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Nadia Stoppani , Federica Raspa , Edoardo Fiorilla , Sandra Maione , Achille Schiavone , Cecilia Mugnai [†] , Dominga Soglia [*,†]

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Feather's RNA: A Non-Invasive Approach for Transcriptomic Profiling in Live Chickens

Nadia Stoppani, Federica Raspa, Edoardo Fiorilla, Sandra Maione, Achille Schiavone, Cecilia Mugnai † and Dominga Soglia *,†

Università degli Studi di Torino, Dipartimento di Scienze Veterinarie, Largo Paolo Braccini 2, 10095 Grugliasco, Turin, Italy

* Correspondence: dominga.soglia@unito.it

† These authors contributed equally to this work.

Simple Summary

This study highlights feathers as a novel, non-invasive tool to investigate metabolic responses in chickens. Using RNA-Seq on feather quill pulp, we clear diet- and sex-dependent molecular differences in a slow-growing chicken breed. The results reveal that feather transcriptomics can effectively capture nutrigenomic responses, offering an ethical alternative to invasive sampling. This approach supports the development of non-invasive biomarkers for precision nutrition and genetic selection, contributing to more sustainable poultry production.

Abstract

This study investigated the feasibility of using **blood feather** transcriptomics to detect sex-differences and gene response to physiological changes in chickens. The identification of molecular markers associated with metabolism in poultry typically requires invasive sampling of tissues, such as liver. Feathers represent a promising non-invasive biological source of RNA: the **quill pulp** of growing feathers contains living cells capable of active transcription. Growing feathers were collected from 150-day-old male and female chickens (Bionda Piemontese, slow-growing breed) raised under a free-range system and fed two finishing diets differing in lipid content: low-lipid (LL, ether extract 3.6%) and high-lipid (HL, ether extract 9.3%). RNA was extracted from quill pulp and subjected to whole RNA-Seq analysis. Differential gene expression and functional enrichment analyses were performed using the RaNA-Seq platform. A total of 17,360 transcripts were detected and used for downstream analyses. Across all individuals, three genes associated with ether lipid metabolism (PLA2G10, PLA2G4F, and ENPP6) were consistently upregulated in chickens fed the HL diet. Sex-specific responses were also observed. In roosters, HL feeding significantly affected genes involved in lipid transport and metabolic regulation within the PPAR signaling pathway, including APOA1 and SLC27A4. In contrast, hens showed differential expression primarily in pathways related to apelin signaling, extracellular matrix remodeling, and cardiovascular function rather than classical lipid metabolism pathways. These findings demonstrate differential responses to dietary treatments between males and females and reveal metabolic differences, confirming the need for sex-specific analyses in this local breed. In conclusion, feather RNA-Seq successfully captured diet-induced molecular responses and revealed sex-specific metabolic adaptations to dietary lipid levels. This study demonstrates that quill pulp represents a practical and ethically favorable alternative to traditional tissue sampling and may support future nutrigenomic and genetic improvement studies. The findings support the development of non-invasive biomarkers applicable to genetic selection and precision nutrition, ultimately supporting more sustainable poultry production.

Keywords: feathers; gene expression; chicken; sex-specific response

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1. Introduction

Growing feathers (blood feather) represent a non-invasive biological sample suitable for genetic analysis in birds, as they can be collected directly from live animals without the need for euthanasia [1]. Structurally, feathers are characterized by a calamus and a rachis, which provides support to the feather. The banner is made up of many barbs from which the barbules originate and are equipped with hooks that give shape to the feather. During feather growth, feather follicles develop: the dermal papilla grows upward, in the calamus, to form the pulp, and endothelial cells give rise to capillary vessels, which transport nutrients to the various parts of the feather [2]. The pulp of growing feathers, often referred to as the "blood feathers", is a vital structure within the calamus formed by mesodermal tissue and containing a rich network of blood vessels and nerves. This tissue nourishes the feather during the anagenic phase, while the epidermal cells of the follicle proliferate rapidly to form the rachis and barbs. The feather follicle is a tissue characterized by the presence of stem cells, which has a strong potential for multidirectional proliferation, division and differentiation. Stem cells can sense changes in microenvironment signals and modulate follicle growth, which is critical for regenerated feather growth cycles [2,3].

