Comparative analysis of whole blood transcriptomics between European and local Caribbean pigs in response to feed restriction in a tropical climate

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Abstract *Feed restriction occurs frequently during pig growth, either due to economic reasons or stressful environmental conditions. Local breeds are suggested to have better tolerance to periods of feed restriction. However, the mechanisms underlying the response to feed restriction in different breeds is largely unknown. The aims of the present study were 1) to compare the transcriptome profile in response to feed restriction and refeeding of two contrasted breeds, Large White (LW), which has been selected for high performance, and Creole (CR), which is adapted to tropical conditions, and 2) to investigate the effect of a moderate feed restriction and refeeding on whole blood transcriptome. Analysis of blood transcriptome allows to study the response to feed restriction and refeeding in a dynamic way. RNAseq was performed on blood samples of growing LW and CR pigs at two time points: after 3 weeks of feed restriction and after 3 weeks of refeeding. The data was compared with samples from control animals offered the same diet on an ad libitum basis throughout the whole experiment. In terms of performance, CR pigs were less impacted by feed restriction than LW. The transcriptional response to feed restriction and refeeding between CR and LW was contrasted both in terms of number of DEGs and enriched pathways. CR demonstrated a stronger transcriptional response to feed restriction whereas LW had a stronger response to refeeding. Differences in the transcriptional response to feed restriction between CR and LW were related to cell stress response (Aldosterone Signalling, Protein ubiquitination, Unfolded Protein Signalling) whereas after refeeding, differences were linked to thermogenesis, metabolic pathways and cell proliferation (p38 MAPK, ERK/MAPK pathway). In both breeds, transcriptional changes related to the immune response were found after restriction and refeeding. Altogether, the present study indicates that blood transcriptomics can be a useful tool to study differential genetic response to feed restriction in a dynamic way. The results indicate a differential response of blood gene expression to feed restriction and refeeding between breeds, affecting biological pathways that are in accordance with performance and thermoregulatory results.*

Keywords Blood transcriptome, feed restriction, refeeding, Creole pig, tropical climate

1. Introduction

During the growing period, pigs may encounter periods of feed restriction due to economic reasons or environmental factors. When facing stressful environmental conditions, such as heat stress, poor sanitary conditions, social stress or disease pressure, pigs reduce their feed intake, leading to feed restriction [1]–[3]. During these periods of feed restriction, the growing pig must adjust its metabolism to maintain homeostasis through changes in nutrient partitioning between growth and maintenance. The animal responses to feed restriction is highly variable within and between populations and part of this variability may have a genetic basis [4], [5]. Our previous work compared the effect of feed restriction on two contrasted breeds, the Creole (CR) breed, a local breed well adapted to tropical conditions and that has not been submitted to genetic selection, and the Large White breed (LW) that has been selected for high growth performance in optimal conditions [6]. Our results suggested that the CR breed may be more tolerant to feed restriction.

In the context of climate change, there is a crucial need of information on local breeds and on their adaptation to specific environmental conditions, as they constitute genetic resources that are essential to maintain livestock systems diversity and ensure food security [7]. The CR breed provides a good model to study the genetic variability in the response to feed restriction in pigs [6], [8], [9].
Advances in high-throughput technologies such as transcriptomics offer opportunities to better understand complex biological mechanisms and to better characterize local breeds lacking this kind of data. The collection of blood samples is relatively easy compared to other tissues and provides the possibility of sampling the same animal at different time points. It is also a technique that would be easily transferable in breeding schemes. A recent study on divergent selected lines of pigs showed that the blood transcriptome is relevant to identify biological processes affected by genetic selection and feeding strategies [10]. In the present study, we used whole blood transcriptome analysis to better understand the molecular mechanisms underlying the differential breed response to feed restriction. The objectives of the current study were 1) to investigate the effect of a moderate feed restriction and refeeding on whole blood transcriptome, 2) to compare the transcriptome profile of two contrasted breeds, CR and LW, in response to feed restriction and refeeding.

2. Methods

All measurements and observations on animals were performed in accordance with the current law on animal experimentation and ethics. The French Ministry of Agriculture authorized the experiment referenced at n°APAFIS#18576-2019011614325318 (after the revision of the Animal Care and Use Committee of French West Indies and French Guyana) on living animals at the INRAE facility under the direction of N. Minatchy (INRAE-PTEA).

