JULIANE DINIZ MAGALHÃES

Estudo do mecanismo molecular da progesterona e do estradiol sobre o início da puberdade em novilhas Nelore

Pirassununga-SP

2014

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Tese apresentada ao Programa de Pós-Graduação em Nutrição e Produção Animal da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para a obtenção do título de Doutor em Ciências

Departamento:

Nutrição e Produção Animal

Área de Concentração:

Nutrição e Produção Animal

Orientador:

Prof. Dr. Luis Felipe Prada e Silva

Pirassununga-SP

2014

DADOS INTERNACIONAIS DE CATALOGAÇÃO-NA-PUBLICAÇÃO

(Biblioteca Virginie Buff D'Ápice da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo)

T.3000
FMVZ

Magalhães, Juliane Diniz
Estudo do mecanismo molecular da progesterona e do estradiol sobre o início da puberdade em novilhas Nelore / Juliane Diniz Magalhães. -- 2014.
73 f. : il.

Tese (Doutorado) - Universidade de São Paulo. Faculdade de Medicina Veterinária e Zootecnia. Departamento de Nutrição e Produção Animal, Pirassununga, 2014.

Programa de Pós-Graduação: Nutrição e Produção Animal.

Área de concentração: Nutrição e Produção Animal.

Orientador: Prof. Dr. Luis Felipe Prada e Silva.

1. Hipotálamo. 2. Neuropeptídeo Y. 3. PCR quantitativo. 4. Reprodução. 5. RNA-Seq. I. Título.

UNIVERSIDADE DE SÃO PAULO

FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

Comissão de Ética no uso de animais

CERTIFICADO

Certificamos que o Projeto intitulado "Estudo do mecanismo molecular da progesterona e do estradiol sobre o início da puberdade em novilhas Nelore", protocolado sob o nº 2475/2011, utilizando 36 (trinta e seis) bovinos, sob a responsabilidade do Prof. Dr. Luis Felipe Prada e Silva, está de acordo com os princípios éticos de experimentação animal da "Comissão de Ética no uso de animais" da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo e foi aprovado em reunião de 16/5/2012.

We certify that the Research "Molecular mecanism study of progesterone and estradiol on the onset of puberty in Nelore heifers", protocol number 2475/2011, utilizing 36 (thirty six) heifers, under the responsibility Prof. Dr. Luis Felipe Prada e Silva, agree with Ethical Principles in Animal Research adopted by "Ethic Committee in the use of animals" of the School of Veterinary Medicine and Animal Science of University of São Paulo and was approved in the meeting of day 5/16/2012.

São Paulo, 17 de maio de 2012.

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Título: Estudo do mecanismo molecular da progesterona e do estradiol sobre o início da puberdade em novilhas Nelore

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Data: ____/____/____

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Instituição: _____ Julgamento: _____

Prof.(a) Dr.(a): _____

Instituição: _____ Julgamento: _____

Prof.(a) Dr.(a): _____

Instituição: _____ Julgamento: _____

Prof.(a) Dr.(a): _____

Instituição: _____ Julgamento: _____

Prof.(a) Dr.(a): _____

Instituição: _____ Julgamento: _____

Dedico à minha família em especial à minha mãe, minha base meu chão.

AGRADECIMENTOS

Agradeço a Deus e a Nossa Senhora por me fazerem forte, me manterem firme, segura dos meus objetivos, e persistente. Agradeço toda a proteção e às bênçãos recebidas.