The calamus represents the only part which contains living cells (quill pulp) during feathers growing, making it useful for genetic analysis. GWAS (Genome-Wide Association Study) research into the development of poultry feather follicles focuses primarily on identifying genetic markers that influence feather traits. Transcriptomics research into the development of poultry feather follicles focuses primarily on changes in gene expression within feather follicle cells and their effect on the formation, development and periodic growth of feather. Gene expression in growing feathers is primarily associated with regenerative and physiological needs and/or in response to trauma, as well as with bird plumage pigmentation [3-8].

Beyond their role in pigmentation and regenerative processes, gene expression in feathers may also reflect systemic metabolic pathways. Feathers are dynamic biological structures that, during their growth phase, integrate signals arising from multiple physiological and environmental dimensions. It is now well established that glucocorticoid hormones, particularly corticosterone, are deposited into the keratin matrix of feathers as they develop, thereby reflecting the endocrine status of the individual over the entire growth period. This process makes feathers a “time-integrated biomarker,” capable of capturing cumulative exposure to both acute and chronic stressors. Beyond physiological processes, a growing body of evidence indicates that feathers also respond to environmental and behavioral stimuli; variations in habitat conditions, including diet composition, can modulate feather follicle stem cells [2,3,9].

Nutrients are essential for the development of the feather follicle and feathers: deficiencies generally lead to severe feather loss or structural abnormalities; but they also play a fundamental role as mediators for triggering signal transduction networks in the feather follicle stem cell microenvironment, modulating their activity. Experimental studies demonstrate that changes in diet quality and food availability directly influence the feather growing, supporting the strong relationship between individual energetic status, highlighting the utility of feathers as indicators of nutritional stress [2].

Collectively, these findings support the concept that feathers are not merely passive structures but rather biological archives that integrate physiological, environmental, and nutritional information over time. This integrative property makes them a powerful tool for assessing animal welfare and for investigating diet interactions.

In addition, feathers can also be exploited for gene expression studies, as the living cells present in the calamus provide RNA suitable for transcriptomic analyses. The tissue within the follicle of a new feather (blood feather) can be extracted and analyzed. Because feathers contain living cells capable of active transcription and blood, the transcriptome of their pulp can provide information on metabolic functions. These living cells are particularly accessible during plumage that occurs during molting or following damage [5].

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Traditionally, RNA expression related to diet effect in chickens has been studied in the liver [9-11], abdominal fat [12], or breast muscle [10,13,14], all of which require euthanizing the birds. Consistent with these findings, was reported that most transcriptomic variations occur in blood rather than in liver or adipose tissue and highlighted a clear sexual dimorphism [10, 11, 15, 16].

In this study, we aimed to evaluate the potential of blood feathers as a non-invasive tissue for assessing genetic responses to physiological stimuli, such as dietary variation, in live male and female chickens.

2. Materials and Methods

The trial was conducted at the poultry facility of the University of Turin. The detailed methods for the rearing of the animals are reported in [15]. Briefly, a total of 60 chickens (30 male and 30 female) of Bionda Piemontese, a slow-growing breed native to North-west Italy, were used for this trial. All chickens were raised in a free-range production system, where they were subjected to natural environmental conditions, including natural temperature condition and photoperiod, and had free access to outdoor areas. Throughout the starter and growing phases, all birds received the same diet.

During the finishing phase (from 120 to 150 days of age), males and females were allocated into two dietary treatments: a control group receiving a standard Low Lipidic content diet (LL, ether extract, EE = 3.6%) and a High Lipidic content diet (HL, EE = 9.3%) prepared adding 6% of palm kernel. HL diet presents an increased in lipid con-tent of 61.6%. The detailed feed formulation is described in Table 1.

Table 1. : Ingredients (g/kg as fed) and formulated chemical composition of the control (low-lipidic; LL) and experimental (high-lipidic; HL) diets during the finisher period.

Ingredients	LL	HL
Corn meal	700	625
Soybean meal	224	239
Palm kernel oil	–	60
Sunflower seed oil	40	40
Calcium carbonate	12	12
Dicalcium phosphate	10	10
Vitamin-mineral premix1	10	10
Soybean oil	5	5
Cane molasses	5	5
Sodium chloride	2	2
Sodium bicarbonate	1	1
Metabolizable energy (kcal/kg)	2983	3264
Chemical composition (formulated)		
Dry matter (%)	88.66	86.51
Crude protein (%)	17.00	17.00
Ash (%)	4.81	4.80
Ether extract (%)	3.85	9.49
Crude fiber (%)	4.06	3.99

Abbreviations: L: low-fat; H: high-fat; DM: dry matter.

