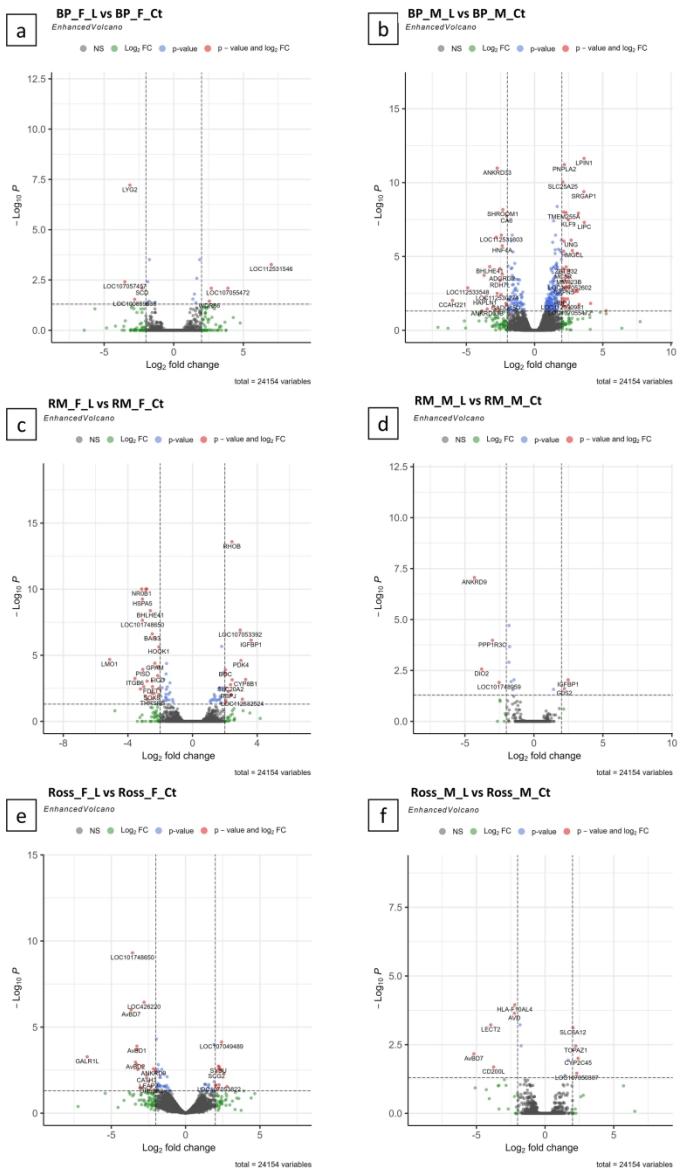


Figure 3. Volcano plot of differentially expressed genes (DEGs) between the Linseed diet (L) and Control diet (Ct) groups. Note: The red dots indicate genes with a P-adjusted value < 0.05 and -2> Log2 fold change >2. The red dots on the right quadrant of the figures are up-regulated in Linseed diet, the ones on the left are down-regulated. The blue dots represent the genes that reached the P-adjusted value < 0.05 but Log2 fold change value is higher than -2 and lower than 2. The green dots represent the genes whose difference in expression level between the Ct and L groups did not reach significance (padj > 0.05).

190x338mm (300 x 300 DPI)

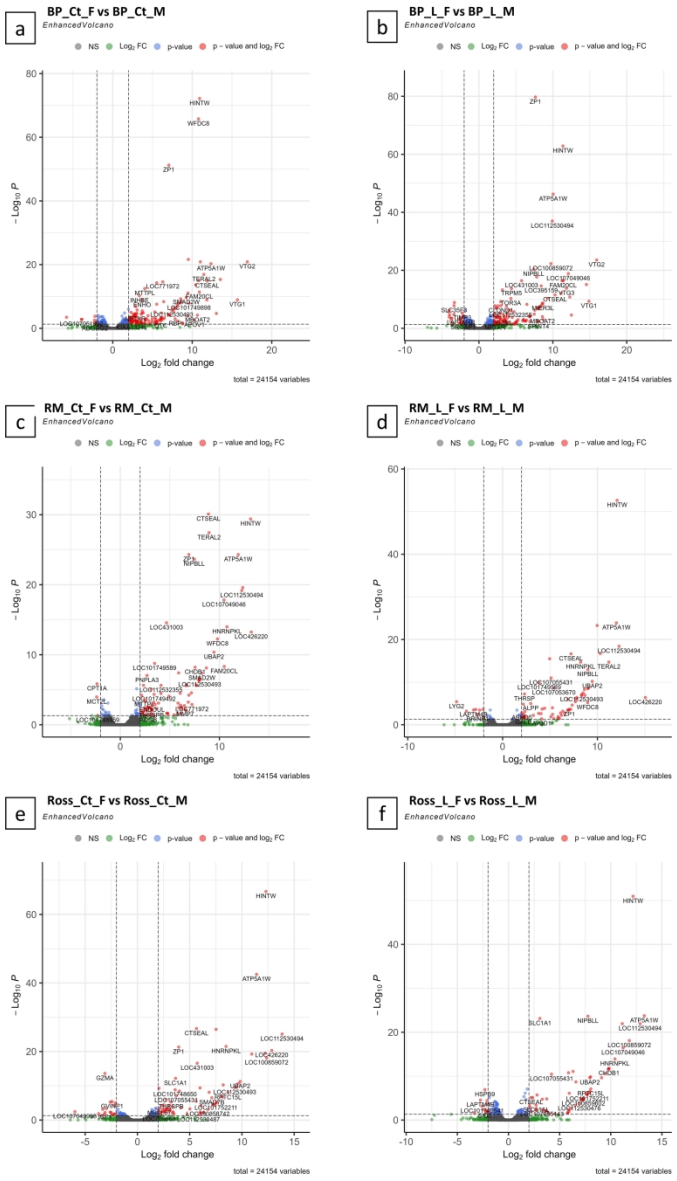


Figure 4. Volcano plot of differentially expressed genes (DEGs) between the Female (F) and Male (M) groups. Note: The red dots indicate genes with a P-adjusted value < 0.05 and -2> Log2 fold change >2. The red dots on the right quadrant of the figures are up-regulated for female, the ones on the left are down-regulated. The blue dots represent the genes that reached the P-adjusted value < 0.05 but Log2 fold change value is higher than -2 and lower than 2. The green dots represent the genes whose difference in expression level between the F and M groups did not reach significance (padj > 0.05).

190x338mm (300 x 300 DPI)