À minha família, meu maior tesouro, meu chão, meu mundo. Em especial à minha mãe, Leila, meu porto seguro e meu maior apoio, a quem tanto amo. Ao meu pai que me apresentou outro mundo e a quem tenho aprendido a compreender. Aos meus avós, Maria e Afonso, fontes de inspiração, alicerces da nossa família, exemplos de união e amores da minha vida. À minha avó Estelita, muitas saudades e ao avô Ernesto que não tive oportunidade de conhecer. Aos meus tios e padrinhos, Keila (amiga inseparável e verdadeira), Marciano (incentivador das minhas idéias mais malucas), tio Júnior (pai dos meus amores), Marlon, Sônia, Netinho, Smênia, Valmira, Xandy, Néia, Vanusa, Alípio, Toninho, Mara, eternos cúmplices, amigos, apoios, pessoas que ajudaram a formar minha personalidade e me ajudaram a ser uma pessoa melhor, obrigada por confiarem em mim. Aos meus irmãos Newton, Daniel, Grégory, parceiros eternos de vida, farras, bons momentos e dificuldades e às minhas irmãs Isabela e Lívia, minhas lindas! Amo a todos vocês! Aos meus primos lindos, Neto, Gu, Bia, Arthur, Lorrane, Igor, Fred, Breno, Grazian, Dani (e família), amo vocês demais apesar da distância. Aos meus dois afilhados Arthurzinho e Gu, que me desmontam a cada passagem por Brasília. E o agradecimento mais especial aos novos presentes de Deus que trouxeram vida nova e alegria à família meus sobrinhos Esther, Enzo e Anna.

A todos os meus amigos de Brasília, que entendem o motivo da minha distância, mas não menor consideração.

À FAPESP pela concessão da bolsa de doutorado e pelo auxílio financeiro à pesquisa, que me possibilitaram ter tranquilidade no desenvolvimento de minhas atividades.

À Prefeitura do Campus da USP de Pirassununga por disponibilizar as instalações do abatedouro escola e parte dos animais.

Ao professor Luis Felipe Prada e Silva, por todo o apoio, confiança, amizade e estrutura, sem os quais não seria possível o desenvolvimento do trabalho. E por todo o aprendizado e vivência fora do projeto de pesquisa, mas dentro do contexto do LPGC, que só me auxiliam a crescer e me fazem uma melhor profissional a cada dia.

Aos meus professores e eternos amigos da UPIS que sempre me apoiaram e colocaram para frente, em especial a todos que me colocaram no caminho da Nutriçao, Reprodução e Biologia Molecular. Dedico ao professor Marco Aurélio Alves de Freitas Barbosa (Marcão,

Titio, Podrão...), triste por não ter lido a última mensagem que deixei para você e por não poder compartilhar mais esta conquista com você. Já me imaginava fazendo parcerias em pesquisas, mas você resolveu nos deixar antes que eu tivesse oportunidade. Os valores e ensinamentos estarão em mim eternamente, saudades.

Aos professores do departamento de Nutrição e Produção Animal da FMVZ-USP por todos os ensinamentos e pelo convívio.

Aos professores José Luiz Moraes de Vasconcelos (Zequinha), Mike Day, Ed Hoffman Madureira, Marcos Santos Veiga, Mario Binelli e Cláudia Leal, pelos ensinamentos, por me auxiliarem no delineamento, tirarem minhas dúvidas, cederem equipamentos, laboratórios e equipe de trabalho sempre que precisei.

A toda a equipe do Laboratório de Pesquisa em Gado de Corte (LPGC), em especial ao prof. Luis Felipe, Bruno Mesquita (Xaxá), Dannylo Oliveira (Denis), Frederich Diaz (Fred), Bárbara Marques (Babita) e Sr. Sérgio; Rafael, Nara, Johnny, Vivi, Marina, Lígia Mesquita (mãe do ano!!!), João Penso (Begônia), Renan (Fimose), Edimarco, Rafaela Bertolini, Júlia Marques, Alexandre (Pégasus) por todo o auxílio, seja a campo, com tabelas, laboratorial e por que não emocional... Ao Antônio Schimidt, nosso mais novo membro, que não participou da execução do experimento, mas participa ativamente de todas as atividades do laboratório e nos ensina um pouco mais sobre os animais todos os dias. Obrigada por tudo!!!

