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Gene expression analyses on adipose tissue of Nellore peripubertal heifers reveal genes that have an impact on reproductive physiology

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Dissertation presented to obtain the degree of Master in Science. Area: Animal Science and Pastures

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SUMMARY

RESUMO

Análise da expressão gênica do tecido adiposo em novilhas Nelore púberes revela

interação entre tecidos reprodutivos

O desenvolvimento púbere é o resultado da interação de genes que produzem sinais moleculares regulando os mecanismos fisiológicos. A compreensão das mudanças genéticas que permeiam o período peripuberal em novilhas Nelore (*Bos indicus*) poderia desvendar o porquê das diferenças em idade a puberdade, pois elas atingem a maturidade sexual mais tarde que o gado europeu (*Bos taurus*). O tecido adiposo regula o metabolismo de energia e é um órgão endócrino que tem participação ativa em processos reprodutivos. O presente estudo teve como objetivo entender as funções do tecido adiposo na época peripubertal em novilhas Nelore. Nossa estratégia foi procurar diferenças em expressão gênica entre animais pré-púberes e púberes. Nós quantificamos a expressão gênica no tecido adiposo de novilhas Nelores precoces ou tardias. A pesquisa foi feita em 30 novilhas Nelore, monitoradas semanalmente a partir do momento em que atingiram 230 kg de peso até a manifestação da puberdade por exame de ultrassom ginecológico. O momento da puberdade foi definido pela presença de corpo lúteo no ovário e concentração sanguínea de progesterona maior que > 1 ng/ml. O tecido adiposo foi coletado por biópsia subcutânea; a primeira biópsia foi feita quando as novilhas alcançaram 230 quilos de peso e subsequentemente uma vez por mês ate que elas entrassem em puberdade. As biópsias foram coletadas de cada novilha que atingiu a puberdade e de sua meia-irmã que não tinha atingido a puberdade. Nós analisamos amostras de seis novilhas púberes precoces e de seis novilhas púberes tardias em dois estágios diferentes: no momento da ovulação e aproximadamente 50 dias antes da ovulação. O RNA foi extraído do tecido das biopsias e a expressão genica quantificada através de sequenciamento de RNA. Encontramos nove genes diferencialmente expressos em novilhas púberes que foram previamente relacionados com características e/ou processos reprodutivos de mamíferos em outros estudos. Esses genes foram APOD, CYP17A1, DNMT3B, AKR1C4, TENM1/ODZ3, HPSE, SMPD3, FAM134B e CYP26B1, já encontrados expressos em adipócitos, hipotálamo, ovários e útero, comprovando conhecimento prévio da conexão existente entre metabolismo de energia, órgãos reprodutivos e influencia neural. Ao medir as mudanças genéticas e correlacioná-las com mecanismos fisiológicos conhecidos em sistema nervoso e órgãos reprodutivos durante a puberdade, nós conseguimos identificar genes expressos em adiposo que são relacionados a modificações no organismo como um todo. Sendo assim, o tecido adiposo pode ser considerado um instrumento para indicar o que está acontecendo durante os processos reprodutivos. .Os resultados apresentados aqui podem ajudar na melhor compreensão de mudanças genéticas durante o período peripuberal.

Palavras- chave: Puberdade; Novilhas; Expressão gênica; Tecido adiposo; Regulação endócrina

ABSTRACT

Gene expression analyses on adipose tissue of Nellore peripubertal heifers reveal genes

that have an impact on reproductive physiology

Pubertal development is an outcome of genes' interaction producing molecular signals that rule physiological mechanisms. Understanding the genetic changes surrounding the peripubertal period in Nellore heifers could unveil certain differences in age at puberty because they reach sexual maturity much later than European heifers. The adipose tissue regulates energy metabolism and is an endocrine organ with active participation in reproductive processes .The present study aimed at understanding the functions of the adipose tissue around pubertal time in Nellore heifers. Our strategy was to search for differences in gene expression between prepubertal and pubertal animals. We quantified gene expression in the adipose tissue for Nellore heifers who reached puberty according to either the early or late pattern. This research was done on 30 Nellore heifers monitored weekly from 230 kg bodyweight until the onset of puberty through gynaecological ultrasound examination. The onset of puberty was defined by the presence of corpus luteum in the ovary and blood progesterone > 1 ng/ml. Adipose tissue was collected through subcutaneous biopsy; the first biopsy was done when heifers reached 230 kilos bodyweight and subsequently once a month until they entered puberty. Biopsies were collected of each heifer that reached puberty alongside its half-sister who had not reached puberty. We analysed samples of six early pubertal heifers and their six late pubertal sisters at two different stages: at the time of ovulation and approximately 50 days before ovulation. The RNA was extracted from the biopsies' tissue and gene expression quantified through RNA sequencing. There were nine genes differentially expressed in pubertal heifers that have been found previously correlated with reproductive traits and/or processes in mammals on other studies. These genes were APOD, CYP17A1, DNMT3B, AKR1C4, TENM1/ODZ3, HPSE, SMPD3, FAM134B and CYP26B1, already found expressed in adipocytes, hypothalamus, ovaries and uterus, corroborating previous knowledge of a link among energy metabolism, reproductive organs and neural influence. By measuring the genetic changes and correlating them with known physiological mechanisms on the nervous system and reproductive organs during puberty, we detected genes expressed here that are related to modifications in the organism as a whole. Therefore the adipose tissue may be regarded as an instrument to indicate what is going on during reproductive processes. The results presented here may aid in a better understanding of genetic changes happening during peripubertal period.