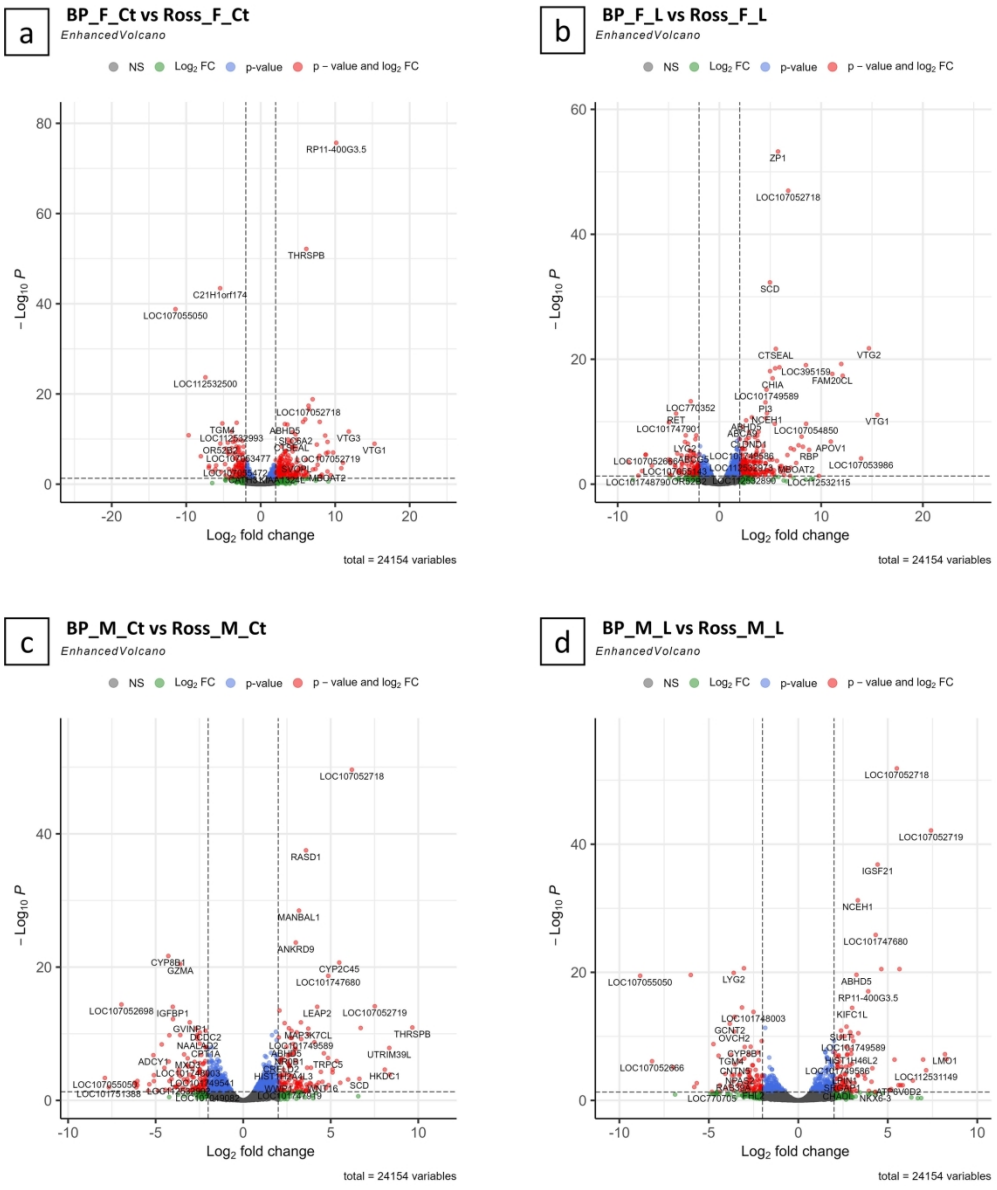


Figure 5. Volcano plot of differentially expressed genes (DEGs) between the Bionda Piemontese (BP) and Ross308 (ROSS) groups. Note: The red dots indicate genes with a P-adjusted value < 0.05 and -2> Log2 fold change >2. The red dots on the right quadrant of the figures are up-regulated in the Bionda Piemontese breed, the ones on the left are down-regulated. The blue dots represent the genes that reached the P-adjusted value < 0.05 but Log2 fold change value is higher than -2 and lower than 2. The green dots represent the genes whose difference in expression level between the BP and ROSS groups did not reach significance (padj > 0.05).

190x228mm (300 x 300 DPI)

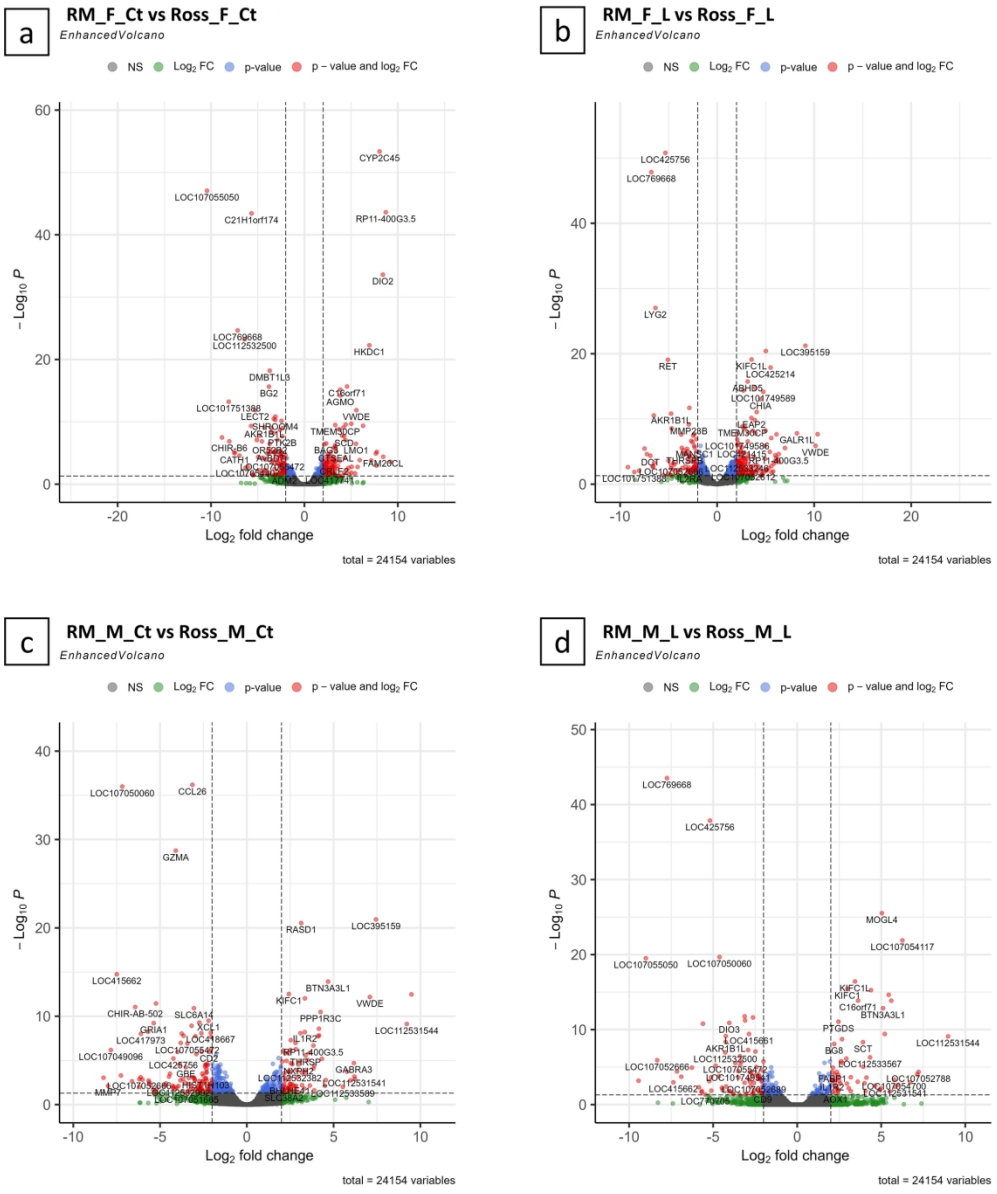


Figure 6. Volcano plot of differentially expressed genes (DEGs) between the Robusta Maculata (RM) and Ross308 (ROSS) groups. Note: The red dots indicate genes with a P-adjusted value < 0.05 and -2> Log2 fold change >2. The red dots on the right quadrant of the figures are up-regulated in the Robusta Maculata breed, the ones on the left are down-regulated. The blue dots represent the genes that reached the P-adjusted value < 0.05 but Log2 fold change value is higher than -2 and lower than 2. The green dots represent the genes whose difference in expression level between the RM and ROSS groups did not reach significance (padj > 0.05).

190x228mm (300 x 300 DPI)

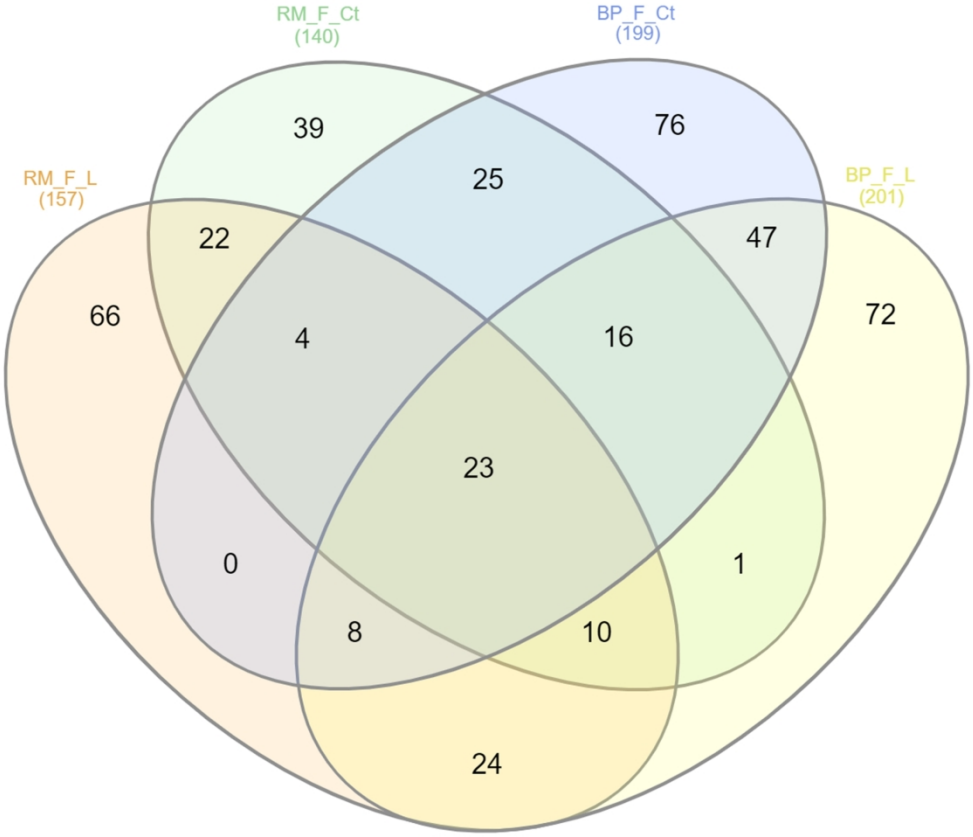


Figure 7. Venn diagram of up-regulated genes in Bionda Piemontese and Robusta Maculata birds compared to Ross308 (ROSS) chickens in female experimental groups.