[1]Premix contained the following nutrients (units are expressed per kg of diet): vitamin A, 15,000 IU; vitamin D3, 3,000 IU; vitamin E, 25 IU; vitamin K3, 5 mg; vitamin B_1, 2 mg; vitamin B2, 7 mg; vitamin B6, 4 mg; vitamin B12, 25 mg; pantothenic acid, 11.04 mg; nicotinic acid, 35 mg; folic acid, 1 mg; biotin, 15 µg; choline chloride, 250 mg; Cu, 1.6 mg; Mn, 60 mg; Zn, 45 mg; Fe, 80 mg; I, 0.4 mg; Se, 0.15 mg.

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Growing feathers were collected from body areas showing active regrowth near the neck and along the dorsal tract, in 60 BP chickens (15 males and 15 females per diet) at 150 days old. All collected feathers were characterized by the presence of blood in the calamus (Figure 1).

Figure 1. Example of growing feathers; 1The feathers are characterized by the presence of blood in the calamus (blood feather).

Feathers were put in RNA later and then stored at -80 °C until extraction.

Total RNA was extracted from feathers (quill pulp) using the FastGene® RNA Premium Kit. For each sample, approximately 2 mg of tissue was collected from the base of the feather calamus containing blood. RNA quantity was assessed using Qubit® (RNA Broad-Range Assay Kit), and RNA integrity was evaluated with the Agilent 2100 Bioanalyzer (RIN).

From the two dietary treatments, 24 RNA samples (12 males and 12 females) were selected based on a minimum concentration of 100 ng/µL and an RNA Integrity Number (RIN) ≥ 7, yielding four balanced experimental groups of six individuals each (HL males, LL males, HL females, LL females). To limit sequencing costs while preserving biological variability, samples were pooled in equal proportions within each group, generating three pools per group and a total of 12 RNA pools submitted for sequencing. An aliquot for each pool was sent to AZENTA (Azenta Life Sciences, US) for RNA profiling. Results were in FASTQ format.

RaNA-Seq open bioinformatics tool (https://ranaseq.eu/) was used to analyze RNA-Seq data. It performs a quantification of FASTQ files by alignment of reference genome, calculates the quality of the reads (Salmon software), performs a normalization test (TPMs, Transcript Per Millions), runs a differential expression analysis (DESeq2) and interprets the results with functional analysis [17]. The flow chart of RaNa-Seq is well explained in [17]. Briefly, the reference genome used for assembly the reads was Gallus gallus-5.0. Once the files in FASTQ format are uploaded in RaNA-Seq software, reads are filtered based on the quality and processed with Salmon software to obtain the quantification of the reads for gene. Reads are normalized as TPMs (Transcripts Per Millions) [18].

According to the flow chart, after the differential expression analysis, a functional enrichment analysis was performed using the goseq R package [19]. By default, the software uses the Kyoto Encyclopedia of Genes and Genomes (KEGG) and WikiPathways databases for pathway

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identification. Molecular pathways were statistically significative with a p-value cutoff less than 0.05. A different gene expression (DEG) was considered for p-adjusted value (padj) < 0.05. The p-value adjustment was made using the Benjamini and Hochberg's approach for controlling the false discovery rate. In addition, the log2 fold change (log2FC) was considered to assess the magnitude and direction of gene expression changes, allowing the identification of upregulated (log2FC > 1) and downregulated (log2FC < -1) genes.

3. Results

This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.