Keywords: Puberty; Heifers; Gene expression; Adipose tissue; Endocrine regulation

1.INTRODUCTION

Puberty is defined by the first estrus with ovulation followed by corpus luteum formation with normal function and duration. The age at which puberty is initiated depends on species, genotype, age and size. Heifers enter puberty when they reach 40 to 50% of the adult's body size. The importance of an adequate start of this event is the fact that a heifer should have its first born at approximately 24 months old [5], so a delayed puberty and first pregnancy bring negative economic consequences for cattle breeders because they decrease the number of calves produced during a cow's reproductive life. Females in reproductive age are responsible for approximately 70% of the costs of the beef cattle production system [2] highlighting the need for them to return this cost by generating offspring.

Associated to age, there is also a larger need in energy, glucose and leptin augmenting the signalling to the hypothalamus to show the energetic balance is propitious for the initiation of reproductive activity [3]. Among the putative modifiers, a determinant for the triggering of puberty in mammals is the energetic body status [6]. Functional coupling between energetic sufficiency and achievement of puberty is especially important in females, where there is a threshold of body fat required to allow the metabolic draining that comes along with pregnancy and lactation [1].

Our choice to use subcutaneous adipose tissue for this analysis goes back to the fact that the adipose tissue is part of the general homeostasis acting as an endocrine organ. In addition to leptin, there is evidence of a strong link between neural influence, expression and secretion of hormones [4]. Leptin receptors were identified in many areas of the brain and in other tissues, including ovaries. In the brain they are located mainly in the hypothalamus and are associated to appetite control, reproduction and growth [7]. Other potential metabolic signals are long chain fatty acids that may signal the availability of nutrients to the central nervous system and change

feed intake and glucose availability. Shifts in the development of these relationships between metabolic signals are considered fundamental for puberty's manifestation [4].

Considering that age at puberty is a phenotype which is difficult and expensive to measure in bovine, the identification of different genetic signals among individuals and their function is important to understand the underlying factors leading to reproductive maturation in cattle. We consider that a study measuring gene expression between heifers at: the same peripubertal stage, related through father side, who ovulated or not, could shed light in terms of understanding these factors.

1.1 Objective

The objective of our study was then to understand differences in gene expression in the adipose tissue over the peripubertal period between Nellore heifers who manifested puberty or did not (measured as first ovulation).

1.2 Hypothesis

There is a difference of gene expression in the adipose tissue between Nellore heifers at the same age, weight and genetic background who reached puberty or did not reach puberty.

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2. Gene expression analyses on adipose tissue of Nellore peripubertal heifers reveal genes that have an impact on reproductive physiology

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2.1 Introduction

The necessary stimulus for puberty to occur is a surge in the secretion of luteinizing hormone (LH) as a consequence of an increase in GnRH (gonadotropin releasing hormone) release by hypothalamus. The period in which this rise occurs is called prepubertal time and includes approximately 50 days preceding puberty. This increase in LH secretion is a result of a decline on estradiol's negative feedback over GnRH [37] mostly due to a decrease in the number of estrogen receptors in the hypothalamus. A larger LH secretion leads to augmented ovarian follicles' growth [8], which in turn increases estradiol concentration inducing the initial preovulatory surge of LH. The LH surge leads to the dominant follicle releasing the oocyte hence causing ovulation; the remaining cells of the follicle in the ovary become luteinised and secrete progesterone, a hormone that will maintain the pregnancy if fertilization is successful.

During sexual maturation in heifers, besides alteration in the hypothalamus sensitivity to estradiol, there are modifications happening to inhibitory and stimulatory neurotransmitters that depend on age and internal availability of energy [55]. The endocrine response to signals coming from adipose tissue has been studied in bovine puberty for a long time now [2;6;71]. This endocrine response enables the adipose tissue to be part of the general homeostasis. Leptin receptors were identified in many areas of the brain and in other tissues, including ovaries and adipose tissue; in the brain they are located mainly in the hypothalamus and are associated with appetite control, reproduction and growth [60]. Investigations have confirmed that human adipose tissue in vivo produces sex-steroids and

glucocorticoids from precursors (androgens to estrogens, cortisone to cortisol)[11 ; 35]. Sexual hormones are secreted and have their receptors on adipocytes leading to an endocrine communication between the hypothalamus, reproductive organs and adipose tissue. Good examples of this crosstalk

are estrogen receptors [10; 49], and enzymes responsible for activation, interconversion, and inactivation of steroid hormones and retinoic acid [36; 67; 68] which were found to be linked to reproductive processes in several mammalian species.

The regulation of adipose tissue metabolism in vivo involves the activity of the autonomic nervous system, the delivery of a mixture of substrates and hormones present in the plasma and, feedback from autocrine and paracrine effectors secreted by adipocytes. The adipose tissue's neuronal control is achieved through sympathetic and parasympathetic innervation [27]. Studies on hamsters and rats have demonstrated direct neural connections between white adipose tissue and several different brain regions involved in the SNS (sympathetic nervous system) regulation of the cardiovascular and other systems [5].

Age at puberty's heritability measured by first detected corpus luteum varied from 0.52 to 0.57 in cattle adapted to the tropics [33].Gene expression studies and gene network analysis have been used to identify complex interactions of genes involved in the fertility of female beef cattle (such as puberty) [64; 65]. Once age at puberty is a heritable characteristic, genetic selection for precocious puberty on Nellore cattle has the potential to impact on production efficiency [21] because the breed reaches sexual maturity later than European cattle. Research that may anticipate the process of genetic improvement related to this characteristic is needed as the time required for phenotype observation is considerable. Considering these circumstances, we gathered data on adipose tissue gene expression of postpubertal versus prepubertal Nellore heifers. We proposed that it is possible to detect differentially expressed genes in the comparison between postpubertal and prepubertal heifers that may improve our understanding of physiological changes linked to manifestation of puberty.