2.1. Animals and experiment design

A total of 30 growing pigs (15 LW and 15 CR) of the same age, with an average BW of 32.3 ± 1.7 kg for LW and 18.2 ± 1.0 kg for CR, were used for the experiment in the semi-open front building of the INRAE experimental farm located in Guadeloupe, French West Indies. At 12 weeks of age, pigs were alloted to 2 or 3 pens with a density of 10 pigs/pen (5 LW and 5 CR).
The experiment consisted of three consecutive periods. Period 1 (**P1**) was the initial period (7 days) where all pigs were fed *ad-libitum*. Period 2 (**P2**) was a 3-week period during which feed restriction was imposed to specific pens. Due to experimental limitations, the two feeding treatments were not balanced in number of animals. During P2, one pen (referred to as NF, 5 LW and 5 CR) continued to be fed ad libitum, whereas 2 pens (referred to as RF, 10 LW and 10 CR) had restricted access to the automatic feeder (from 7:00 to 17:00). Period 3 (**P3**) constituted the following 3-week period and corresponded to the refeeding period during which all animals were fed ad libitum.

2.2. Measurements

Blood samples were collected at the end of P2 (week 15) and at the end of P3 (week 21) at 08:00 in the morning. Jugular vein blood was obtained (10-mL BD K_2 EDTA Vacutainers tubes (BD, Franklin Lakes, NJ)) via venepuncture. For samples dedicated to RNA extraction, one volume of blood sample was mixed with one volume of lysis buffer from the Nucleospin RNA blood kit (Macherey-Nagel, Lyon, France). The obtained mixture was then stored at -80°C for later analyses.

2.3. RNA extraction and quality analysis

Total RNA was extracted from frozen blood samples of 28 animals from the first replicate [9 NF (4 CR, 5LW) and 19 RF (10 LW, 9 CR)] using the NucleoSpin RNA isolation kit (Macherey-Nagel, Hoerdt, France) in accordance with the manufacturer's instructions. The total RNA concentration was measured with NanoDrop 2000 (ThermoScientific TM, France) and the quality was quantified using an Agilent 2100 Bioanalyzer (Agilent Technologies, France). The extracted total RNA was stored at -80˚C until use.

2.4. Library preparation and sequencing

High-quality RNA (RIN > 7.5) was used for the preparation of cDNA libraries according to Illumina's protocols (Illumina TruSeq RNA sample prep kit for mRNA analysis). Briefly, poly-A mRNA was purified from 4μg of total RNA, fragmented and randomly primed for reverse transcription to generate double stranded cDNA. The cDNA fragments were then subjected to an end repair process, consisting of the addition of a single 'A' base, and the ligation of indexed Illumina adapters at both ends of cDNA. These products were then purified and enriched by PCR to create the final bar-coded cDNA library. After quality control and quantification, cDNA libraries were sequenced on 2 lanes on the NovaSeq6000 S4 (Illumina® NEB, USA) to obtain approximatively 48 million reads (100 bp paired-end) for each sample.

2.5. Quality control and read mapping to the reference genome

The quality control check on raw reads in FASTQ format were processed using FASTQC and the Q30, GC content and length distribution of the clean data were calculated. The sequences obtained by RNA-Seq were splice-aligned for each library, using STAR (version 2.3.0e with standard parameters) [11]. The reads were mapped to the *Sus Scrofa* genome (assembly 11.1). HTSeq (http://pypi.python.org/pypi/HTSeq) [12] was used to calculate the number of sequence reads aligned to all protein-coding genes from the ENSEMBL v74 annotation of the *Sus scrofa* genome. The Bioconductor package DeSeq2 [13] was then used to identify differentially expressed genes (DEGs). Two treatment comparisons were tested for DEGs for each breed: (i) RF v. NF at the end of Period 2; (ii) RF v. NF at the end of Period 3. Statistically significant ($P<0.05$) DEGs with a Benjamini-Hochberg false discovery rate of < 0.05 were deemed to be significant. Analysis of canonical pathways and regulatory effects as well as network analysis were performed using Ingenuity pathway analysis (IPA) software (Ingenuity Systems, Redwood City, CA) for DEGs in each comparison. IPA identifies known regulators, including genes and other molecules that may affect the expression of DE genes, then it calculates a z-score, which is a statistical measure of the match between the expected relationship direction between the regulator and its targets, and the observed gene expression [14]. Moreover, KEGG pathway and Gene Ontology enrichment analyses were performed using ShinyGO [15].