Ao Milton Maturana Filho pela amizade e presença constante e fundamental em toda a execução do experimento a campo. Você é um mala, mas eu te adoro!

A toda a equipe do professor Ed Hoffman Madureira, os pós-graduandos: Milton Maturana, Thiago Santin (Arroz), Ju Naves (Mega) e os estagiários: Tiago Nakamura, Fidel Grabert, David, Virgílio (Papa), Thiago (Kumi), Rômulo Germano, Gabriela Fonseca, Mariana, Roberto e Miguel, pela indispensável parceria durante toda a fase de campo e aos novatos Klebão, Augustin, Ismael e Felipe por todo o auxílio nas IATFs do LPGC e por aguentarem meus esresses em dias de manejo.

A todos os amigos que participaram dos abates: Denis, Xaxá, Babi, Fred, prof. Luis Felipe, Lari, Ju Barreiro, Qui, Bettha, Tácia, Zé Alípio, Xibas, Frodo, Rodrigo (Gorgonzola), Henrique (Bronha) e ao meu dessecador de úteros oficial Tiago Tomazi. À Lígia e ao Pedrinho que cuidaram dos ovários, à Moana e ao Fernando que dissecaram os endométrios e me deixaram tranquila durante os abates. Obrigada por me emprestarem seu tempo e contribuírem muito com nosso trabalho.

Um agradecimento especial à Lígia pelo auxílio e por dividir dias super descontraídos e todo o conhecimento no laboratório de Genômica. Parabéns mamãe!

Aos colegas de pós, sejam do VNP, VRA, VCI ou FZEA, que fizeram e fazem parte da caminhada diária.

À Carol Tobias, pessoa que admiro muito. Obrigada por sua amizade e presença frequente em minha vida e em nossas conquistas. Um dia evoluo o suficiente para manter a calma e a razão como você. Te Adoro!!!

Impossível esquecer quem conviveu e convive diariamente comigo, a família que construímos em Pirass! Chatinho (Johnny) que participou e dividiu o mestrado comigo, você é o cara!!! Saudade de vc sempre. Após quatro anos de convivência o Re nos abandonou, mas faz parte, tava na hora de acabar a faculdade, né Carioca? Nosso último ano de convivência fez crescer ainda mais a amizade, adoro vc. Felipe Syuffi (Arma) aprendi a gostar de você logo de cara, foi uma convivência única, ano intenso naquela casa que deixou saudades... Mineirinho, amigo mais que especial e presente, bom demais acompanhar sua evolução. Aí vem a nova fase da casa... Quem diria Ju Mega, depois de 4 anos se tornou uma república de meninas? Ju, não é segredo para ninguém o quanto é importante em minha vida, amizade verdadeira, cúmplice e duradoura!!! Lari, um presente morar com você, nossa trajetória aqui dentro é muito semelhante e o convívio com você, sua família e o Marcão, só me fazem crescer. Mi, a mascote da casa, protegida e cuidada por todos, é um privilégio conviver com você e contar com sua amizade. Meninas, a vida me deu de presente 3 irmãs, AMO vocês!!!

Aos eternos agregados, amigos antigos que sempre pedem asilo em casa quando voltam a Pirass, sempre bom receber os amigos.

Às pequenas e não menos importantes moradoras Periquita, Heineken, Belinha e Cacau e aos demais animais agregados, por tornarem meus dias menos pesados.

Às novilhas, motivos do meu estudo, às vacas, aos bezerros, aos gatos e todos os animais que nos geraram e geram mais conhecimento todos os dias.

Às pessoas mais especiais que conheci e convivi aqui: Ju Barreiro, Pauleta, Dani Donato, Ju Pin, Ju Mega, Lari, Mi, Eriketa e Julinha, Jeffão, Henry, Johnny, Mineirinho, Boi, Tenente, Lucas, Xibas, Frodo, Arroz, Perna, Rafael Françoso, Iaça, Henrique, Qui, Bettha, Mona, Dudu, Flavinho, Van, Bre, Doci... Guardo vocês em um lugar mais que especial. Obrigada pela oportunidade de conviver com vocês e desfrutar desta amizade forte e verdadeira.