2.2 Material and methods

2.2.1 Animals and sampling methodology

We assessed a set of 30 Nellore heifers sired by five different bulls kept under the same lodging system and high-gain feeding diet. The diet contained 12% of raw sugar cane bagasse, 70% of ground corn, 16% of soybean meal, 1% of urea, and mineral mixed ad libitum (16% of crude protein, 23% of

neutral detergent fiber, and 78% of total digestible nutrients). Heifers were weighed weekly from 10 months old. Heifers were managed and handled as per approval of the Committee of Animal Ethics at ESALQ - University of São Paulo - Brazil.

Whenever heifers reached 230kg bodyweight, they started being monitored every week through transrectal ultrasonography to detect the presence of corpus luteum in the ovary, and hence the initiation of puberty. Also, to confirm the pubertal status, concentration of blood progesterone had to be > 1ng/ml. Each heifer went through a subcutaneous biopsy to collect adipose tissue every four weeks from alternate sides of the tail head until the occurrence of puberty was confirmed. Subsequently, when a heifer's puberty was confirmed by the presence of corpus luteum, a biopsy was done to sample her pubertal status and another biopsy performed on her sister who had not ovulated (age and sire match control).The animals that did not ovulate (late puberty) were followed by weekly ultrasound for at least another 5 months to ensure they were late puberty control. These latter samples were used in the analysis to eliminate the bias caused by growth and development that had occurred during the time that passed between the pre-pubertal and pubertal status (Fig. 1). With this strategy, we had adipose tissue samples from all animals approximately 50 days before puberty, at puberty and from age and sire match control that did not reach puberty.

A subcutaneous biopsy to collect adipose tissue was performed after the heifer's tail head area was shaved, cleaned with antiseptic solution and local anaesthesia performed using 2% 10 ml lidocaine. Adipose tissue samples were snap frozen immediately in liquid nitrogen and stored at −80°C until extraction of RNA was done.

FIG.1. Animals and sampling methodology

Group E = Early pubertal heifers   **Group L = Late pubertal heifers**

E_a = early pubertal after puberty L_a = late pubertal after puberty

E_b = early pubertal before puberty L_b = late pubertal before puberty
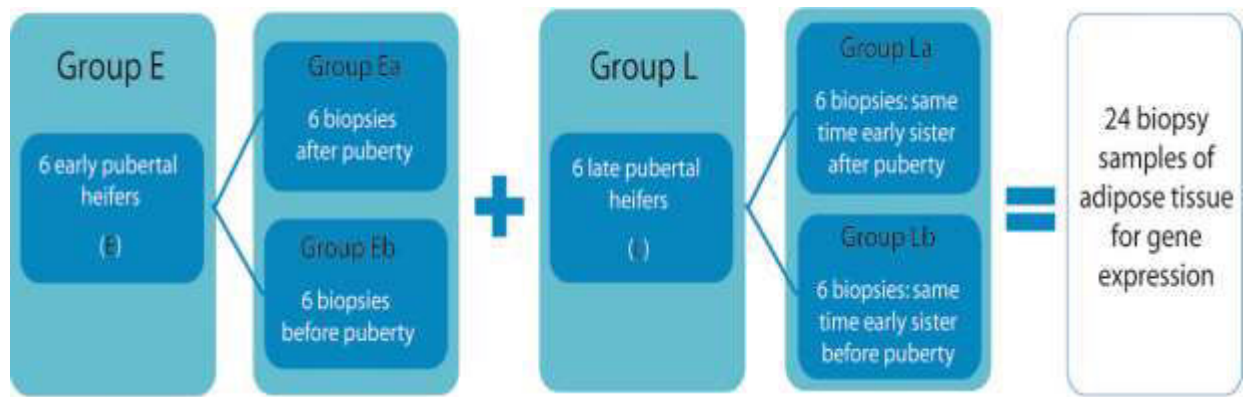
2.2.2 RNA extraction

A sample of 0.5 g of adipose tissue was used to extract RNA from each of the six early pubertal heifers and their six late pubertal sisters at two different moments: at the time of ovulation and approximately 50 days before ovulation. The frozen samples' tissue was grounded with mortar and pestle in liquid nitrogen and then processed according to Trizol reagent's protocol (Invitrogen, Carlsbad, CA). Quality was evaluated through gel electrophoresis and microfluidic electrophoresis Bioanalyser 2100 (Agilent, Santa Clara, CA, USA) to determine the integrity of the extracted RNA.

2.2.3 Library preparation and Sequencing

The libraries were prepared following Illumina TruSeq Stranded mRNA Sample Preparation Guide (Illumina, San Diego) kit instructions.

Libraries were quantified by KAPA SYBR FAST Roche Light Cycler 480 qPCR (Kapa Biosystems) so an optimal cluster density was created in each sequencing flow cell to prevent accumulation or excessive space among clusters. The 24 samples were pooled together and then put in two sequencing flow cells. To avoid a flow cell bias and obtain an appropriate coverage level, each sample was sequenced twice on each flow cell. Sequencing was done on an Illumina HiSeq 2500 (Illumina San Diego) analyser that yielded 100 - base paired end reads.

2.2.4 Quality control, mapping and read counts

Quality control of the raw reads was done with FASTQC v. 0.10.1 software (Babraham Bioinformatics Institute). Adapters' removal and filtering of the reads were done with SeqyClean software v.1.9.10 [70], reads were filtered according to a minimum length of 65bp and quality of the sequence 24.

Mapping of the reads to the bovine genome and their count were done with STAR v 2.5.2a [23] software. Sequence reads were assembled to the annotated Bos taurus reference genome UCSC bosTau8 downloaded from Illumina iGenomes website (http://support.illumina.com/sequencing/sequencing_software/igenome.html).Reads mapping to multiple features or overlapping genes were discarded.