240x248mm (300 x 300 DPI)

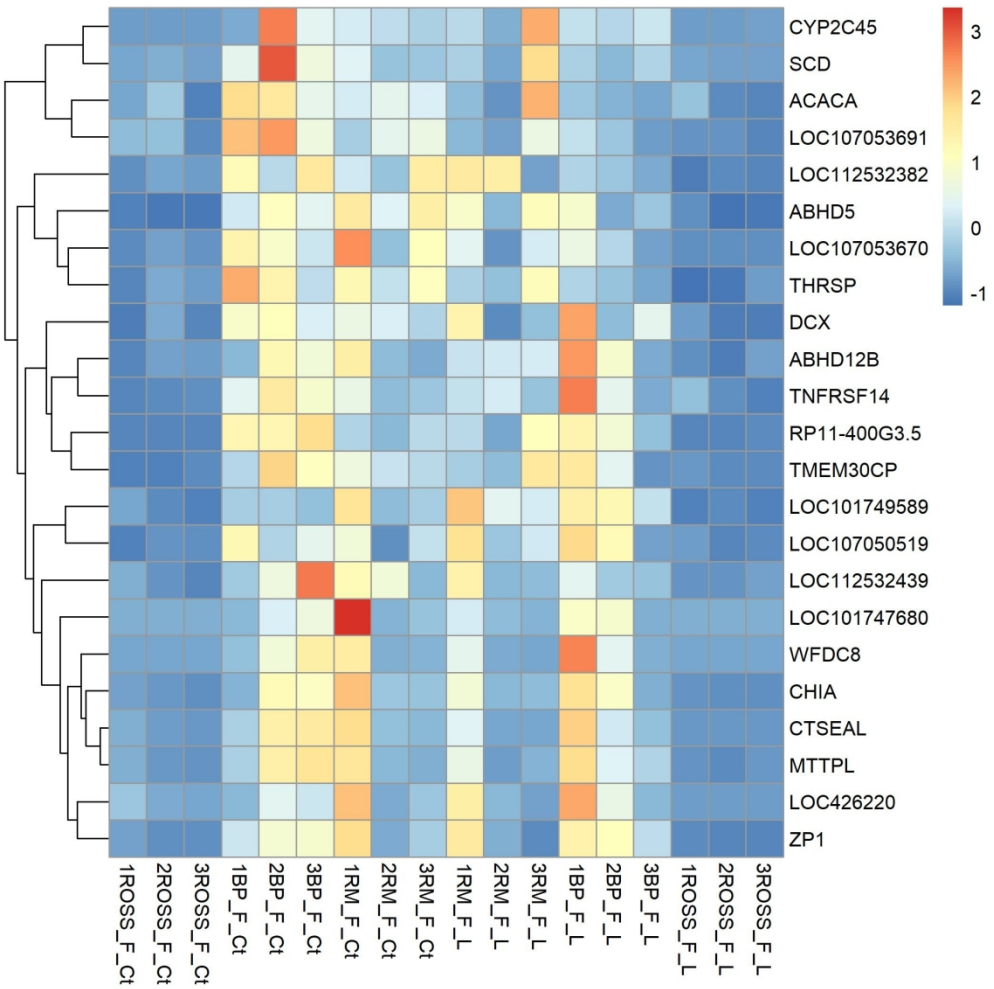


Figure 8. Heatmap showing the K-means clustering of transformed expression values for the 23 genes reported in common between female experimental groups in Figure 7. Red represents higher expression and blue represents lower expression.

177x177mm (300 x 300 DPI)

Table 1: Differentially expressed genes shared by BP and RM female groups when compared to ROSS birds. Genes are related to Figure 7, Figure 8 and Appendix S6.

Gene ID	Gene name	Functions	Reference
ABHD12B	*Abhydrolase Domain-Containing Protein 12B*	Hydrolyzation of the very long chain lysophosphatidylserine lipids	(Li *et al.*, 2021)
ABHD5	*Abhydrolase Domain Containing 5*	Crucial gene for fat mobilization and functions as a protein cofactor of ATGL	(Ouyang *et al.*, 2016)
ACACA	*Acetyl-CoA Carboxylase Alpha*	Catalysing carboxylation of acetyl-CoA to malonyl-CoA, which is the rate-limiting step in fatty-acid synthesis	(Pirany *et al.*, 2020)
CHIA	*Chitinase*	Chinase activity	(Tabata *et al.*, 2017)
CTSEAL	*Cathepsin E-A-like protein*	Process egg yolk proteins	(M. Bourin *et al.*, 2012)
CYP2C45	*Cytochrome P450 Family 2 Subfamily C Member 45*	Promotes hepatic steatosis by inducing glycolysis-related genes and PPAR pathway	(Zhao *et al.*, 2019)
RP11-400G3.5	*cytochrome P450 2C21-like (CYP2C21L)*	Unknown	(Watanabe *et al.*, 2013)
DCX	*Doublecortin*	Member of a family of microtubule-associated proteins that are required for neuronal migration during cortical development	(Vermillion *et al.*, 2014)
LOC101747680	*C-factor-like*	-	
LOC101749589	-	-	-
LOC107050519	-	-	-
LOC107053670	-	-	-
LOC107053691	-	-	-
LOC112532382	-	-	-
LOC112532439	-	-	-
LOC426220	*Avidin-related protein 6-like*	Correlated with egg yolk	(Gloux *et al.*, 2019)
MTTPL	*Microsomal triglyceride transfer protein-like*	Secretion and lipid metabolism	(Gloux *et al.*, 2019)
SCD	*Stearoyl-CoA desaturase*	Desaturation of palmitic and stearic acid to palmitoleic acid (C16:1) and oleic acid (C18:1), respectively	(Nematbakhsh *et al.*, 2021)
THRSP	*Thyroid hormone responsive*	Transcription factor involved in control of lipogenic enzymes	(Cui *et al.*, 2018)
TMEM30CP	*Transmemebrane protein 30C*	Unknown	-
TNFRSF14	*Tumor necrosis factor receptor superfamily, member 14*	Mediated apoptosis, leading the cancer cells to escape during the immune process	(Guo *et al.*, 2020)
WFDC8	*WAP four-disulphide core domain protein 8*	Correlated with egg yolk	(Gloux *et al.*, 2019)
ZP1	*Zona pellucida sperm-binding protein 1*	Female fertility	(Bausek *et al.*, 2004)

RNAseq reveals modulation of genes involved in fatty acid biosynthesis in chicken liver according to genetic background, sex and diet

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Keywords: fatty acid metabolism, transcriptome, poultry, local breeds, differentially expressed genes.

ABSTRACT

Increases in chicken production are mainly due to specialised breeds. However, of increasing importance are the local breeds, known for their ability to adapt to the environment and for their unique products. Conventional poultry products contain lower levels of n-3 fatty acids compared to those obtained from local breeds, therefore the aim of this study was to evaluate the modulation of expression of genes involved in LC-PUFA biosynthesis pathways according to genetic background, diet conditions, and sex. Animals from two local breeds and a commercial line were fed different diets: control and experimental diet (10% linseed supplementation). For each breed and diet group, both males and females were reared. The RNA was extracted from 36 liver samples and was sequenced by RNAseq method. Bioinformatic analysis was carried out to find differentially expressed genes (DEGs) from different comparisons between experimental groups. Results showed low impact of diet on DEGs related to fatty acid biosynthesis, but linseed diet increased percentage of n-3 fatty acids of liver. Sex , particularly the female groups, and genetic background determined the differential expression of genes related to LC-PUFA biosynthesis. Specifically, females of local breeds shared 23 up-regulated genes when compared to their respective commercial line groups. Some of the shared genes had a role in *de novo* triglyceride biosynthesis (*MTTPL* and *GPAM*), and in genes involved in *de novo* FA biosynthesis (*ACACA* and *SCD*) was detected. In conclusion, sex and local genetic background appear to have influence on the expression of genes related to LC-PUFA synthesis.

INTRODUCTION

To date, commercial chicken lines have dominated the poultry market so far, thanks to the fast growth and high production performances. Besides the standard commercial chicken lines, of increasing importance are the local breeds which are able to adapt to the environment where they live (Perini *et al.*, 2021), and for their different quality products compared to conventional ones (Franzoni *et al.*, 2021). Unfortunately, the productivity traits are critical for local breeds, which are instead appreciated for the diversity of their products, which could include economically exploitable traits (Moula *et al.,* 2010). Currently, it is still

difficult to obtain economic feedback from breeders who decide to raise local breeds because of low competitiveness on productive performances.

A potential valorisation strategy is the enrichment in terms of nutritional value of meat and eggs. Enhancing the quantity of n-3 fatty acids in chicken meat and eggs through the diet can have important impacts: i) supplementing the fatty acid intake in the human diet, as it is usually rich in n-6 and lacking in n-3 fatty acids (Mariamenatu & Abdu, 2021); ii) chickens are able to convert precursors of n-3 in long chain (> 20 carbon atoms) polyunsaturated fatty acids (LC-PUFA); iii) finally, eggs and meat enriched with n-3 would have increased market potential compared to standard animal products.

The LC-PUFAs, eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) are the main form of n-3. The fatty acid profiles of poultry meat and eggs can be readily enriched in EPA and DHA through the diet (Cartoni Mancinelli *et al.*, 2022). Although conventional poultry meat contains low levels of EPA and DHA, several factors such as sex, feed, and genetic background may influence their quantity. In particular, genetic background is reported in the literature as a crucial factor: higher concentrations of n-3 LC-PUFA are synthesized by local breeds compared to commercial lines (Cartoni Mancinelli *et al.*, 2021).