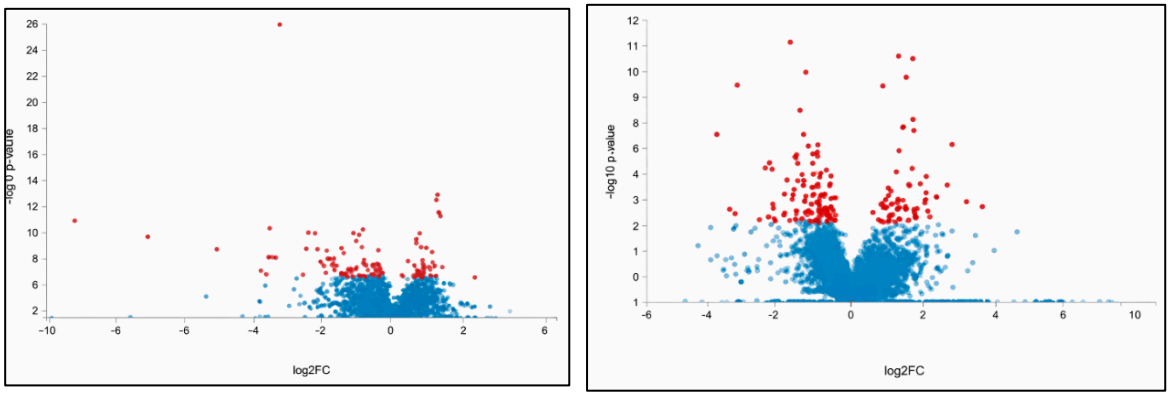
3.1. RNA Extraction from Feathers

A mean RNA extraction yield of 456 ± 234 ng/µL was obtained from feather's samples. In total, about 2.5 × 107 reads were sequenced from each library and 92% were aligned with Gallus gallus-5.0 genome. Quality control results showed that the expression was distributed homogeneously in all samples, and this means that no sequencing or sample collection problems have been caused. Therefore, all samples were included in a differential expression analysis between dietary treatments (HL vs. LL) and the two sexes (Males vs. Females) using DESeq2, as implemented in the RaNA-Seq software platform. A total of 17,360 transcripts were detected.

3.2. Diet Effect on Gene Expression in Feathers

Differential expression analysis between the two dietary treatments detected 316 (263 annotated) significant genes (padj < 0.05). For Gene Ontology (GO) analysis, 4,266 genes were successfully annotated, including 89 DEGs. The highest proportion of genes was annotated to the Epidermal Growth Factor Receptor (EGFR) signaling pathway, which regulates growth, survival, proliferation, and differentiation [20]. Functional enrichment analysis, performed using the software, identified significantly altered pathways (P < 0.05). Three significant genes were linked to ether lipid metabolism, including PLA2G10, ENPP6, and PLA2G4F. All genes showed a trend of up-regulation in the HL diet compare to the LL diet, independent of sex effect (padj = 0.04, 0.03, and 0.04; log2FC = 0.46, 0.60, and 0.30, respectively). However, these results may be confounded by the strong effect of sex, which can also impact differential gene expression.

Consequently, a separate analysis was conducted by sex; when sex was considered, 118 DEGs were detected in roosters and 153 in hens (Figure 2).



(**a**) (**b**)

Figure 2. Volcano plot of significant different expressed genes (DEGs) between the two diets in Roosters (a) and Hen (b). In red, significant DEGs (padj < 0.05) between high-lipid (HL) and low-lipid (LL) diets. X-axis shows the log2 scaled fold change (FC) of each gene. Y-axis shows the minus log10 p-value calculated by the selected analysis method for each gene.

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Functional enrichment analysis revealed that in roosters differential gene expression is associated with key pathways regulating metabolism, growth, and cellular homeostasis, including the cell cycle, AGE-RAGE signaling, insulin signaling, p53, and PPAR pathways. Within PPAR pathways, involved in energy and lipid metabolism, APOA1 and SLC27A4 were significantly upregulated in rooster fed the HL diet (padj = 0.01, and 0.02, respectively), with a markedly stronger effect for APOA1 (log2FC = 1.69) compared to SLC27A4 (log2FC = 0.34).

While in female most altered pathways are involved in apelin signaling pathway, extracellular matrix (ECM) remodeling mediated by metalloproteinases (MMPs), and cardiac muscle contraction. No pathways directly related to lipid metabolism were identified.