2.2.5 Differential gene expression analysis

Differential expression analysis was carried out with the Bioconductor software package edgeR v. 3.3.1[61] with the R statistical programming language. Genes were kept in the analysis only if they had more than 2 counts per million reads mapped in at least six samples. Normalization factors for library size were computed using edgeR's TMM method [62] and incorporated into the differential expression analysis. Our intention with this experiment was to identify genes differentially expressed in adipose tissue at the onset of puberty. For that, we compared gene expression obtained at puberty vs 50 days before puberty. Based on the experimental design described in the section animals and sampling methodology, differences in gene expression were tested for the six early pubertal heifers (Group E) between the measurements undertaken before (Eb) and after (Ea) the onset of puberty. To take into account effects not specifically introduced by the onset of puberty, but rather as a consequence of growth and development that occurred over the time between the first biopsy (before puberty) and the second biopsy (after puberty), we contrasted the analysis of Group E with the measurements undertaken in late pubertal heifers (Group L) at corresponding time points (Lb and La, respectively).This resulted in a multi-level design as described in edgeR User's Guide, Section 3.5: Comparisons both between and within subjects [16]. The model was accordingly constructed by incorporating an effect for group assignment (Group E versus Group L), interactions between group

assignment and heifer ID (to account for sample pairing within groups), and interactions between group assignment and time point of biopsy. From the fitted model, we correspondingly extracted genes responding differently to the onset of puberty in Group E versus Group L heifers. Multiple testing correction was applied using the method of Benjamini and Hochberg [7].

2.2.6   KEGG Pathways and GO Categories enrichment analysis

Gene-set enrichment analysis was performed to detect significantly enriched KEGG pathways and GO categories using the R package GAGE version 2.22.0 [42]. KEGG pathways were downloaded on 2016-11-17 using the "kegg.gsets" function from GAGE, and GO categories were obtained from the org.Bt.eg.db package version 3.4.0 [15]. KEGG pathways found to be significantly enriched were visualized with Pathview package version 1.12.0 [41].

2.3 Results

The six heifers that were considered early pubertal were at least five months more precocious (average age at puberty 14 months-standard deviation 0,89) than their half-sisters (average age at puberty 20 months, standard deviation 0,70 - Table 1).

Table 1: Age at puberty, bodyweight and sire for Early pubertal heifers (E) and their Late pubertal (L) sisters. sd = standard deviation.

Early Heifers (group E)	Age at Puberty	Weight	Sire	Late Heifers (group L)	Age at Puberty	Weight	Sire
36	13 months	314 kg	L	17	19 months	333 kg	L
51	15 months	364 kg	J	60	20 months	369 kg	J
71	13 months	319 kg	S	69	20 months	296 kg	S
64	14 months	301 kg	S	89	Did not ovulate	288 kg	S
33	14 months	309 kg	L	26	20 months	326 kg	L
120	15 months	293 kg	S	169	21 months	322 kg	S
	mean= 14 months	mean= 317 kg			mean= 20 months	mean = 322 kg	
	sd = 0.9	sd = 25			sd = 0.7	sd = 29	

2.3.1 Gene expression analysis

After quality control, RNA sequencing generated an average amount of 19.5 million paired end reads per heifer and STAR mapped uniquely 17.3 million paired end reads on average per sample/heifer/stage. On average 11600 genes were expressed in each library.

We have found, before multitesting correction, 83 genes differentially expressed on a significance level of 0.01(P-value). We have focused our search among the top 25 genes on the differentially expressed genes list with raw p-value under 0.01(Table 2) for a possible relationship with puberty. There were nine genes among the top 25 that have been found previously associated with reproductive processes in mammals on other studies. These genes were APOD, CYP17A1, DNMT3B, AKR1C4, TENM1/ODZ3, HPSE, SMPD3, FAM134B and, CYP26B1. All of these genes had already been found expressed in adipocytes, hypothalamus, ovaries and uterus, corroborating previous knowledge of a link between energy metabolism, reproductive organs and neural influence [2; 6;17 ;60].

Table 2 – Top 25 differentially expressed genes in Early pubertal heifers compared to their Late pubertal sisters

Gene ID	log Fold Change	raw PValue
APOD	-2,59	1,43E-05
SLC38A3	-2,28	6,53E-05
DNMT3B	2,14	0,000319
AKR1C4	-1,94	0,000503
IER3	-1,57	0,000522
CYP17A1	-3,47	0,000574
PTGES	-2,15	0,000597
TENM1	2,42	0,000692
IFI27	-1,6	0,000734
LY75	2,74	0,000966
P2RX5	1,22	0,00101

IKZF1	2,61	0,00125
HPSE	2,73	0,00129
FAM134B	1,44	0,00155
ITGA4	2,4	0,00166
ADAM19	1,39	0,00173
ITPR1	1,33	0,00176
LBR	1,19	0,00222
C17H5orf52	-2,03	0,00229
RAB19	3,02	0,00243
CLDN4	-2,39	0,00244
DEFB1	-2,78	0,00254
MDN1	1,45	0,00283
SMPD3	2,35	0,00286
CYP26B1	2,91	0,00291

2.3.2   Gene Set and Pathways analysis

Despite not finding individual genes with a significant difference of expression, KEGG pathway analysis identified 39 pathways enriched for up-regulated genes with q-value < 0.05 (Supplemental table 1) and six pathways enriched for down-regulated genes with q-value <0.02 (Supplemental table 2). This significance of the pathways is possible because little coordinated changes of gene expression in a pathway may have a substantial biological effect even if these changes are not significant for any individual genes. Among the pathways enriched for up-regulated genes associated with puberty, there were important pathways involved in reproductive processes: oxytocin signaling pathway, estrogen signaling pathway, oocyte meiosis, GnRH signaling pathway,progesterone-mediated oocyte maturation, cAMP signaling pathway,. ErbB signaling pathway and MAPK signaling pathway. Also present were neuronal communication related pathways: cholinergic synapse and axon guidance.