The application of a diet rich in precursors of n-3 (α-linolenic acid: 18:3n-3, ALA) can affect the amount of LC-PUFA in meat and eggs, and may change the expression level of genes involved in the process of lipid biosynthesis and elongation. In mammals, these two metabolic pathways, mainly occur in the adipose tissue, while in chicken the majority (90%) takes place in the liver (Nematbakhsh *et al.*, 2021). To elucidate the complete expression profile of liver genes, RNAseq provides a useful tool. This transcriptomic approach can accurately study tissue transcriptomes with high resolution and depth. Increasingly, the nutrigenomic approach is used to investigate the effect of diets on metabolic processes also in poultry (Soglia *et al.*, 2022). A recent study of the liver transcriptome after folic acid supplementation in the diet of broiler chickens found that peroxisome proliferator activated receptor (PPAR) signalling is the pathway most activated by the enriched diet (Zhang *et al.*, 2021).

In recent years, many studies tried to elucidate which genes are principally involved in the biosynthesis process of LC-PUFA. Studies focused on the evaluation of the presence/activity of elongase of very long

chain fatty acid (*ELOVL*) and fatty acid desaturase (*FADS*) enzymes (Cartoni Mancinelli *et al.*, 2022; Lee *et al.*, 2016). Although recently many studies have highlighted some pathways involved in the biosynthesis of LC-PUFAs (e.g. *PPAR* signaling), there remains a lack of scientific research in this area (Mihelic *et al.*, 2020). The aim of this study was to determine the modulation of expression of genes involved in LC-PUFA biosynthesis pathways, according to genetic background (local breed *vs* commercial line), diets, and sex.

MATERIALS AND METHODS

Animal ethics

Birds were raised, handled and processed according to the European legislation for the protection of chickens kept for meat production (European Commission, 2007), the protection of animals at the time of killing (European Commission, 2009) and the protection of animals used for scientific purposes (European Commission, 2010). The experimental protocol was positively evaluated and approved by the Ethical Committee of the University of Perugia (ID: 62700_15/07/2020).

Experimental design

The trial was carried out in the experimental section of the Department of Agricultural, Food and Environmental Sciences (University of Perugia, Italy). Specifically, three different chicken genetic backgrounds were used: Robusta Maculata (RM) and Bionda Piemontese (BP) as local breeds and Ross 308 (ROSS) as a commercial line. Both RM and BP are dual-purpose breeds, in particular BP shows higher aptitude for laying eggs (200 eggs/year *vs* 150 eggs/year of RM) (www.pollitaliani.it). One-day old chicks of each sex were housed in an environmentally-controlled poultry facility and vaccinated against coccidiosis, infectious bronchitis, Marek's Disease, Newcastle Disease and Gumboro. Until 21 days of age, all the chickens received the same starter diet. Then each genetic background was divided into two groups: one fed a standard diet (control group, Ct), the second fed the standard diet supplemented with 10 % extruded linseed (experimental group, L). The two diets were formulated to meet the nutritional recommendations of Ross 308 birds (Table S1) (Aviagen, 2019). Three replicates/group were formed, each representing both

male and female chickens (5 males + 5 females/each replicate). Chickens were raised in different indoor pens at the same stocking density (5 chickens/m^2) and the temperature was set according to the age of the birds (20-32°C; Relative Humidity: 65-72%). Water and feed were provided *ad libitum*. At 81 days of age, live body weight was recorded (g) for all the birds which were later slaughtered in a commercial slaughterhouse. From each replicate, two birds (one for each gender) were selected. Table S2 schematically explains the experimental design used in the present study.

Tissue collection and RNA extraction:

Liver tissue was collected from all the animals. Around 1 g of tissue was collected in a 2 mL cryogenic vial (Corning® Inc., Corning, NY), instantaneously frozen in liquid nitrogen, and stored at -80° C until RNA extraction. The remaining part of liver tissue was collected and stored at -20°C for further analysis of fatty acids (FA) profile.

At this stage, RNA was extracted only from liver tissue. Samples were prepared from 36 animals, with three biological replicates for each experimental group (Table S2). RNA isolation was performed with a NucleoSpin RNA Mini kit for RNA purification (Macherey-Nagel, Germany) as recommended by the manufacturer, starting from 30 mg of tissue. A homogenization step was carried out using an Omni Tissue Homogenizer (TH) - Omni, Inc) in ice. DNA contamination was removed with a DNase enzyme (included in the kit) during the incubation step. RNA concentration was measured with a Qubit 3.0 fluorometer (Life Technologies) and Qubit RNA HS Assay Kit (Life Technologies) according to manufacturer instruction. Total RNA (~1.5 µg/sample) was sent to Genewiz (South Plainfield, NJ, USA) for quality check, library preparation and Illumina sequencing. For testing RNA quality, the Agilent (Santa Clara, CA) 2100 Bioanalyzer Nano Kit was used, and all the samples showed RNA integrity (RIN) numbers ≥6.5 (data not shown). Library preparation was carried out through polyA + selection and paired-end (PE) sequencing was run on an Illumina NovaSeq System that generated 150 bp PE reads.

Bioinformatic analysis

RNAseq data were quality checked by FastQC software (Brown *et al.*, 2017). Paired qualified reads were mapped to the chicken reference genome (GRCg6a, accession number: PRJNA13342) using STAR software (Dobin *et al.,* 2015) with default parameters. The mapping rates for each sample were assessed using Samtools 'flagstat' command (Heng Li *et al.*, 2009). The mapped BAM file was then used in featureCounts software that, assigning raw alignments to annotated genes, generates the raw expression count file used for gene expression quantification (Liao *et al.*, 2014). An overview of genomic distance in the dataset was performed through Principal Component Analysis using the ggplot2 R package. Differential expression (DE) analysis was performed using the DESeq2 R package (Love *et al.*, 2014). The P-value adjustment was made using Benjamini and Hochberg's approach for controlling the False Discovery Rate (FDR). Genes with a P-adjusted value < 0.05 and -2> Log2 fold change (LFC) >2 were used as thresholds for significant DE by DESeq2. Visualization of contrast between different experimental groups was performed by EnhancedVolcano package in R (Blighe *et al.,* 2022). The overlap of significant genes among experimental groups was assessed with Venn diagrams built with the Interactivenn web tool (Heberle *et al.*, 2015). Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity) was used to identify gene ontology pathways, and regulatory networks to which DE genes belong, as well as upstream regulators. Ingenuity Pathway Analysis can transform a set of genes into a number of relevant networks based on comprehensive records maintained in the Ingenuity Pathways Knowledge Base. According to DEG input, IPA software calculates a z-score value for each biological pathway. When a pathway has been categorized with a z-score > 2, it resulted as an activated pathway. Moreover, to confirm the IPA results, gene ontologies (GO) were examined using the Panther (v17.0) database according to the Statistical over-representation test. Fisher's test was used to correct for False Discovery Rate (Figure S1) (Mi *et al.*, 2019). Differential expression (DE) analysis was performed in individual contrasts according to diet, sex and genetic background, separately. In particular we evaluated six diet contrasts, six contrasts for sex and four contrasts for each local breed against the ROSS birds (Table S3).

Fatty acids (FA) profile

The fatty acids profile was determined from the same samples used for the RNAseq. Lipids were extracted from 5 g of liver/sample based on the methods described in Folch *et al.* (1957). The fatty acids were identified in the form of their methyl esters using a Varian Gas Chromatograph (CP-3800) and a DB wax capillary column (25 mm ø, 30 m long). The establishment of each fatty acid was done in relation to the retention time with respect to fatty acid methyl ester standards (FAME, Sigma-Aldrich, Bellefonte, PA). The relative quantity of each fatty acid present in the liver was calculated using heneicosanoic acid (C21:0; Sigma-Aldrich) as the internal standard. Data were expressed as % of total FA. The average amount of each FA was used to calculate the sum of total PUFA of the n-3 and n-6 series. After using the function of the Shapiro test (for normality test), data were analysed with ANOVA and with Tukey's *post-hoc* test in R software. All results are expressed as mean ± SEM, with the level of significance set at $p < 0.05$.

RESULTS

Sequencing data analysis

Average mapping rate was 85.11% across all samples, 98% of which were seen to be properly paired. Among all the samples, the average quality score for each base was > Q30 and the average GC content in sequenced samples was around 52% (data not shown). After all quality checks, all 36 samples were taken forward for further analysis. In order to shed light on genetic similarity, a principal component analysis (PCA) plot was computed and visualized using R software. This showed genetic distance between the samples according to the comparison of Principal Component 1 and 2 (PC1 and PC2) (Figure 1). Genetic diversity was appreciable, especially between the two main clusters seen in Figure 1 representing male and female birds. The male cluster showed less genetic variance compared to the female one, and the ROSS birds grouped closest to each other within each cluster, because of their standard genetic background. On the contrary, the two local breeds showed more genetic variance within the experimental groups and were clearly separated from the ROSS cluster.