A uma portuguesa que entrou na minha vida e se tornou parte dela. Ana Carina (só para irritar você). Adoro você Qui e o meu sobrinho fujão (Mingau). Nossa caminhada só começou! Nem um oceano é capaz de nos separar, rs.

A três presentes que ganhei este ano Van, Bre e Doci, vocês tornaram meus dias menos monótonos e garantiram boas risadas, além de uma nova amizade que chegou em uma hora perfeita e necessária. Só tenho uma coisa para dizer, os momentos princesas jamais serão esquecidos.

Aos funcionários do VNP, em especial à Alessandra (Lelê), à Fábia e ao João que seguram nossos rojões, muito obrigada. Aos funcionários da Bromato, da TPOA, da faxina e da segurança, muito obrigada.

Aos funcionários do STAPIR, Marcão, Paula, Alex, Magali e Adones por todo o auxílio e dedicação. Ao Júnior e ao Nei por todo auxílio técnico. À Míriam e à Dona Dalva, que sempre cuidaram do nosso ambiente de trabalho, obrigada.

Aos funcionários do Gado de Corte, Ismael, Paulinho, Ricardo e Macarrão que tanto me ajudaram no manejo das novilhas nas fugas e nos abates.

A todos os funcionários do matadouro escola, pela disponibilidade e apoio, obrigada.

Aos queridos amigos do vôlei, que me ajudaram a controlar o estresse do dia-a-dia e me proporcionaram momentos inesquecíveis, tá difícil ficar longe, mas a causa é nobre. Ao Margutti e à Rafa, por todo o apoio e estímulo. Foi difícil demais ter que abrir mão, saudade.

A todos que fizeram parte desta caminhada, agradeço de coração.

RESUMO

DINIZ-MAGALHÃES, J. **Estudo do mecanismo molecular da progesterona e do estradiol sobre o início da puberdade em novilhas Nelore**. [Study of the molecular mechanism of progesterone and estradiol on the onset of puberty in Nellore heifers]. 2014. 73 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, 2014.

A elucidação dos mecanismos moleculares pelos quais tratamentos hormonais alteram o início da puberdade é de fundamental importância para o desenvolvimento de estratégias que reduzam a idade ao primeiro parto, e consequentemente a taxa de desfrute do rebanho Nelore. Foram investigados os efeitos do uso de dispositivos de progesterona, e do estradiol endógeno, sobre mecanismos moleculares controlando a obtenção da puberdade de novilhas Nelore peripúberes. Especificamente, como as diferenças na expressão de genes relativos à reprodução em duas áreas do hipotálamo. Trinta e cinco novilhas Nelores não púberes, e com idade entre 13 e 14 meses, foram divididas em quatro tratamentos experimentais (nove ou oito por tratamento): dispositivo de P4 sem estradiol (SP); dispositivo de P4 com estradiol (PE); sem dispositivo de P4 e sem estradiol (SS); e sem dispositivo de P4 e com estradiol (SE). As novilhas foram alimentadas no cocho pós desmame até atingirem 295 ± 11 kg, com fornecimento de água à vontade. Ao término do tratamento hormonal as novilhas foram abatidas e as porções de hipotálamo colhidas para processamento e armazenagem a -80 ºC. O RNA total do tecido hipotalâmico foi extraído, tratado com DNAse I e submetido à síntese de cDNA para estudo da expressão gênica por PCR em tempo real (qRT-PCR). Foram formados pools de RNA para a realização de um estudo abrangente da administração de progesterona e do efeito do estradiol endógeno e das diferenças entre áreas do hipotálamo, realizado por sequenciamento de nova geração (RNA-Seq), de forma a identificar possíveis genes candidatos no hipotálamo. Foram encontrados genes diferencialmente expressos alterados pelos tratamentos e entre as áreas do hipotálamo relativos à obtenção da puberdade.