No significantly enriched GO categories for biological processes were found on GAGE analysis.

2.4 Discussion

To our knowledge, our study was the first to investigate puberty with better controlled genetic and environmental variation in heifers. Our early and late pubertal heifers are related through the father's side, bearing similar genetic backgrounds, are age matched and their body weights were highly homogeneous. Moreover, their accommodation conditions and feeding diet were exactly the same. Thus the gene expression of the heifers in our study should provide an accurate illustration of developmental events surrounding pubertal timing.

2.4.1 KEGG pathway analysis

2.4.1.1 Progesterone-mediated oocyte maturation and estrogen signaling

Steroid hormones are required for a normal reproductive activity. Estradiol signaling through estrogen receptor alpha appears to be necessary for the changes required for pubertal development [17].It was demonstrated that conditional ablation of estrogen receptor α in kisspeptin neurons results in advancement of puberty onset in female mice [43] showing the importance of estrogen signaling in the timing of puberty. The fact we found this pathway up regulated in adipose tissue in our heifers is yet another evidence for an existing correlation to neuronal influences during peripubertal period.

Proper development of the oocyte starts just prior to ovulation, when its maturation occurs[32]. In our pubertal heifers the oocyte maturation pathway is up regulated , a physiologically expected phenomena in order for ovulation to happen. This adds up more certainty to our hypothesis that the adipose tissue is reflecting very specific physiological changes related to sexual maturation.

2.4.1.2 GnRH signaling pathway

In our pubertal heifers the GnRH signalling pathway is up regulated. The hypothalamic release of pulsatile GnRH is considered the trigger for mammalian puberty because it acts upon its receptor in

the anterior pituitary to regulate the production and release of the gonadotropins, LH and FSH(follicle stimulating hormone), hormones required for gonadal activity and gametogenesis[25].

2.4.1.3 Axon guidance signaling pathway

Our pubertal heifers had the axon guidance signalling pathway up regulated. Axon guidance represents a key stage in the formation of neuronal network. Genes implicated in the formation and physiology of the central nervous system play a role in reproduction through influence on neuronal formation, differentiation, communication and reproductive hormone synthesis [48].

2.4.1.4 Oxytocin signaling pathway

Oxytocin (OT) is a nonapeptide synthesized in the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus. Its most well-established roles are stimulation of uterine contractions during parturition and milk release during lactation. Oxytocin also influences various sexual behaviors in some mammals [44] .Oxytocin was found to facilitate sexual maturation in female rats. This effect is mediated by a mechanism involving production of PGE_2 (prostaglandin E_2) from hypothalamic astrocytes which accelerates pulsatile GnRH release [50].The oxytocin gene was up regulated in adipose tissue of pubertal Brangus heifers [14]. Adipose tissue is not the main local of synthesis or action of oxytocin and, our finding that its signalling pathway could be identified as up regulated in adipose tissue in pubertal heifers, is another indication of communication between reproductive tissues.

2.4.2   Genes already found associated with reproductive processes

2.4.2.1 Genes Involved in Steroid hormone regulation and bioactivity

In our pubertal heifers, an enzyme that metabolizes progesterone CYP17A1(cytochrome P450 17A1) - had its transcription level down regulated after ovulation (when the LH surge happens). This is consistent with findings that the enzyme was down regulated in bovine follicles (theca cells) after the LH surge preceding ovulation which also augmented chromatin condensation in its promoter [46]. There have been reports of delayed puberty in humans, for example in a case with a homozygous

mutation on the sequence of this gene [19] and also complete failure regarding the onset of puberty development in instances when the gene sequence has been deleted [13]. In our heifers that reached puberty we have found this enzyme down regulated which may result in circulating progesterone being available for a longer period of time because it is not being metabolized. A corresponding mechanism was confirmed by Eriksson et al [26] in a study that found a polymorphism in an estrogen metabolizing enzyme (catechol-o-methyltransferase-COMT). This mutation rendered the COMT enzyme less active, heightening levels of circulating estrogen and was associated with early pubertal development in girls. Moreover, Anderson et al [3] proposed that progestin administration induces puberty in heifers by accelerating the peripubertal decrease of estradiol negative feedback on LH secretion, highlighting the importance of adequate progesterone levels in puberty manifestation.

AKR1C4 (aldo-keto reductase 1C4) was found down regulated in our early pubertal heifers. AKRs regulate the occupancy and transactivation of several steroid receptors in target tissues leading to transcription of hormone -responsive genes [52] regulating processes involved in development, homeostasis and reproduction. The products of AKR activity have been implicated in obesity, polycystic ovary disease and a delay in the onset of puberty in humans [9; 54; 58]. Two SNP in AKR1C4 were associated with age at puberty and nipple number in a study assessing the AKR1C gene cluster for effects on reproductive traits in swine Meishan crosses [47]. The neurosteroid 3alpha-hydroxy-4-pregnen-20-one (3-α-HP) produced from progesterone by AKR1C inhibits GnRH activity on gonadotropes and suppresses FSH release from pituitary cells [28]. Modulation of the GnRH pulse frequency could therefore cause variation in the timing of puberty. Knowing that in heifers reaching puberty AKR1C4 is down regulated, one could assume that a possible consequence would be less 3-α-HP being produced from progesterone thus not inhibiting GnRH activity leading to normal release of FSH.