Body weight and fatty acids profile of animals

The effect of diet was evaluated according to the results from live body weight (Figure S2) and the percentage of n-3 and n-6 PUFA, and LC-PUFA (Figure 2). The diet had no statistical impact on the body weight at slaughtering of the two local breeds, either in males or females. Instead, the L diet significatively decreased the body weight in ROSS (male and female).

Figure 2 represent the n-6 and n-3 PUFA proportion in liver, and the sum of LC-PUFA. Figure 2a shows the percentage of LC-PUFA was not statistically affected by the diet. On the contrary, the n-6 PUFA (ranging from 18% to 34% approximately) showed higher level than n-3 PUFA (from 4% to 12). Moreover, the control diet generally increased the n-6 percentage in both sexes of ROSS and in RM_M. On the contrary, the L diet significantly enhanced the n-3 level in all groups, excepted for RM females.

Differentially expressed genes (DEGs)

Each experimental group was compared against one another to check differential gene expression. This was done on the basis of: diet, sex, and genotype (Table S3). In particular, we were interested in comparing local breeds to the broiler (ROSS) genotype. Appendix S1 lists all significantly differentially expressed genes in each comparison. Moreover, all genes related to FA metabolism, and which were found to be differentially expressed, are reported in Table S4.

Diet contrast

For the evaluation of the possible effect of diet on gene expression between sexes and genetic background, the linseed diet group was compared to the control diet group within each sex and within each genetic background. Experimental groups formed by “genetic background” and “sex” were taking in consideration individually. Linseed diet groups have been used as experimental groups, hence the up and down-regulated genes and the GO analyses referring to them. Figure 3 shows the volcano plots resulting from each comparison. Figures 2a-b showed the DEGs detected for the female (BP_F) and male (BP_M) Bionda Piemontese experimental groups respectively. In BP_F, the diet played a limited role in differentiating gene expression level, indeed Figure 3a had only eight differentially expressed genes with a *P*-value < 0.05, of

which some involved in fatty acid metabolism (e.g. *LYG2*, *SCD*), being found down-regulated with the L diet. Other important genes involved in FA synthesis (*ACACB*, *ACACA*, *FASN*) had a significant *P*-value but had lower LFC values (between -2 and 2) (Appendix S1).

In the BP_M group, the diet contrast showed 280 significant genes, and the Gene Ontology study confirmed that the most influenced pathways were cellular lipid metabolic process (GO:0044255) and lipid metabolic process (GO:0006629) (Appendix S2). Regarding the Robusta Maculata breed, volcano plots for DEGs in females (RM_F) and males (RM_M) are shown in Figures 2c-d respectively. Contrary to the BP birds, the RM_F comparison showed more DEGs than the RM_M group, namely 83 and 13 genes. Gene ontology analysis in each group showed that the RM_F group had biological processes which were significantly enriched in the L diet, although not directly related with FA biosynthesis, and males did not show anything significant (i.e. 'de novo' protein folding (GO:0006458)) (Appendix S2). Finally, the last genetic background evaluated for the effect of diet was the commercial ROSS hybrid (Figure 3e-f). In both female and male groups, a limited number of DEGs were identified (72 and 13, respectively). This is similar to the GO biological annotations associated with FA metabolism. None of the six diet contrasts resulted in a significantly activated biological pathway in IPA analysis.

Effect of sex

The effect of sex on gene expression was assessed as follows: the experimental groups compared within the same genetic background and diet groups but differing by sex. Female groups were taken as the experimental group, hence the up and down regulated genes and GO analysis are referred to “F” group in each contrast. Looking at Figure 4a, it is easy to appreciate the numerous DEGs up-regulated in females. For each BP comparison, as shown in Figures 4a-b, the GO annotations on up-regulated genes clarify the sexual dimorphism in the most typical way: cellular response to estrogen stimulus (GO:0071391) and response to estrogens (GO:0043627) (Appendix S3). When comparing the ROSS broiler birds, and examining the linseed diet group (L), significant biological enrichment was found in organo-nitrogen compound biosynthetic process (GO:1901566), for example (Appendix S3). With regard to FA pathways, in BP birds there was

activation of lipid pathways in females, namely lipid transport (GO:0006869) and lipid localization (GO:0010876). This result from Panther was also confirmed using IPA software. Fatty acid metabolism was seen to be significatively enhanced in the two female groups of the BP breed. The significance of fatty acid concentration pathway in RM chickens treated with linseed diet was also indicated (Appendix S3). In particular, the data in Appendix S3 shed light on the genes that were differentially expressed in females within the experimental group. The females expressed some genes strictly related to FA metabolism differentially to males within respective groups. For examples: *ELOVL2* in BP_Ct, *ELOVL2*, *FADS1* and *FADS2* in BP_L, and *SCD* in RM_L.

Genetic background comparison

One of the aims of this study was to assess the impact of breed and genetic makeup on the profile of LC-PUFA expression. In particular, the comparison between local breeds and the commercial line (same sex and diet) was examined.

Figure 5 represents the DEGs from the BP *vs* ROSS comparison. BP breed represented the experimental group, which up and down regulated genes and GO analysis are referred to. Figures 5a-b show females treated with control and linseed diet respectively. According to results from Panther, many pathways related to lipid metabolism were up-regulated in the BP breed, such as fatty acid biosynthetic process (GO:0006633) and long-chain fatty acid metabolic process (GO:0001676) (Appendix S4). In the female control diet group, the up-regulated genes found through IPA were related to FA metabolism: *SCD*, *CYP1A1*, *FASN*, *ACACA*, *THRSP*, *FAR1*, *FADS1* (Appendix S4). Figure 5b showed the results for a genetic background contrast with females fed the linseed diet. The results from GO analysis showed a large number of lipid related pathways, the most important being lipid metabolic process (GO:0006629). Some of the genes related to this, which were more highly expressed in BP relative to the commercial line include *FABP3*, *SCD*, *GPAM*, *LPIN1*, *SREBF2* and *ACACA* (Appendix S4). Meanwhile, Figures 5c-d exhibit the contrast of BP with ROSS birds, in males fed the two different diets. With the control diet, BP males showed higher expression of genes involved in fatty acid metabolic process (GO:0006631) in comparison with ROSS. BP males fed the

linseed diet do not show DEGs significant in FA metabolism (e.g. *FASN*, *ACACA*, *ACACB)* when compared with ROSS (Appendix S4), but amino acid biosynthetic processes are highlighted in the GO analysis.

We next wanted to compare Robusta Maculata (RM) *vs* Ross308 (ROSS) groups. The experimental groups consisted of the RM breed, hence up and down regulated genes and GO analysis are referred to as "RM" breed groups. The volcano plots are presented in Figure 6, of which the first two (Figure 6a-b) represented the contrast in females, with control and experimental diets, respectively. Figure 6a showed 391 genes with a P-value lower than 0.05, of which 234 genes had a value of -2 > LFC >2, and 121 genes in RM with LFC >2. The Panther GO analysis clearly showed that within the up-regulated genes, fatty acid metabolic process (GO:0006631) was clearly enhanced, with similar pathways activated in female linseed diet groups. IPA analysis showed significance of LC-PUFA synthesis in RM birds fed the control diet and synthesis of FA in RM birds fed the linseed diet (Appendix S5). The RM female groups shared DEGs clearly involved in FA pathways, such as *SCD*, *ACACA*, and *THRSP*. On the contrary, in RM males (Figures 6c-d), no pathways related to FA appeared as significantly involved in birds fed either diet. Instead, immune pathways were active in Ct diet birds, while there were no significant pathways in birds fed the supplemented diet (Appendix S5).

Comparison of local vs commercial females

According to the results from the sex comparison previously described, females, for the most part, appeared more specialized for FA production compared to males. Moreover, in the comparison of genetic backgrounds, all the female groups from local breeds showed higher activation of FA-related pathways compared to commercial hybrid birds. In order to understand which DEGs were common to the local breeds, a Venn diagram was generated (Figure 7). Here we took into consideration only the female birds, and only the genes differentially expressed in local breeds (BP and RM) compared to ROSS. Interestingly, 23 genes were commonly up-regulated among local breeds (Appendix S6). A heat map was generated to graphically represent the 23 shared genes and their expression level among all female samples (Table 1) (Figure 8). GeneMania software was used to identify any connection between them, with *GPAM* being

suggested as being directly related to *THRSP* and *SCD*. In fact, we saw *GPAM* as being up-regulated in both BP female groups and in RM females fed the control diet, but not the linseed diet (Figure S3).

DISCUSSION

Many studies have reported how the diet can modulate the expression of genes in different tissues (Sevane *et al.*, 2014; Szalai *et al.*, 2021). Here, we fed animals with a diet rich in the precursor of n-3 LC-PUFA. We designed the experiment assuming that diet plays a key role in changing lipid metabolism in the liver. Indeed, vitamin supplementation (Niu *et al.*, 2009) taurine (He *et al.*, 2019) and zinc oxide nanoparticles (Ramiah *et al.*, 2019) have been used to modulate gene expression of FA related genes, whereas the supplementation of dietary PUFA is widely used to increase LC-PUFA concentration in animal products of different animal species, such as dairy cows, rabbit and turkey (Castellini *et al.*, 2022; Kliem *et al.*, 2019; Szalai *et al.*, 2021). Chickens are usually an ideal target for diet supplementation, especially linseed based supplements (Head *et al.*, 2019; Jing *et al.*, 2013; Sevane *et al.*, 2014; Zhang *et al.*, 2021).