The Venn diagram (Figure 3) illustrates the number of differentially expressed genes (DEGs) identified in the comparison between LL and HL dietary treatments in males and females. Overlapping areas indicate genes commonly differentially expressed between two groups: a total of 10 DEGs were shared between roosters and hens, indicating that their differential expression was unaffected by sex (overlapping areas). These include genes involved in extracellular matrix remodeling (MMP11), neural development (SEMA3A, PTN), immune signaling (ZAP70), and ion transport (SCNN1B). The remaining five DEGs were annotated with Ensembl transcript IDs, indicating transcripts not yet assigned to official gene symbols in the current Gallus gallus annotation. These transcripts have been reported as novel in the Red Junglefowl, a local chicken breed from India, although further characterization studies are still lacking. In contrast to the limited overall effect of diet, most gene expression differences emerged when sex was analyzed within each dietary condition, suggesting sex-specific responses to diet in roosters and hens. Accordingly, sex exerted a strong influence on gene expression and was further evaluated in blood feathers across the two dietary groups.

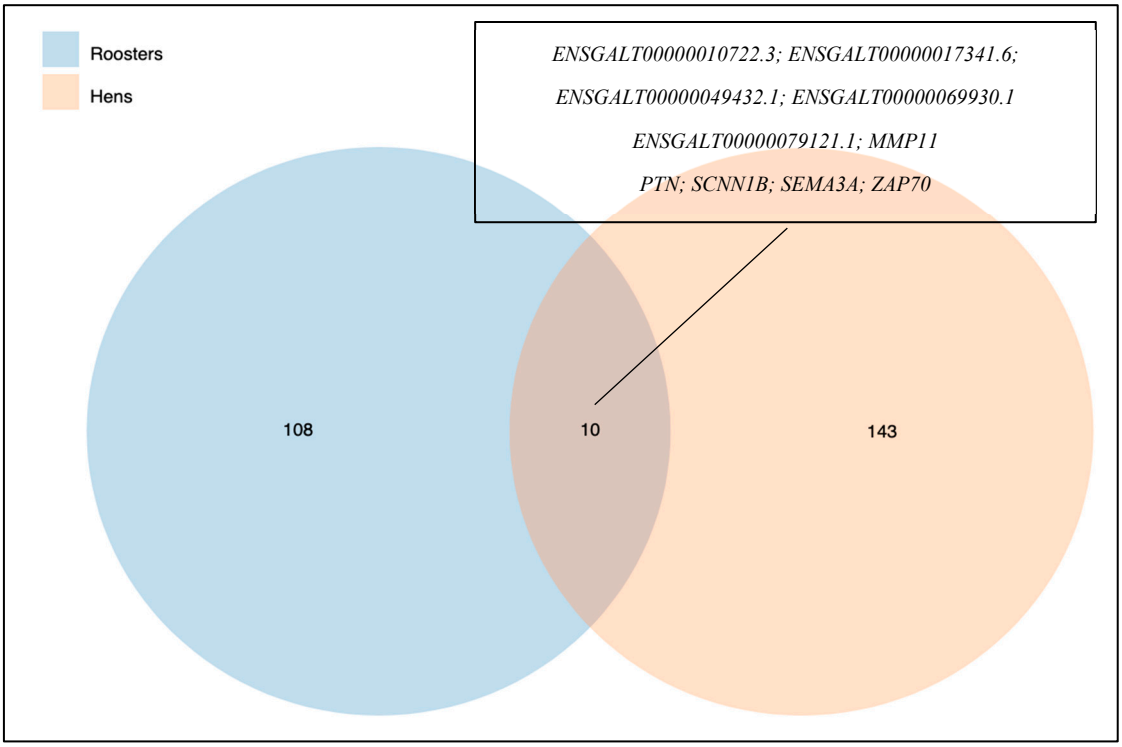

Figure 3. Venn diagram of significant different expressed genes (DEGs) between the two dietary treatments in roosters and hen: Number of DEGs (padj < 0.05) between high-lipid (HL) and low-lipid (LL) diets in Roosters – in light blue, and Hens – in light orange. The overlapping region highlights 10 genes showing diet-dependent expression changes independent of sex.