3. Results

3.1. mRNA read alignment and differential gene expression

Following the period of feed restriction, at the end of P2, 648 genes were differentially expressed (DE) in CR, whereas 198 were DE in LW (Figure 1a). Of the 648 DEG in CR, 193 were up-regulated and 455 down-regulated. In LW, of the 198 DEG, 62 up-regulated and 136 down-regulated. CR and LW shared 51 DEGs in response to feed restriction, with 45 down-regulated and 6 up-regulated. Following refeeding, the opposite pattern was found, with a higher number of DEG in LW than CR (1538 in LW vs. 187 in CR) (Figure 1b). After refeeding, in both breeds, the majority of DEG were up-regulated (55% and 61% upregulated, in LW and CR, respectively) whereas after restriction, DEG were mostly down-regulated (69% and 70% downregulated, in LW and CR, respectively). Few DEG were shared by both breeds, with 28 upregulated and 19 down-regulated. An additional 21 genes were shared by both breeds but the direction of the fold change was reversed between the two breeds.

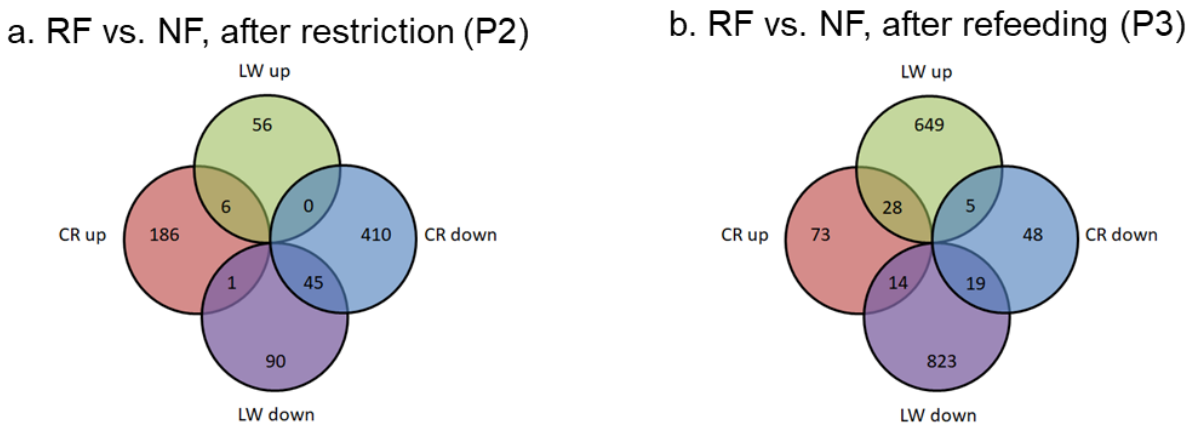

Figure 1. Venn diagrams displaying the number of differentially expressed genes (DEG) in Large White (LW) and Creole (CR) pigs for each comparison. RF: Restricted Feeding, NF: Normal Feeding. P2: restriction period, P3: refeeding period. Numbers in overlapping areas represent DEGs shared by both breeds.

3.2. Gene Ontology and Pathway analysis

The DEG from each comparison were submitted to ShinyGO [15] for Gene Ontology (GO) analysis. Pathway analysis based on the KEGG database revealed 39 enriched pathways at the end of P2 for CR and 18 at the end of P3 for LW (Top 10 shown in Fig. 2). However, the smaller number of DEG identified at the end of P2 for LW and at the end of P3 for CR did not allow to reach any significant KEGG pathway enrichment.