2.4.2.2 Genes Involved in Regulation of developmental processes in reproductive organs

We found a methyltransferase enzyme responsible for the de novo methylation - DNMT3B up regulated in early puberty - which targets unmethylated and hemimethylated substrates and, is

essential for the establishment of DNA methylation patterns during development. De novo metilation is a mechanism which when blocked pharmacologically in the hypothalamus leads to a delay in the onset of puberty. This observation suggests that interruption of this mechanism activates repressor genes whose expression would diminish at puberty [48].This argument is supported by a study of genome wide analysis showing entire regions of different chromosomes becoming hipomethylated or hipermethylated with the advent of puberty [48]. DNMT3B methylates the transcription promoters during meiosis in mouse oocytes, ceasing aberrant transcription [40; 69] demonstrating its function of reproductive relevance in the ovary as well. DNMT3A, DNMT3B, and DNMT3L mRNA and protein expression were detected in growing bovine oocytes illustrating that the methylation machinery necessary for establishing de novo methylation patterns is present during the period of bovine DNA methylation imprint acquisition [24]. Cyclical methylation of the E2 (estrogen) responsive ps2 promoter DNA mediated by DNMT1, DNMT3A and DNMT3B occurs on a timescale of tens of minutes[34] demonstrating the degree of plasticity of this epigenetic response to a steroid hormone.

CYP26B1(cytochrome P450 26B1) is an enzyme essential for postnatal survival and development of germ cells and, one of its mechanisms of action is to degrade retinoic acid which in turn initiates meiosis [12;68]. In our pubertal heifers the released mature egg is arrested in metaphase II, and should complete meiosis II after fertilization. Up regulation of CYP26B1 could be one of the factors inhibiting premature entry into meiosis through augmented degradation of retinoic acid. The relevance of receptors for retinoic acid (RAR) on puberty timing was shown in a genome wide analysis that found signals near RAR-related genes. These signals are significantly enriched for associations with age at menarche in women [20]. Another function found for CYP26B1 enzyme is to mediate the autocrine/paracrine functions of activin in regulating ovarian follicle formation and development in the postnatal ovaries [38].

Heparan sulfate proteoglycans are major components of the basement membrane and extracellular matrix. Heparanase (HPSE) is an enzyme that cleaves heparan sulfate proteoglycans to permit cell movement through remodeling of the extracellular matrix. It is up regulated in pubertal heifers. Transient levels of HPSE were evident in granulosa cells and macrophages of human and murine

ovaries during the luteal phase and luteal regression, supporting a role for HPSE in extracellular matrix and basement membrane remodeling in the ovary [29]. In an association study for reproductive traits for a swine Landrace-Duroc-Yorkshire population using SNPs in HPSE, significant polymorphisms were found for age at puberty, ovulation rate and number of piglets born alive [56].

2.4.2.3 Genes Involved in Reproductive mechanisms originating in the nervous system

Teneurin C-terminal-associated peptide (TENM1 or ODZ3) plays a role in the regulation of neuroplasticity in the limbic system, and acts as a bioactive neuroprotective peptide on limbic neurons of the brain. The limbic system, among other functions, controls sexual and emotional behavior. A significant SNP in TENM1 for conception and age at first birth was found in Nellore heifers on a genomic association study for reproductive traits [18].Evidence has been found of an association between estrus behavior and reproductive efficiency in dairy cattle [59]. Consequently, genes found expressed in both adipose tissue and limbic system could be further explored to understand how this connection interferes in pubertal development.

SMPD3(sphingomyelin phosphodiesterase 3) is an enzyme that catalyzes the hydrolysis of sphingomyelin to form ceramide and phosphocholine, predominantly expressed in CNS(central nervous system) neurons. It plays a critical role in the control of late embryonic and postnatal development. The knockout mutant mouse SMPD3 -/- develops an unique form of dwarfism and delay in pubertal development as part of a deficiency in anterior pituitary hormones caused by absent secretion of GnRH in the hypothalamus .Lack of SMPD3 production also led to impaired ovary growth [66], reinforcing the idea that its action is crucial for pubertal development; this coincides with our data showing its expression up-regulated in early pubertal heifers.

FAM134B –is a protein that functions as a receptor in the endoplasmic reticulum(ER) . It is necessary for long-term survival of nociceptive and autonomic ganglion neurons. A non-sense mutation of this gene in humans causes hereditary sensory and autonomic neuropathy [45] - a disease found connected to central precocious puberty condition [30] (Fig. 2). Central precocious puberty in humans is caused

by a mutation in the MKRN3 (makorin ring finger protein 3) gene [1], MKRN3 also had a SNP associated with the early pregnancy phenotype in Nellore cattle[31].Our suggestion is that the relationship regarding FAM134B and MKRN3 participates in pubertal mechanisms.

FIG. 2. Relationship between FAM134B and MKRN3 genes[72]
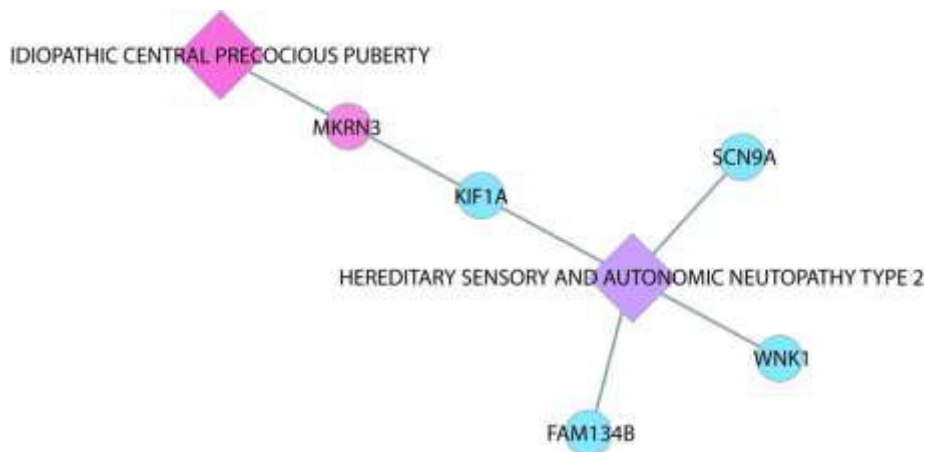
2.4.2.4 Genes involved in energy homeostasis and development