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3.3. Metabolic Alteration Between Sexes Evaluated in Feathers

According to the high sex effect evaluated in the previous findings, a differential expression analysis was performed between the two sexes. Overall, it was identified a total of 474 (295 annotated) significant genes (padj < 0.05) between males and females. For Gene Ontology (GO) analysis, 4,266 genes were successfully annotated, including 123 DEGs. Functional enrichment analysis, performed using the software, identified significantly altered pathways (P < 0.05). Sex-related differential gene expression analysis revealed that the observed differences between males and females were not restricted to a single biological process but instead involved multiple interconnected pathways. In particular, strong enrichment was observed in central metabolic processes, including glycolysis, gluconeogenesis, oxidative phosphorylation, and carbon metabolism, indicating sex-dependent regulation of energy production and metabolic flux. In parallel, pathways related to ribosome biogenesis, RNA processing, and spliceosome activity were significantly represented, suggesting differences in transcriptional and translational capacity. Additionally, cell cycle regulation, DNA replication, and proteostasis pathways, including proteasome and lysosome activity, were enriched, supporting a global remodeling of cellular growth and protein turnover. Key signaling pathways, such as MAPK, insulin, and calcium signaling, further indicate coordinated regulation of cellular responses. Overall, these findings highlight a systemic effect of sex on gene expression, affecting core processes related to metabolism, gene regulation, and cellular homeostasis.

4. Discussion

The present results highlighted the possibility of using "blood feathers" as a non-invasive tissue to study the genetic response to physiological stimulus, as diet effect, in live chickens. Quill pulp proved effective for detecting both broad and nuanced gene expression patterns associated with dietary lipid exposure: this study focused on feather-derived transcriptomes from chickens exposed to high-lipid (HL) or low-lipid (LL) diets. Despite the limited sample size, the reported results are in agreement with what has previously been reported in the literature, which reported clear transcriptomic differences between the transcriptome of males compared to females in different feeding regimes. Across all individuals, the HL diet consistently up-regulated PLA2G10, PLA2G4F, and ENPP6, indicating a generalized enhancement of phospholipid turnover. PLA2G10 and PLA2G4F are members of the phospholipase A2 (PLA2) family, which plays an essential role in signal transduction, phospholipid remodeling, membrane homeostasis, and energy production for fatty acid β-oxidation in mammals [21]. Specifically, PLA2G10 gene is involved in the selective hydrolysis of phospholipids and plays a crucial role in fat metabolism and energy utilization in pigs [22], while PLA2G4F has been linked to intramuscular fat deposition in chicken breast muscle [23], and its up-regulation in duck liver has likewise been associated with higher intramuscular fat content [24], further supporting its involvement in lipid accumulation. In this study, intramuscular fat content was not evaluated, but previous results showed that males fed the HL diet had a lower thawing loss compared with those fed the LL diet [15], indicating a possible improvement in muscle water-holding capacity that may be partly driven by small differences in lipid distribution or membrane phospholipid remodeling. In contrast, ENPP6 is highly expressed in liver where it acts as a crucial choline-supplying enzyme by breaking down phosphocholine-containing substrates to provide vital choline for hepatocyte function, especially for synthesizing phosphatidylcholine for lipoprotein secretion, and preventing fatty liver [25]. However, ENPP6 may act in concert with PLA2 family to generate phosphocholine from the matrix vesicle membrane during skeletal mineralization [26]. This dual functionality highlights the enzyme's involvement not only in hepatic phospholipid metabolism but also in extracellular matrix dynamics. Therefore, the upregulation observed in the present study could reflect a more complex physiological adjustment, where membrane phospholipid remodeling—potentially triggered by the dietary lipid level—affects both metabolic and structural pathways. Taken together, the simultaneous modulation of ENPP6 and PLA2-related genes suggests

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the activation of a coordinated response involving phospholipid turnover, choline metabolism, and possibly matrix-associated processes.

Despite this shared initial response, males and females exhibited distinct secondary transcriptional programs. In males, the HL diet altered the insulin signaling and PPAR pathways, both of which are involved in energy and lipid metabolism [27,28]. In particular, it was observed an increased expression of genes associated with lipid uptake, transport, and utilization, most notably APOA1, SLC27A4. These changes imply an enhanced capacity for fatty acid activation, HDL (high-density lipoprotein) biogenesis, and reverse cholesterol transport [2,13]. In chickens, APOA1 gene has been reported to be down-regulated in abdominal adipose tissue [29] and in the liver [30] of fat broiler, consistent with elevated fat deposition and increased blood cholesterol. In local breeds, reared under extensive farming system, the regulation of this gene may differ, potentially reflecting breed-specific genetic characteristics and adaptive metabolic response. In fact, in our study, APOA1 was up-regulated in rooster fed the high lipid diet suggesting a possible adaptive mechanism to maintain cholesterol homeostasis under increased dietary lipid intake [31]. Regarding SLC27A4, this gene is already recognized as a candidate for traits associated with fat deposition in liver and breast muscle in chickens [32]. The up-regulation of SLC27A4 observed in chickens fed the high-lipid diet may reflect an increased requirement for fatty acid uptake and activation in response to the elevated dietary lipid load. This pattern differs from the down-regulation reported in chickens with naturally high intramuscular fat [32], suggesting that SLC27A4 expression may respond primarily to the immediate availability of dietary lipids rather than to the final level of lipid deposition in muscle. Thus, its up-regulation in our study likely represents a metabolic adaptation aimed at handling higher fatty acid flux, which does not necessarily translate into increased intramuscular fat accumulation.