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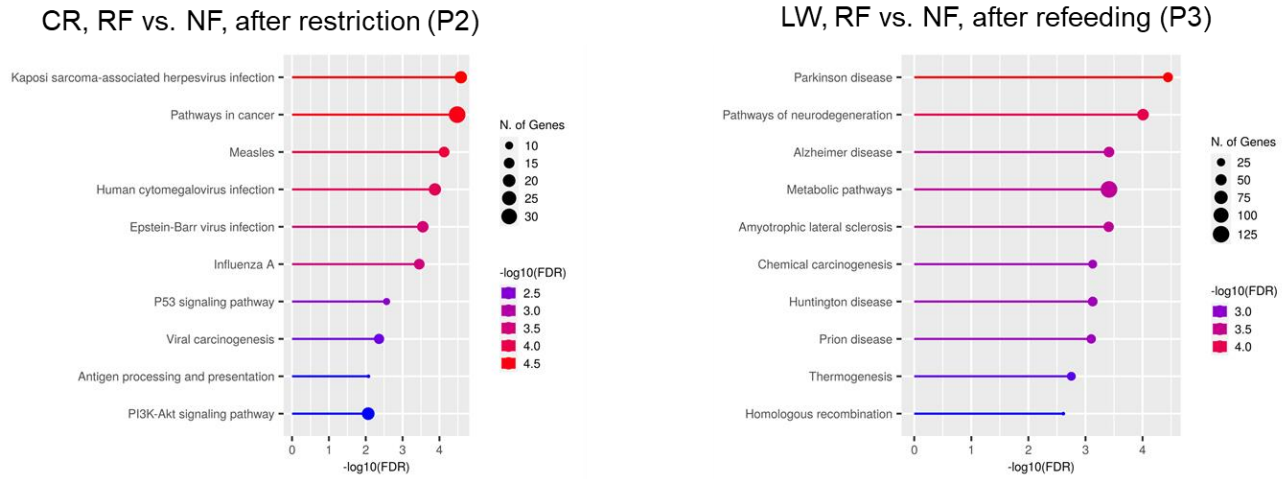

Figure 2. Top 10 significant KEGG pathways identified by ShinyGO [15]–[17] **using DE genes between treatments.** CR: Creole, LW: Large White, RF: Restricted Feeding, NF: Normal Feeding.

3.3. Ingenuity Pathway Analysis (IPA)

After feed restriction, at the end of P2, Ingenuity Pathway Analysis (IPA) identified 29 significant canonical pathways for LW and 179 for CR. Whereas after refeeding, at the end of P3, IPA found 30 canonical pathways for LW and 27 for CR. IPA was also used to compare results from the different comparisons in the 2 breeds between treatments (NF vs. RF), over time (after restriction and after refeeding). The top 10 canonical pathways and the top 10 diseases and biological functions were compared (Figure 3). When comparing the 2 breeds after restriction, synaptogenesis signalling was the only pathway to be significantly inhibited (z-score < 2) in both breeds and it was no longer inhibited after refeeding. In CR, after restriction, enriched pathways were inhibited and mostly related to the immune response (natural cell killer signalling, neuroinflammation

signalling, production of nitric oxide). When comparing results after restriction and after refeeding, all pathways and disease and biological functions had a z-score closer to 0 (lower activation) after P3 than after P2. For disease and biological functions, "organismal death", "anemia", "polycythemia" were activated in both breeds after restriction but it was no longer the case after refeeding. "Quantity of lymphocytes" was inhibited in both breeds after restriction. After refeeding, "quantity of lymphocytes" was still inhibited in LW to a lower extent but not in CR. "Immune response of cells" was inhibited in CR after restriction and to a lower extent after refeeding.