APOD (apolipoprotein D) is widely distributed in the mammalian organisms ,occurring in macromolecular complexes, predominantly with APOA2 in HDL and APOB-100 in LDL and VLDL. Balbin et al [4] & Dilley et al [22] found that the amino acid sequence of a progesterone-binding protein, GCDFP-24, present in high concentration in gross cystic disease fluid [51], matched perfectly with the sequence of the human APOD protein. As a consequence, a putative role of APOD in the transport of steroid hormones has been proposed. Hypothalamic levels of mRNA APOD increase and are positively correlated with body fat and circulating leptin in rats submitted to high-fat diet. Furthermore, APOD and the leptin receptor Ob-Rb were seen colocalized in hypothalamic neurons, and a binding interaction between them was confirmed [39]. It was observed that in rabbits the expression of APOD mRNA in the epididymis could be modulated during sexual maturation in epididymal connective tissue [53]. Moreover, APOD was also found interacting with VAPA (vesicle-

associated membrane protein)[63] (Fig.3), which had a SNP associated with pregnancy status in Brahman cows [57].Although the relationship among these genes are not fully understood, we conjecture a bond that regulates energy homeostasis fundamental to the achievement of pubertal status because APOD was found differentially down-regulated in our early pubertal heifers.

FIG. 3. Identified interactions among genes APOD, LEPR and VAPA [73]
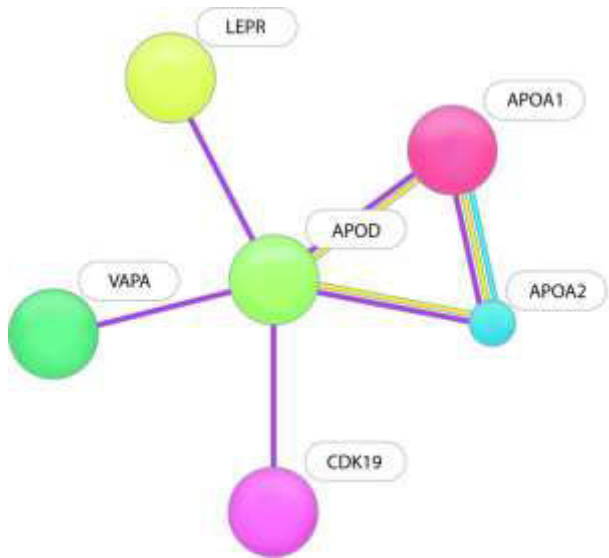
The evidence obtained in our study combined with data from previous research, implies that the adipose tissue presents an intrinsic expression of genes that regulate the activity of neural and sexual hormone factors, thus determining more autonomy in its regulatory functions on the manifestation of puberty. It seems sensible that functional studies follow up on our research to confirm these findings; for example a study to measure the expression level of these genes at different time points during pubertal development and their related protein expression. However, we believe these studies should be conducted in the context of the organism as a whole and not in isolated adipocytes because the expression changes of such factors depend on the immersion of the adipose tissue in this blend of substrates and hormones delivered via blood flow, plus the action of the autonomic nervous system and feedback from autocrine and paracrine effectors [5; 27]. In addition, the transformation of a given hormone to another by steroid-converting enzymes may modulate metabolic pathways and other adipose tissue functions [36;67].

The genes found differentially expressed in adipose tissue and its functions on reproductive processes suggest that expressed genes in the adipose tissue work in tune with the physiological mechanisms observed in the ovary, uterus, hypothalamus and pituitary during the peripubertal period. It was particularly interesting to discover genes that are mainly expressed in these tissues also differentially expressed in adipose tissue. The significance of our findings resides in the genes and pathways found and how these discoveries may be useful to better understand how the adipose tissue behaves during the pubertal period.

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73. Figure 3. Gene Cards website[http://www.genecards.org/cgi-bin/carddisp.pl?gene=APOD]