In females, the HL diet did not strongly induce classical lipid-metabolism genes. Instead, differential expression was observed in pathways related to apelin signaling, ECM remodeling mediated by MMPs, and cardiac and vascular function. None of these pathways is directly involved in lipid metabolism. In chickens, apelin signaling pathways is primarily associated with the initiation of sexual maturity [33], ECM remodeling with oviduct development [34], and the cardiac/vascular pathway with heart contraction. These results, which should be confirmed by a larger study, suggest that in females the response of HL diet may affect reproductive and cardiovascular physiology rather than classical lipid metabolism pathways. This highlights the importance of considering sex-specific metabolic and transcriptional programs in nutritional studies. Collectively, these findings support the view that males and females employ fundamentally distinct adaptive strategies to manage dietary lipid challenges.

The distinct responses to dietary treatments observed between sexes highlight the strong influence of sex on metabolic processes, underscoring the importance of considering males and females separately when studying this local chicken breed. Consistently, the analyses demonstrated a sex-dependent regulation of energy production and metabolic flux. Moreover, sex also affected gene expression at a broader level, impacting fundamental processes related to metabolism, gene regulation, and cellular homeostasis.

Taken together, these results demonstrate that feathers are a robust and sensitive tissue for detecting diet-induced metabolic and sex-specific transcriptional responses. A high-lipid diet activates a conserved phospholipid-remodeling mechanism across sexes, while downstream responses diverge strongly between males and females due to inherent physiological and endocrine differences. Males exhibit increased lipid transport and catabolism, whereas females showed alterations in their reproductive and cardiovascular physiology.

The results demonstrate the potential of using quill pulp to investigate molecular responses to physiological stimuli, such as varying levels of dietary lipid intake, in live chickens. These findings provide a foundation for future studies aimed at identifying molecular biomarkers that can be assessed non-invasively from blood feathers in live birds. Such biomarkers could be valuable tools in poultry genetic selection, contributing to improved growth performance and feed efficiency, as well

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as enabling precision nutrition strategies to enhance efficiency and sustainability. Overall, blood feather transcriptome analysis represents an innovative genomic approach for poultry improvement, with the potential to support more sustainable poultry production.

5. Conclusions

This study demonstrates that quill pulp represents a viable and ethically favorable source of RNA for transcriptomic analyses in live chickens, offering a non-invasive alternative to traditional tissue sampling. The consistent upregulation of PLA2G10, PLA2G4F, and ENPP6 across dietary groups indicates a shared phospholipid remodeling response to increased dietary lipid intake, while the divergent downstream responses, lipid transport and catabolism in roosters (APOA1, SLC27A4; PPAR pathway) versus apelin signaling and extracellular matrix remodeling in hens underscore the necessity of sex-disaggregated analyses in nutritional studies on local breeds. The non-invasive nature of this approach is especially advantageous for local breeds such as Bionda Piemontese, whose limited population size and protected status preclude the routine use of tissue biopsies, making feather-based transcriptomics a particularly fitting tool for both conservation and genetic improvement purposes. Collectively, these findings establish blood feather transcriptomics as a sensitive tool for capturing diet- and sex-specific molecular signatures in live poultry. Future studies with larger sample sizes are warranted to validate these findings and to develop feather-based biomarkers applicable to genetic selection and precision nutrition programs.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The RNA-seq dataset supporting the conclusions of this article is depositated in the GEO DataSets (https://www.ncbi.nlm.nih.gov/gds) Accession: NNNNNN ID: NNNNNN.

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