Palavras-chave: Hipotálamo. Neuropeptídeo Y. PCR quantitativo. Reprodução. RNA-Seq.

ABSTRACT

DINIZ-MAGALHÃES, J. **Study of the molecular mechanism of progesterone and estradiol on the onset of puberty in Nellore heifers**. [Estudo do mecanismo molecular da progesterona e do estradiol sobre o início da puberdade em novilhas Nelore]. 2014. 73 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, 2014.

The understanding of the molecular mechanisms by which nutrition, genetics and hormonal treatments affect the beginning of puberty is of great importance for developing strategies aiming to reduce the age at first calving, and therefore increase the slaughter rate in Nellore cattle. The effects of progesterone device and of endogenous estradiol on the molecular mechanisms controlling the attainment of puberty in Nellore heifers were investigated. Specifically, the molecular pathways of progesterone and estradiol were studied in the hypothalamus. Thirty five non-pubertal heifers, between 13 and 14 months of age, were divided into four treatment (nine or eight per treatment): P4 device without estradiol (SP), P4 device with estradiol (PE), without P4 device and without estradiol (SS), and without P4 device and with estradiol (SE). The heifers were fed after weaning until reach 295 ± 11 Kg, with water access. At the end of the hormonal treatments all heifers were slaughtered and the hypothalamus areas were harvested, processed and then also stored at -80°C. Total RNA of hypothalamus were extracted, treated with DNase I and submitted to cDNA synthesis for gene expression quantification by real time PCR (qRT-PCR). RNA samples were pooling to realize a comprehensive study of the effects of progesterone administration and endogenous estrogen on attainment of puberty by next-generation sequencing (RNA-Seq), in order to identify possible candidate genes in the hypothalamus. Genes diffentially expressed between hypothalamic areas and affected by treatments were found.

Key words: Hypothalamus. Neuropeptide Y. Quantitative PCR. Reproduction. RNA-Seq.

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1 INTRODUCTION

Currently, Brazil is the second largest beef producer, being behind only of the United States, and the world's second largest beef exporter (USDA, 2012). However, Brazilian meat production is on a wide stretch of land, with low stocking rates and productivity (ANUALPEC, 2012). One reason to the low efficiency of beef production system is the delayed onset of reproductive activity that has a big impact in calf production. The heifers are the basis of the replacement herd, however if they become pregnant at high ages, they will provide delay and damage to the productive system.

The Nellore breed (*Bos indicus*), although well adapted to tropical conditions, reaches puberty at more advanced age than European breeds, such as Angus, Hereford and Shorthorn, even when reared under similar conditions (RODRIGUES; KINDER; FITZPATRICK, 2002). But more specialized and genetically improved systems produce sexually precocious Nellore females, which in an appropriate plane of nutrition and health conditions, reach puberty at 14 months. Explore genetic characteristics, combined with nutrition and behavioral characteristics, allows the insertion of young heifers in the breeding herd, increasing productive efficiency.

Puberty is defined as the acquisition of reproductive competence, and is directly linked to the ability of hypothalamic neurons to produce gonadotropin-releasing hormone (GnRH) in sufficient quantities to promote and support gametogenesis (SENGER, 2012). The hypothalamic maturation and the attainment of puberty occurs through feedback mechanisms involving the hypothalamic-pituitary-gonadal axis (KINDER et al., 1995). Prior to puberty, hypothalamic GnRH neurons were under estradiol inhibition (negative feedback), and the pulses of luteinizing hormone (LH) are not frequent. The hypothalamic maturation and the increase in the estradiol synthesis and release by ovarian follicles, results in hypothalamic desensitization to gonadal steroids (positive feedback), increasing the gonadotropins secretion : follicle stimulating hormone (FSH) and LH, allowing the onset of puberty (SCHAMS, et al. 1981; KINDER, et al. 1995; MELVIN, et al. 1999).