In the present study, the different diets affected the body weight of commercial birds. This result is explained by the fact that during the experimental trial, the ROSS chickens showed problems of adaptation to the linseed diet, consuming around 7% less quantity of the L diet with respect to the Ct diet, both in males and in females. In other studies was already reported how extruded linseed diet could lead to lower body weight gain and lower feed intake in broiler (Avazkhanloo *et al.,* 2020; Anjum *et al.*, 2013). This outcome indicated once again how the local breeds can easily adapt themselves to different environmental factor (e.g. alternative diet sources), while commercial lines encounter difficulties. In the present study, n-3, n-6 and LC-PUFA of liver were examined. LC-PUFA, which are directly related to the desaturation and elongation ability, was not affected by diet, conversely to n-6 and n-3 levels. The percentage of n-6 was generally higher in the Ct diet because the Ct diet is higher in linoleic acid (LA), which is the precursors of n-6 PUFA. On the other hand, the L diet positively affected the n-3 PUFA, and indeed the n-3 is significantly higher in the L diet than in the corresponding Ct groups (Figure 2). Both these results are commonly found in the literature (Hang *et al.*, 2018; Head *et al.*, 2019; Meineri *et al.*, 2018). Head *et al.* (2019) described the

modulation of FA profile in a given tissue through the diet, and also provided insights into low effect of the diet on the gene expression. The fact that the trend of LC-PUFA in the different experimental groups was similar confirms this assertion. Thus, it seems that the entity of LC-PUFA produced by liver is mainly due to the level of dietary precursors. On the same time, the type of precursor furnished α-linolenic (ALA, n-3) or linolenic (LA, n-6) determined an alternative accumulation of the same PUFA series in the liver. Indeed, we found that the dietary supplementation of linseed affected expression of genes involved in FA metabolism in only one group (BP_M group – Appendix S2). The most highly expressed genes in the linseed diet group included *PPARGC1A,* and *LPIN1*. *PPARGC1A* is a co-activator of PPARγ which is a transcription factor that participates in induction and stimulation of fat-specific genes and fatty acid bio-synthesis (Wang *et al.*, 2017). *LPIN1* is involved in synthesis and transport of triacylglycerol, a major constituent of chicken lipids (Desert *et al.*, 2018), and also found to be over expressed in a chicken line selected for intramuscular fat content together with *PPARγ* (Liu *et al.*, 2020). The remaining contrasts (n=5) for diet did not show enriched pathways, and for this reason, we can assert that diet is the factor with lowest impact on gene expression of fatty acid metabolism. Although no GO enrichment was discovered regarding FA metabolism in the other 5 diet contrasts, there are some interesting genes down-regulated with the L diet belonging to the BP_F and RM_F groups. Indeed, in BP_F the L diet showed down-regulation of *LYG2*, *ACACA*, *FASN*, *SCD* and in RM_F, down-regulation of *PLIN2* and *GPAM*, all important genes in FA metabolism and discussed later in this section. This is also in agreement with Head et al. (2019) who ascertained that the effect of linseed on the expression of 14 genes involved in FA metabolism in chicken liver was low. We therefore saw differing modulation of FA metabolism in liver between genetic backgrounds and sexes through diet.

This study also evaluated the effect of sexual dimorphism and its impact on FA metabolism. A clear separation between male and female birds is appreciable in Figure 1, underlying a divergent level of expression in genes between the two groups. The background of sex effect on FA metabolism was investigated by Poureslami *et al.* (2010b) where they described the poor effect of sex on final concentration of MUFA and PUFA of the n-3 and n-6 series (Poureslami *et al.,* 2010a). Elsewhere, Lopez-Ferrer *et al.* used dietary linseed oil, finding that abdominal fat percentage in male chickens was significantly lower than in

the female chickens (López-Ferrer *et al.*, 2001). This was corroborated in human where the differential ability to activate PPARα together with the well-documented effect of sex hormones on lipid metabolism may highlight sex as a significant factor in plasma FA levels (Thifault *et al.*, 2013). Differences between male and female chickens have never been thoroughly described and hence our interest in including sex effect in our analysis. BP_F, in both diet groups, showed a greater enrichment for estrogen pathways because it was the most egg-layer type chicken in the dataset. Indeed, estrogen in laying hens stimulates the liver to synthesize fatty acids for egg enrichment (Hanlon *et al.*, 2022). As reported by Ayres *et al.* (2013), differential expression of genes related to the female W chromosome are essential for sex determination in chickens. Some of them (*NIPBL* and *UBAP2*) have also been found to be central to sex determination in Japanese quail (Caetano-Anolles *et al.*, 2015). Regarding lipid metabolism, some genes were found to be more highly expressed in females with respect to males. This is the case for *ELOVL2* (in both BP contrasts), *FADS1*, *FADS2* (in BP_L) and *SCD* (in RM_L, and in both ROSS groups) that code for enzymes directly involved in desaturation and elongation of FA in the diet, such as ALA (18:3n-3), resulting in LC-PUFA (Head *et al.,* 2019). Interestingly, the results can be appreciated in the comparison of females with males in the BP breed. Besides the finding of elongases and desaturase-related genes being more highly expressed in females, other genes directly involved in FA metabolism were also identified. For instance, *APOB* plays a vital role in the assembly and secretion of triacylglycerol-rich lipoprotein in the liver of egg-laying chickens (Ma *et al.,* 2017), in line with the BP breed having a major aptitude in laying, especially when compared to ROSS (meat type) and RM birds (dual-purpose). Moreover, in the BP_L group, the female highly expressed genes of the fatty acid-binding protein (*FABP*) family, particularly *FABP3* and *FABP1*, which are considered biomarkers for intramuscular fat content. Bongiorno *et al.* (2022) reported that the saturated fatty acid (SFA) of breast meat was mainly influenced by gender. On the other hand, in the RM breed the higher female expression of *THRSP* strongly suggests a control of lipogenic targets (Resnyk *et al.*, 2017). Activated estrogen pathways could explain differences in genes related to FA in females. In 2017 Zhang *et al.* found three miRNAs involved in down-regulation of genes related to FA metabolism. More specifically, estrogen eliminates the suppressive effect of miRNAs on the target gene *ELOVL5*. Interestingly, estrogen suppresses

the host *SLIT2* gene, thus decreasing the expression of intronic miR-218-5p to promote hepatic synthesis of long-chain polyunsaturated fatty acids in the liver (Zhang *et al.,* 2017). Although *ELOVL5* was not found to be directly overexpressed in females in the present study, many genes functionally related to it (i.e. *ELOVL2*, *SCD*, *FADS1*, *FADS2*) were identified. It is possible that estrogen pathways could regulate FA metabolism in general through the down-regulation of miR-218-5p, which regulates the genes related to FA metabolism, thus enhancing FA biosynthesis. Interestingly, it is the same miRNA (miR-218-5p, regulated by estrogen) that has a role in *FADS1* regulation in liver (Hong Li *et al.*, 2016), and *ELOVL2* regulation in chicken muscle (Zhang *et al.,* 2018). A more comprehensive evaluation of estrogen modulation via miRNA of FA metabolism should be investigated in further studies.

In the present study, we observed that the genetic background was the variable having the largest effect on differential gene expression. Initially, we expected differing expression of genes for FA metabolism between the local breeds and in particular when compared to ROSS broilers. In the BP breed, a huge effect was identified in all four experimental groups when compared against ROSS birds. According to gene expression rates, the two female groups for each diet were the most divergent. These results suggested not only that the females had higher expression of genes involved in FA metabolism, but also that the BP female exhibited higher expression of genes responsible for FA metabolism, when compared to the ROSS females. In particular, the female control diet group showed an enrichment for genes involved in the long-chain fatty acid metabolic process (GO:0001676) supported by both GO analysis and the related genes *ELOVL2*, *SCD*, *FADS1*, *FADS2*, *THRSP*, *FABP3* and *LPIN1*.