SUPPLEMENTAL DATA

Supplemental table 1: KEGG pathways enriched for up-regulated genes

Kegg Pathway	p.geomean	stat.mean	p.value	q.value	set.size	exp1
bta04022 cGMP-PKG signaling pathway	9,51E+09	379.151.086.570.735	9,51E+09	0.0075037	119	9,51E+09
bta04810 Regulation of actin cytoskeleton	9,93E+09	3.771.648.408.908	9,93E+09	0.0075037	139	9,93E+09
bta04510 Focal adhesion	0.000117315066433149	372.839.412.089.561	0.000117315066433149	0.0075037	137	0.000117315066433149
bta04660 T cell receptor signaling pathway	0.000151590510566051	369.558.180.109.717	0.000151590510566051	0.0075037	79	0.000151590510566051
bta04151 PI3K-Akt signaling pathway	0.000277324964108044	347.757.215.475.108	0.000277324964108044	0.0094755	229	0.000277324964108044
bta04070 Phosphatidylinositol signaling system	0.000325704847596932	348.551.144.042.879	0.000325704847596932	0.0094755	73	0.000325704847596932
bta04068 FoxO signaling pathway	0.000357454477428662	344.021.706.861.535	0.000357454477428662	0.0094755	96	0.000357454477428662
bta04630 Jak-STAT signaling pathway	0.00038285020557561 3	343.066.859.660.475	0.000382850205575613	0.0094755	82	0.000382850205575613
bta04010 MAPK signaling pathway	0.000562484104663098	328.424.215.929.187	0.000562484104663098	0.0123746	179	0.000562484104663098
bta04919 Thyroid hormone signaling pathway	0.00063449194472704	328.057.820.719.625	0.000634491944727039	0.0125629	82	0.00063449194472704
bta04611 Platelet activation	0.000739423340453723	322.757.122.116.193	0.000739423340453722	0.0133096	93	0.000739423340453723
bta04662 B cell receptor signaling pathway	0.000826886285524604	322.496.146.806.773	0.000826886285524603	0.0136436	57	0.000826886285524604
bta04921 Oxytocin signaling pathway	0.00134058062336574	304.072.659.610.983	0.00134058062336575	0.0184695	101	0.00134058062336574
bta04015 Rap1 signaling pathway	0.0013963099114737	301.433.926.190.864	0.00139630991147371	0.0184695	153	0.0013963099114737
bta04725 Cholinergic synapse	0.0013992091579536	304.633.696.714.653	0.00139920915795359	0.0184695	69	0.0013992091579536
bta04666 Fc gamma R-mediated phagocytosis	0.00159326440232322	300.033.167.795.263	0.00159326440232323	0.0197166	72	0.00159326440232322
bta04014 Ras signaling pathway	0.00199710723177895	290.021.126.862.815	0.00199710723177894	0.0230556	157	0.00199710723177895
bta04020 Calcium signaling pathway	0.00209596541321151	289.509.036.149.807	0.00209596541321151	0.0230556	106	0.00209596541321151
bta04520 Adherens junction	0.00235299484990644	288.871.429.267.817	0.00235299484990644	0.0245206	54	0.00235299484990644
bta04110 Cell cycle	0.00262214633240405	282.255.067.260.669	0.00262214633240405	0.0259592	102	0.00262214633240405
bta04750 Inflammatory mediator regulation of TRP channels	0.00293721488090761	280.021.439.587.923	0.00293721488090761	0.0268301	67	0.00293721488090761
bta04144 Endocytosis	0.00309182461748982	275.344.465.082.256	0.00309182461748983	0.0268301	191	0.00309182461748982
bta04915 Estrogen signaling pathway	0.00311663664487327	277.735.359.133.297	0.00311663664487327	0.0268301	71	0.00311663664487327
bta04114 Oocyte meiosis	0.00367885745948595	271.340.845.293.104	0.00367885745948596	0.0300006	85	0.00367885745948595
bta04012 ErbB signaling pathway	0.00396063788263413	27.015.134.260.796	0.00396063788263413	0.0300006	60	0.00396063788263413
bta04550 Signaling pathways regulating pluripotency of stem cells	0.00415319453712828	267.101.114.707.328	0.00415319453712828	0.0300006	85	0.00415319453712828
bta04360 Axon guidance	0.00417002770914036	265.985.857.862.896	0.00417002770914037	0.0300006	122	0.00417002770914036
bta04970 Salivary secretion	0.00424251558796204	268.762.398.999.515	0.00424251558796203	0.0300006	50	0.00424251558796204
bta04540 Gap junction	0.00458793388920192	26.491.020.300.797	0.00458793388920192	0.0313249	60	0.00458793388920192
bta04914 Progesterone-mediated oocyte maturation	0.00485291794143231	26.249.110.403.819	0.00485291794143231	0.0320292	66	0.00485291794143231
bta00562 Inositol phosphate metabolism	0.00619779471195411	254.681.884.898.276	0.00619779471195411	0.0390330	51	0.00619779471195411
bta04730 Long-term depression	0.00642122121005879	254.887.910.022.109	0.00642122121005879	0.0390330	39	0.00642122121005879
bta04024 cAMP signaling pathway	0.00650550238040324	250.370.874.560.327	0.00650550238040324	0.0390330	112	0.00650550238040324
bta04340 Hedgehog signaling pathway	0.00679654197479026	254.283.392.374.259	0.00679654197479026	0.0395798	31	0.00679654197479026
bta04912 GnRH signaling pathway	0.00805509405455913	244.110.821.703.511	0.00805509405455913	0.0433117	61	0.00805509405455913
bta04971 Gastric acid secretion	0.00806868761789906	245.096.476.325.602	0.00806868761789908	0.0433117	47	0.00806868761789906
bta04310 Wnt signaling pathway	0.00817504245923219	242.408.559.838.906	0.00817504245923219	0.0433117	90	0.00817504245923219
bta04261 Adrenergic signaling in cardiomyocytes	0.0083554640864285	241.392.831.442.968	0.00835546408642851	0.0433117	98	0.0083554640864285
bta04514 Cell adhesion molecules (CAMs)	0.0085311010650622	240.888.870.732.685	0.00853110106506221	0.0433117	91	0.0085311010650622

SUPPLEMENTAL table 2: KEGG pathways enriched for down-regulated genes

Kegg Pathway	p.geomean	stat.mean	p.val	q.val	set.size	exp1
bta03010 Ribosome	3,53E-12	-125.380.245.471.066	3,53E-13	6,99E-11	123	3,53E-12
bta00190 Oxidative phosphorylation	4,21E-02	-877.823.422.561.122	4,21E-02	4,17E+00	109	4,21E-02
bta03050 Proteasome	9,59E+08	-468.912.116.165.026	9,59E+08	0.0006329	40	9,59E+08
bta04260 Cardiac muscle contraction	0.0002482219162941 54	-363.539.657.603.111	0.0002482	0.0122869	40	0.000248221916294154
bta00980 Metabolism of xenobiotics by cytochrome P450	0.000377986040373062	-364.861.856.353.205	0.0003779	0.0149682	21	0.000377986040373062
bta00590 Arachidonic acid metabolism	0.000626916302219679	-337.914.525.035.377	0.0006269	0.0206882	33	0.000626916302219678