Nutritional plan, exposure to environmental and social stimuli, and genetics can be considered influencers of the ability of presynaptic transmission to GnRH neurons (SENGER, 2012). Both live weight and body condition has effect at age to puberty (MORAN; QUIRKE; ROCHE, 1989). Weight gain and adipose tissue mass (adiposity) plays an important role on this process it (KENNEDY; MITRA, 1963). Many authors reported that diets of chronic

energy restriction retarded the growth and delayed puberty in rodents (BARASH; CHEUNG; WEIGLE, 1996), ovines (FOSTER; OLSTER, 1985) and bovines (DAY; IMAKAWA; ZALESKY, 1986).

Endocrine mechanisms have been proposed as modulators of the onset of puberty in heifers. Among them may be cited the hormone leptin and the inhibitory action of neuropeptide Y (NPY). Hypothalamic neurons detect moment-to-moment changes in blood glucose and fatty acids by signaling mechanisms. Leptin "notifies" the adiposity to hypothalamic neurons and can be considered an important indicator of nutritional status, because its blood concentration is directly related to the amount of body fat (SENGER, 2012). Leptin is a potential inhibitor of NPY action; both have antagonistic roles in the attainment of the puberty.

In an experiment conducted by our research group, comparing heifers fed with high- or low-energy diets, it was observed that hypothalamic NPY expression was not altered by diet, however one of its receptors, the NPY-Y1, was less expressed in heifers fed high energy diet (DINIZ-MAGALHÃES, 2010). A similar effect was reported by Vaiciunas et al. (2008), which verified that precocious heifers had lower expression of hypothalamic NPY receptors. These results suggested that both nutrition (more energetic diets) and genetics (precocious animals), could attain puberty sooner by reducing the hypothalamic sensitivity to the NPY inhibitory effects.

Another way to hasten the occurrence of the puberty in heifers is hormonal manipulation, using protocols that pattern the physiological action. Administration of progesterone (P4) or progestagens during the prepubertal period can induce puberty in heifers (LAMB et al., 2006; TAUCK et al., 2007). Patterson et al. (1992) observed that this ability seems to be influenced by age and nutritional status. Day and Anderson (1998) suggested that the exposure to progestagens reduced the estradiol receptors expression, reducing the estradiol negative feedback on GnRH secretion. However, progesterone stimulates the synthesis of the mRNA of NPY in hypothalamus (O'CONNER et al., 1995), this statement contradicts the puberty induction by P4 or progestagens in heifers, but the puberty induction protocols only become concrete after the device removal.

Thus, there are three main factors able to induce puberty in prepubertal heifers: 1) the genetic factor, 2) the increased energetic consumption, and 3) the exposure to exogenous progestagens. The elucidation of the molecular mechanisms by which these different factors can influence the attainment of puberty is fundamental to understanding puberty and to

develop strategies for nutrition, genetic selection or new drugs that will help in reducing age at first calving in zebu heifers.

2 I CHAPTER – DIFFERENTIAL GENE EXPRESSION ON ANTERIOR AND PREOPTIC HYPOTHALAMIC AREAS IN PREPUBERTAL NELLORE HEIFERS

2.1 INTRODUCTION

The hypothalamus is situated at the base of the brain and control the endocrine synthesis and secretion of small peptides to the pituitary gland. Aires (2008) described the hypothalamus division in areas: preoptic, anterior, tuberal, and mammillary.

Preoptic and arcuate nucleus are the most important hypothalamic nuclei related to reproduction. On this study, the arcuate nucleus (ARC) was harvested with anterior hypothalamus. Both hypothalamic areas, expressing genes identified as regulators of the GnRH neurons function, or other reproductive modulators, related to the attainment of puberty. Puberty can be defined as the ability to accomplish reproduction successfully.