With males belonging to the local breeds, an effect was only observed within the BP breed, suggesting the importance of FA processing in this breed. RM females also showed interesting results with regards the FA process. On the contrary, the comparison between RM_M and ROSS_M did not show significant differences in genes involved in the FA processes. This lower effect in males of local breeds could be due to the estrogen stimulus that was strongly activated in females from local breeds and results in the difference in expression of genes related to LC-PUFA. Comparing all the results from contrasts of BP and RM female groups against the ROSS birds highlights 23 genes that are shared between the female local breeds (Figure

7 and Figure 8, Appendix S6). Some of these are still uncharacterized (*LOC101747680*, *LOC107053670*, *LOC426220*, *LOC101749589*, *LOC112532382*, *LOC107050519*, *LOC107053691*, *LOC112532439*), but three of them are already reported in the literature with regards FA metabolism related functions. The *LOC101747680* gene codes for a C-like protein and it is located on chromosome 11, and *LOC107053670* is a non-coding RNA situated on chromosome 6, each with roles which are still unclear. *LOC426220* is located on the W sex chromosome, and is an avidin-related protein 6-like, and with all other avidin-like molecules localized on chromosome Z, suggesting a sex-specific regulation. Moreover, two studies have found this gene strongly correlated with egg yolk, vitelline membrane, and white coloured eggs (Gloux *et al.*, 2019; Niskanen *et al.*, 2005). Regarding eggs and fertility, another three genes were found differentially expressed in local breed females: *WDFC8*, *CTSEAL*, and *ZP1* (Table 1). *WDFC8* is clearly related to "WAP four-disulphide core domain proteins". Members of this family are involved in various aspects of mucosal immunity (Wilkinson *et al.,* 2011). *WDFC8* was also found highly overexpressed in the liver of laying hens (Gloux *et al.*, 2019) and has been shown to protect egg yolk precursors from proteolytic activities/inactivation in the plasma, on their way from the liver to the growing oocytes (Marie Bourin *et al.*, 2012). Moreover, *WFDC8* has been found as a unique protein in egg yolk of chicken (Farinazzo *et al.*, 2009). The avian-specific *CTSEAL* gene was found to be overexpressed in liver of laying hens and the translated protein shares large sequence similarity with cathepsin D, suggesting *CTSEAL* as an accessory of cathepsin D in the processing of egg yolk precursors (Bourin *et al.*, 2012; Gloux *et al.*, 2019). *ZP1* gene was also up-regulated in the liver of hens, where the protein is synthesized and then transported to the ovary to be inserted in the perivitelline membrane surrounding the oocyte (Gloux *et al.*, 2019). Here, it plays a fundamental role in the first interactions between spermatozoa and the oocyte (Bausek *et al.*, 2004). These three genes were differentially expressed in liver of BP and RM birds, with an explanation being that these are multi-purpose breeds and are thus more suitable chickens for egg production than the ROSS broilers. Table 1 and Figure 8 also show other genes not known to be involved in a particular pathway or showing a functional relationship with others. An example is chitinase (*CHIA*), which is a gene coding for a major protease-resistant glycosidase with a physiological role as a digestive enzyme that breaks down chitin-

containing organisms in the chicken gastrointestinal tract (Tabata *et al.*, 2018). Another example is Doublecortin (*DCX*) – a member of a family of microtubule-associated proteins that are required for neuronal migration during cortical development (Vermillion *et al.*, 2014). Tumor Necrosis Factor Receptor Superfamily, Member 14 (*TNFRSF14*) was also found differentially expressed. It mediates apoptosis signalling, and can allow cancer cells to escape the immune process (Guo *et al.*, 2020).

The remainder of genes found differentially expressed in females of local breeds (compared to ROSS), are involved in FA metabolism. The *ABHD12B* and *ABHD5* genes are part of the α/β hydrolase domain-containing family appointed to mobilization of lipids. In particular, *ABHD12B* has been highlighted by Li et al (2020) as a hydrolyser of very long chain lysophosphatidylserine lipids in human cells (Li *et al.*, 2021). *ABHD5* the most well-characterized gene of its family codes for a protein cofactor of the ATGL enzyme and stimulates triacylglycerol hydrolase activity. Ouyang et al (2016) reported that overexpression of *ABDH5* markedly decreased the triglyceride content of preadipocytes in chicken (Ouyang *et al.*, 2016). This mechanism is modulated via the two above-mentioned genes and might explain the question of why native breed hens raised in free-range systems have less fat but higher polyunsaturated fatty acids in their meat muscles (Sokołowicz *et al.*, 2016). We discovered other genes differentially expressed in local female breeds belonging to the cytochrome P450 family were also highlighted: *CYP2C45* and *RP11-400G3.5*. Cytochrome P-450 2C45 (*CYP2C45*) was the most highly expressed cytochrome P-450 isoform in chicken liver and is a unique isoform in avian species (Watanabe *et al.*, 2013). Moreover, Zhao *et al.* (2019) have shown that *CYP2C45* was overexpressed in liver of overfed geese, hence promoting hepatic steatosis. Eventually, this resulted in the up-regulation of some genes involved in FA metabolism (i.e. *PK* and *ALOX5*) acting via *PPAR* pathways (Zhao *et al.*, 2019). *RP11-400G3.5* is reported as a *CYP2C21*-like pseudogene and is the closest gene to *CYP2C45* on chromosome 6 (Watanabe *et al.*, 2013). In fact, both of these genes showed similar function, especially in arachidonic and linolenic acid metabolism in KEGG pathways (data not shown). Furthermore, in local breeds we found significantly more expression in two genes having a role in de novo triglyceride biosynthesis (*MTTPL* and *GPAM*), two genes involved in *de novo* FA biosynthesis (*ACACA* and

SCD), confirmedly involved in desaturation of FA in comparison to ROSS birds. All those genes have been seen to be involved in FA or lipid metabolism (Figure S3).

In Table 1 we report the microsomal triglyceride transfer protein-like (*MTTPL*) gene located on chromosome 6, which is an orthologue of *MTTP* on chromosome 4. *MTTP* is responsible for the assembly and subsequent secretion of very low density lipoproteins from hepatocytes and controls the incorporation of triglycerides into apolipoprotein B (Liu *et al.*, 2016). Moreover, *MTTPL* was found overexpressed in liver of chickens fed with a diet rich in LC-PUFA precursor (Liu *et al.*, 2019; Tesseraud *et al.*, 2014). In the present study, *MTTPL* was differentially expressed in the liver of local breeds, underlining their capacity for FA metabolism. This was not the first time that genetic background has been shown to be a factor in liver expression of *MTTPL*: in Hérault 2010 it is differentially expressed in Muscovy duck with respect to Pekin duck fed *ad libitum* (Hérault *et al.*, 2010). Another gene involved in triglyceride metabolism is Glycerol-3-phosphate acyltransferase (*GPAM*), not shown in Table 1, but found differentially expressed in 3 of the 4 comparisons depicted in Figure 7 (Appendix S1). In fact, *GPAM* was found significantly up-regulated in RM_F_Ct, BP_F_Ct and BP_F_L groups, and plays a central role in *de novo* lipogenesis, particularly of triglycerides (Figure 8) (Claire D'Andre *et al.*, 2013). It was observed that *THRSP* (alias *SPOT14*) was discovered to have a reported role in *de novo* lipogenesis. Indeed, the *THRSP* expression level in liver is correlated with its ability to synthesize lipids (Desert *et al.*, 2018). Furthermore, *SPOT14* has been shown to be a direct target of the key lipogenic *SREBF1* transcription factor (Wu *et al.*, 2013) with its expression levels being under control of estrogens in chicken (Ren *et al.*, 2017). However, the biochemical mechanism linking *SPOT14* to *de novo* lipogenesis remains unclear. Another gene regulated by *SREBF1* is acetyl CoA carboxylase (*ACACA*) also identified in this study, it is critically important for the synthesis of long chain fatty acids (Resnyk *et al.*, 2017). The *ACACA* gene encodes for an enzyme which catalyses the conversion of acetyl-CoA to malonyl- CoA, the substrate of the *de novo* lipogenesis (Nematbakhsh *et al.*, 2021). We also identified stearoyl-CoA desaturase (*SCD*) that plays an important role in biosynthesis of LC-PUFA via the PPAR signalling pathway. Along with *ACACA*, *SCD* could be regulated by *SREBF1*, with both genes being involved in FA *de novo* biosynthesis (Resnyk *et al.*, 2017). *SCD,* thanks to its Delta-9 desaturase activity, can

convert palmitic acid (C16:0) and stearic acid (C18:0) to palmitoleic (C16:1 n-7) and oleic acid (C18:1n-9), respectively. *SCD* is recognized as a gene responsible for FA metabolism and having a significant role in intramuscular fat deposition. The third gene regulated by *SREBF1* is not presented in Table 1, but was significant in the comparison between BP_F_Ct and ROSS birds. This is the *FASN* gene and it is involved in *de novo* FA biosynthesis along with *ACACA* and *SCD* (Nematbakhsh *et al.*, 2021).

In conclusion, we provide a comparison of differential gene expression in two native dual-purpose slow-growing chickens, namely RM and BP, compared to commercial ROSS, fed a diet high in n-3 PUFA and assert that high levels of PUFA precursors in the diet do not result in significant changes in expression of genes involved in FA metabolism, whichever the genetic background or sex studied. The diet had an effect on phenotype as body weight, especially in ROSS birds which were significantly lighter in the L diet. On the contrary, it is clear that sex is an important factor in FA processes. Indeed, females differentially express elongases and desaturase genes (*ELOVL2*, *FADS1*, *FADS2,* and *SCD*) with a central role in LC-PUFA production, particularly in the BP breed. The BP breed is also suggested to be the genetic background with better capacity for modulation of expression of genes involved in FA metabolism. The two local breeds compared with ROSS commercial birds, have shown a significantly higher modulation of gene expression in liver, which underlines a greater aptitude of local breeds in FA metabolism and final LC-PUFA production. Finally, this study has shed light on the capability of local chicken breeds to modulate the expression of some of the genes involved in FA metabolism, as well as the gene expression in females compared to males. Furthermore, the local breeds had the ability to adapt easily to a different diet without change in final weight, enriching the tissue (liver in this case) with n-3, hence having the potential for the establishment of high-quality products.

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DATA AVAILABILITY STATEMENT

RNAseq raw data are available in NCBI (BioProject ID PRJNA865899).