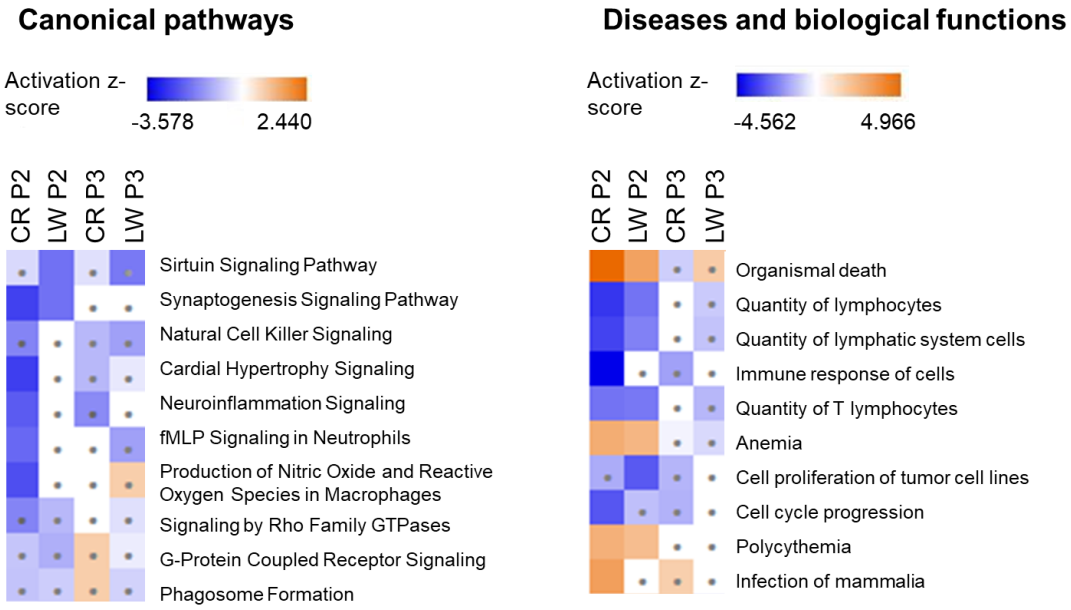

Figure 3. Heat map of canonical pathways and diseases and biological functions identified by Ingenuity Pathway Analysis using DE genes between treatments (RF vs. NF). CR: Creole, LW: Large White, RF: Restricted Feeding, NF: Normal Feeding. P2: restriction period, P3: refeeding period. Squares with dots indicates pathways for which activation/inhibition was not significant (z-score <|2|).

4. Discussion

Periods of feed restriction may occur during pig growth due to economic reasons or external factors, such as heat waves, inflammatory stress, feed transition or social stress [3]. Few studies have investigated the effect of feed restriction and refeeding on livestock transcriptome [18]–[20] and to our knowledge, there is no comparative analysis of the transcriptomic response to feed restriction and refeeding in different pig breeds. The present study aimed to investigate the effect of feed restriction and refeeding on the blood transcriptome of growing pigs from two contrasted breeds.

RNAseq analysis comparing the two feeding groups (RF vs. NF) show that after restriction there were more DEGs in CR than LW, suggesting that the response elicited by feed restriction is stronger in CR than LW. Consequently, after restriction, we also identified more enriched pathways in GO and IPA analysis for CR than LW. KEGG enrichment showed that the main pathways triggered after feed restriction in CR were related to immunity. Similar results were found after IPA analysis regarding canonical pathways after restriction in CR. The most enriched pathways were related to the immune response and viral infection (Interferon signalling, Th1 pathway), cancer (Pancreas adenocarcinoma signalling, Rac signalling) and Ephrin receptor signalling, which is involved in the maintenance of several processes including angiogenesis, stem cell differentiation and cancer. Finding many genes related to immunity in the blood transcriptome is not surprising as blood cells constitute one of the first lines of immune defence [21]. Similar findings were found in pig studies on blood transcriptome response to genetic selection for feed efficiency and nutritional status [10], [22]. Moreover, genes involved in the immune response were also found to be differentially expressed after dietary restriction in beef cattle jejunal epithelium [18]. Reports in mice, human and rats have also described improved immune