The ARC is a target of leptin activity, which is a puberty stimulator (GARCIA; AMSTALDEN, 2002). However, leptin is a potent inhibitor of neuropeptide Y (NPY), which is an appetite stimulator and a GnRH inhibitor (GONZALES et al., 2003). The comprehension of hypothalamic areas and mechanisms controlling the onset of puberty is essential. The objective of this study was to verify whether genes related to the attainment of puberty are differently expressed in two hypothalamic areas in peripubertal Nellore heifers.

2.2 MATERIALS AND METHODS

All experimental procedures are in agreement with the Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching (CONSORTIUM, 1999), and approved by the Animal Bioethics Committee of the Universidade de São Paulo (protocol number 2475/2011).

2.2.1 Experimental Site

The experiment was conducted at the Beef Cattle Research Laboratory (LPGC) of the Universidade de São Paulo, located in Pirassununga (São Paulo state, southeast of Brazil, 21°59'46'' S, 47°25'33'' O, 627 m above sea level), from September to November of 2011. According to the Köppen classification, in Pirassununga the climate is Cwa type, characterized by hot and wet summer (mean temperature of the hottest month higher than 22°C), dry winter (mean temperatures of the coldest month below to 18°C) and ~ 1100 mm of annual rainfall. The rain season lasts from October to March and the dry season from April to September.

2.2.2 Animals description

Ninety weaned Nellore heifers were weighed and body condition score (BCS) was evaluated. They were fed *ad libitum* once a day, with concentrate diet and corn silage, formulated to promote average daily gains of 0.9 kg/day. After four months of supplementation the heifers reached 295 ± 11 kg and BCS 5.9 ± 0.7 in a 1 to 9 scale. Six days before the beginning of the experimental period, heifers were submitted to transrectal ultrasonography (USG) to evaluate the reproductive status. The heifers had dominant follicles with 10 ± 1.2 mm of diameter and were considered able to the experiment. Heifers with *corpora lutea* were removed from the experiment.

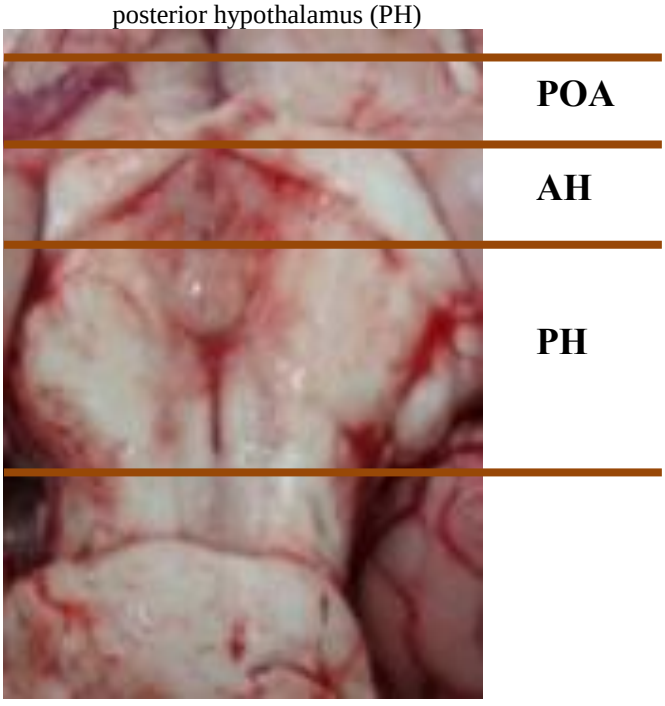
2.2.3 Reproductive evaluation, slaughter and hypothalamus collection

Thirty five peripubertal heifers, between 13-14 months of age, were slaughtered at the experimental abbatoir of the Universidade de São Paulo and the hypothalamus was harvested and processed for analysis. The heifers were divided into 3 groups according to body weight, and slaughtered on 3 different days to reduce the harvest time of the hypothalamus.