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TABLES

Table 1: Differentially expressed genes shared by BP and RM female groups when compared to ROSS birds. Genes are related to Figure 7 and 8 and Appendix S6.

FIGURES

Figure 1. Principal component analysis (PCA) of all samples. Colours define different genetic background (BP = Bionda Piemontese; RM = Robusta Maculata; ROSS = Ross 308); shapes represent different diet (Ct = Control diet; L = Linseed diet). The two clusters represent the sex: males (M) in the smallest cluster on bottom right, and females (F) in top left.

Figure 2. Effect of diet on the percentage of total n-6 (a), total n-3 (b) and total PUFA (c) in relation to total FA in liver tissue. All the comparisons were assessed by experimental groups (same genetic background and sex, but different diet). *P < 0.05, ***P < 0.001 (post hoc Tukey's test). The data are presented as mean ± standard error.

Figure 3. Volcano plot of differentially expressed genes (DEGs) between the Linseed diet (L) and Control diet (Ct) groups. Note: The red dots indicate genes with a P-adjusted value < 0.05 and -2> Log2 fold change >2. The red dots on the right quadrant of the figures are up-regulated in Linseed diet, the ones on the left are down-regulated. The blue dots represent the genes that reached the P-adjusted value < 0.05 but Log2 fold change value is higher than -2 and lower than 2. The green dots represent the genes whose difference in expression level between the Ct and L groups did not reach significance (padj > 0.05).

Figure 4. Volcano plot of differentially expressed genes (DEGs) between the Female (F) and Male (M) groups. Note: The red dots indicate genes with a P-adjusted value < 0.05 and -2> Log2 fold change >2. The red dots on the right quadrant of the figures are up-regulated for female, the ones on the left are down-regulated. The blue dots represent the genes that reached the P-adjusted value < 0.05 but Log2 fold change value is higher than -2 and lower than 2. The green dots represent the genes whose difference in expression level between the F and M groups did not reach significance (padj > 0.05).

Figure 5. Volcano plot of differentially expressed genes (DEGs) between the Bionda Piemontese (BP) and Ross308 (ROSS) groups. Note: The red dots indicate genes with a P-adjusted value < 0.05 and -2> Log2 fold change >2. The red dots on the right quadrant of the figures are up-regulated in the Bionda Piemontese breed, the ones on the left are down-regulated. The blue dots represent the genes that reached the P-adjusted value < 0.05 but Log2 fold change value is higher than -2 and lower than 2. The green dots represent the genes whose difference in expression level between the BP and ROSS groups did not reach significance (padj > 0.05).

Figure 6. Volcano plot of differentially expressed genes (DEGs) between the Robusta Maculata (RM) and Ross308 (ROSS) groups. Note: The red dots indicate genes with a P-adjusted value < 0.05 and -2> Log2 fold change >2. The red dots on the right quadrant of the figures are up-regulated in the Robusta Maculata breed, the ones on the left are down-regulated. The blue dots represent the genes that reached the P-adjusted value < 0.05 but Log2 fold change value is higher than -2 and lower than 2. The green dots represent the genes whose difference in expression level between the RM and ROSS groups did not reach significance (padj > 0.05).

Figure 7. Venn diagram of up-regulated genes in Bionda Piemontese and Robusta Maculata birds compared to Ross308 (ROSS) chickens in female experimental groups.

Figure 8. Heatmap showing the K-means clustering of transformed expression values for the 23 genes reported in common between female experimental groups in Figure 7. Red represents higher expression and blue represents lower expression.

SUPPLEMENTARY MATERIALS:

Table S1. Formulation and chemical analysis of chicken diet

Table S2. Outline of experimental design. Birds reared for each experimental group. In brackets the liver samples used for the RNA extraction.

Table S3. Summary of contrasts made in differential expression analysis divided by Diet, Sex, Genetic Background. In bold the groups used as experimental, with up and down regulated genes referred to them.

Table S4. Representation of all genes involved in fatty acids metabolism found as differentially expressed in all experimental groups and divided by the belonging contrast

Figure S1. Step by step bioinformatic procedures adopted in the present study.

Figure S2. Effect of diet (Ct = control, L = linseed) on Live Body Weight in g. ***P < 0.001 (post hoc Tukey's test).

Figure S3. GeneMANIA report of 23 genes (dashed circles) reported in common between female experimental groups in Figure 7. Non-dashed circles represent genes commonly co-expressed with the 23 belonging to our dataset. Legends explain biological function in which the genes are involved.

Appendix S1. DEGs of all the comparison. Six Diet comparisons (up and down regulated genes refer to the Linseed group), six Sex comparisons (up and down regulated genes refer to the female group), four BP *vs* ROSS comparisons (up and down regulated genes are referred to the BP group) and four RM *vs* ROSS comparisons (up and down regulated genes refer to the RM group),
In each comparison is reported just genes with a P-value > 0.05. All sheets report Gene ID; baseMean as the average of the normalized count values, dividing by size factors, taken over all samples; log2FoldChange indicates how much the gene or transcript's expression seems to have changed between the experimental and control groups. This value is reported on a logarithmic scale to base 2; lfcSE as the standard error estimate for the log2 fold change estimate; stat as the value of the test statistic for the gene or transcript; P-value of the test for the gene or transcript; and padj as Adjusted P-value for multiple testing for the gene or transcript.

Appendix S2. Gene Ontology for DEGs in Diet contrasts with significant values in Panther. Gene Ontologies refer to up-regulated genes in the Linseed diet group. The considered contrast for each gene ontology is reported in the title of each sheet. The GO tables are formed by *Gallus gallus - REFLIST* column that shows how many genes are involved in a given pathway. *Name of the contrast column* shows number of genes differentially expressed in the contrast belonging to a given pathway. *Over/under represented* column represents whether the pathway is enriched or down-regulated. *Fold enrichment* column explain how many times the pathway is enriched or down-regulated. *Raw p-value* and *FDR* (False discovery Rate) columns represents the statistical output of the Statistical overrepresentation test in Panther.

Appendix S3. Gene Ontology for DEGs in Sex contrasts with significant values in Panther and in IPA software. Gene Ontologies refer to up-regulated genes in the female sex group. The considered contrast for each gene ontology is reported in the title of each sheet. The GO tables are formed by *Gallus gallus* -

REFLIST column that shows how many genes are involved in a given pathway. *Name of the contrast column* shows number of genes differentially expressed in the contrast belonging to a given pathway. *Over/under represented* column represents whether the pathway is enriched or down-regulated. *Fold enrichment* column explains how many times the pathway is enriched or down-regulated. *Raw p-value* and *FDR* (False discovery Rate) columns represents the statistical output of the Statistical overrepresentation test in Panther. IPA analysis used the Sex comparison for each group. Only pathways with a z-score > 2 (activated) are reported. Titles of each sheet explain the contrast under study and the pathways detected as activated. Each IPA sheet reports ID and full name of genes involved in a given pathway, prediction of the behaviour of a given gene when the pathway is activated, and log2 Fold Change value for every gene in that contrast.

Appendix S4. Gene Ontology for DEGs in Genetic background contrasts (Bionda Piemontese *vs* Ross308) with significant values in Panther and IPA analyses. Gene Ontologies refer to up-regulated genes in the BP breed group. The considered contrast for each gene ontology is reported in the title of each sheet. The GO tables are formed by *Gallus gallus - REFLIST* column that shows how many genes are involved in a given pathway. *Name of the contrast column* showed number of genes differentially expressed in the contrast belonging to a given pathway. *Over/under represented* column represents whether the pathway is enriched or down-regulated. *Fold enrichment* column explain how many times the pathway is enriched or down-regulated. *Raw p-value* and *FDR* (False discovery Rate) columns represents the statistical output of the Statistical overrepresentation test in Panther. IPA analysis used the Breed comparison for each group. Only pathways with a z-score > 2 (activated) are reported.. Titles of each sheet explain the contrast under study and the pathways detected as activated. Each IPA sheet reports ID and full name of genes involved in a given pathway, prediction of the behaviour of a given gene when the pathway is activated, and log2 Fold Change value for every gene in that contrast.

Appendix S5. Gene Ontology for DEGs in Genetic background contrasts (Robusta Maculata *vs* Ross308) with significant values in Panther and in IPA analyses. Gene Ontologies refer to up-regulated genes in the BP breed group. The considered contrast for each gene ontology is reported in the title of each sheet. The GO tables are formed by *Gallus gallus - REFLIST* column that shows how many genes are involved in a given pathway. *Name of the contrast column* shows number of genes differentially expressed in the contrast belonging to a given pathway. *Over/under represented* column represents whether the pathway is enriched or down-regulated. *Fold enrichment* column explain how many times the pathway is enriched or down-regulated. *Raw p-value* and *FDR* (False discovery Rate) columns represents the statistical output of the Statistical overrepresentation test in Panther. IPA analysis used the Breed comparison for each group. Only pathways with a z-score > 2 (activated) are reported. Titles of each sheet explain the contrast under study and the pathways detected as activated. Each IPA sheet reports ID and full name of genes involved in a given pathway, prediction of the behaviour of a given gene when the pathway is activated, and log2 Fold Change value for every gene in that contrast.

Appendix S6. Gene lists of Venn diagram groups reported in Figure 7.