function after periods of caloric restriction [23]–[25]. The main hypothesis is that the immune response may be involved in nutrient partitioning, allowing activation of tissue mobilisation during dietary restriction [26].
GO analysis for LW after restriction comparing RF to NF did not allow to reach any enrichment, probably due to the low number of DEG. Nevertheless, disease and biological functions found with IPA in LW and CR after restriction were mainly related to the immune response (quantity of lymphocytes and T-lymphocytes, immune response of cells). Interestingly, only 3 disease and biological functions were activated after restriction in both breeds, which were "organismal death", "anemia" and "polycthemia", suggesting that feed restriction may also trigger genes associated with organismal death and blood defects. The canonical pathway comparison between breeds led to only one common pathway with a z-score < 2 in both LW and CR, which was synaptogenesis. Chronic stress exposure in rats and non-human primates have been shown to induce atrophy of dendrites and decreased glia and neurogenesis in the adult hippocampus [27], [28]. The mechanisms that control food intake also involve communication between gut, adipose tissue and the central nervous system through hormones and peptides circulating in the blood. We could therefore hypothesize that feed restriction generates stressful signals that may affect synaptogenesis.
The Top 5 canonical pathways found with in IPA in the two breeds after restriction did not overlap, suggesting differential response to feed restriction between breeds. In LW, several DEGs in the Top3 enriched pathways found in IPA encodes for Heat Shock Protein (HSPs): DNAJA1, DNAJC17, DNAJC9, HSP90AA1, HSPA12B. HSPs are highly conserved proteins playing an essential role in the cellular stress response [29]. The expression of HSP could be linked to the fact that the present experiment takes place in a tropical climate, with a mean temperature of 25.5°C, which is above growing pig thermoneutral temperature [2]. However, the differential expression of HSP was found comparing RF and NF after restriction, indicating that the response observed is related to the feed diet. Proteomic studies on short-term heat stress (12h) using pair-feeding controls showed that pigs with a reduced plane of nutrition in thermoneutral conditions had increased HSP70 [30]. HSP are also part of the common over-represented pathways Aldosterone Signaling in Epithelial Cells, Protein ubiquitination pathway and Unfolded Protein Signaling. Genes encoding for HSPs and involved in the aldosterone pathway have been identified as over-expressed in the liver and duodenum of pigs with low FE compared to high FE pigs [31]. Interestingly here, upregulation of HSP after feed restriction is detected in LW and not in CR, indicating that HSP are not triggered upon feed restriction in that breed. This evidence suggest that LW have higher stress response than CR, which is supported by the performance results obtained and previous studies comparing LW and CR [32]. In line with these results, a study comparing HSP90 mRNA expression levels after heat stress in peripheral blood mononuclear cells of LW and CR found an increase of HSP90 mRNA expression in both breeds after 6h, but a significant decrease in CR pigs after 9h [33]. The authors suggested that the difference observed after 9h could be due to a reduced impact of heat stress on protein conformations in CR pigs.
After refeeding, the number of DEGs was higher in LW than CR, suggesting stronger response to refeeding in LW than CR. In LW, the KEGG pathways identified after refeeding were related to the immune response but also to thermogenesis. Thermogenesis could be triggered during refeeding due to increased feed intake compared to the restriction period, which may generate increased metabolic heat [34]. The immune response has also been shown to be triggered upon refeeding in beef cattle jejunum transcriptomic profile and could allow more dietary derived energy to be partitioned towards growth during re-alimentation [18]. However, despite the greater number of DEGs found in LW than CR after refeeding, the difference in terms of performance between the 2 breeds after refeeding were not significant (data not shown). In none of the breeds do we observe compensatory growth, i. e. a period of accelerated growth following periods of feed restriction, during refeeding. Compensatory growth in pigs depends on the onset, severity and duration of the restriction period and the onset and duration of refeeding [35]. In the present study, despite a long period of feed restriction and refeeding, the severity of the feed restriction was probably not sufficient to induce compensatory growth. Consistent with this result, pathways and disease and biological functions enrichment in IPA after refeeding led to lower z-score than after restriction, suggesting lower response for both breeds after refeeding than after restriction.

5. Conclusions

In conclusion, the present study indicates that blood transcriptomics can be a useful tool to study differential genetic response to feed restriction in a dynamic way throughout the different periods of stress of the animal life. In both breeds, major transcriptional changes after restriction and refeeding were related to the immune

response. Nevertheless, the transcriptional response to feed restriction and refeeding between CR and LW was contrasted both in terms of number of DEGS and enriched pathways. CR demonstrated a stronger transcriptional response to feed restriction whereas LW had a stronger response to refeeding. Most differences in the transcriptional response to feed restriction between CR and LW were related to cell stress response, whereas after refeeding, differences were linked to thermogenesis, metabolic pathways and cell proliferation. Additional research on local breeds and potential structural variants that could increase the transcriptional response to feed restriction while maintaining performance would contribute to deepening our understanding of post-absorptive metabolism differences between breeds.

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