To avoid hypothalamus damage, captive bolt stunning was used to render animals insensible with a single shot on the spinal cord. After stunning, the jugular vein was clean severed to allow bleeding, the head was removed, and then the brain. To locate the hypothalamus structures from diencephalon were used as delimiters: dorsally to the median eminence, caudally to optic chiasm, frontally to mammillary body and ventrally to third ventricle. The preoptic area and anterior hypothalamus were harvested, following Glass, Amann and Nett (1984) description (Figure 1).

Samples from preoptic area and anterior hypothalamus were collected, frozen in liquid nitrogen, and stored at -80°C for subsequent RNA extraction.

Figure 1 – Description of hypothalamic dissection preoptic area (POA), anterior hypothalamus (AH) and posterior hypothalamus (PH)



Fonte: (DINIZ-MAGALHÃES, J., 2014).

2.2.4 Sample preparation

Hypothalamus samples were submitted to total RNA extraction using TRIzol (Life Technologies, Brazil) reagent protocol based on Chomczynski and Sacchi (1987). These samples were used to next-generation RNA-sequencing (RNA-Seq) and gene expression quantification by real time PCR (qPCR). The RNA concentration was quantified on NanoDrop 2000 (Thermo Scientific, USA) and some pools of samples were used to verify the RNA integrity on Bioanalyser 2100 (Agilent Technologies).

To RNA-Seq analyses twelve pools of each hypothalamus area were formed by mixing the RNA sample of two or 3 heifers according to slaughter date, body weight, BCS and follicle diameter before slaughter. RNA concentration of each sample was normalized before pooling.

To conduct the qPCR analyses the RNA samples of the 35 heifers were submitted to cDNA synthesis. Before synthesis, 2.75 µg of total RNA was treated with DNase I (Life Technologies, Brazil), to avoid genomic contamination. The cDNA syntheses were did with 2.0 µg of treated RNA in a reverse transcriptase reaction using Superscript II cDNA synthesis kit (Life Technologies, Brazil).

2.2.5 Next-generation sequencing (RNA-Seq)

A comprehensive and comparative study of the differences between genes on preoptic or anterior hypothalamic areas was conducted through next-generation sequencing (RNA-Seq), in order to identify possible candidate genes in each area that can be related to the attainment of puberty. The sample preparation and bioinformatics analyses were performed at the Genomic Facility, located at ESALQ-USP in Piracicaba city, Brazil.

The methodology is based on whole-genome sequencing using RNA as template, eliminating the use of probes. Expression analysis of large or highly repetitive genomes, such as that of bovine genome, requires paired-end sequencing reads of 100 or 150 bp to accurately map the reads to the genome. To RNA sample prepare were used the TruSeqTM RNA Sample Prep Kit v2 Set A (48rxn) (Illumina, USA). Then the samples were clustering on flow

cells using the TruSeq PE Cluster Kit v3 cBot HS, and sequenced using TruSeq SBS Kit v3-HS (Illumina, USA). The sequencing was performed using the HiSeq 1000 (Illumina, USA).

2.2.6 Bioinformatics analyses

Around 20 million sequence mRNA reads were obtained in each sample, and then 16 million were mapped against a bovine (*Bos taurus*) reference genome. Indexing of RNA-Seq libraries with 12 bp barcodes allows for sequencing of multiple samples in the same sequencing reaction. The indexed reads need to be demultiplexed in order to assign each read to the corresponding sample. These indexes were generated a in a Fastq format which is a text-based format for storing a nucleotide sequence and its corresponding quality scores. The quality of the obtained raw Fastq files was checked and adjusted with a tool to quality control for high throughput sequence data the FastQC. Then the reads were direct mapped against a reference bovine (*Bos taurus*) genome, using Bowtie2, a tool for aligning sequencing reads to long reference sequence (LANGMEAD; SALZBERG, 2012). The count data needed to be normalized according to the paired-end exon model FPKM (Fragments Per Kilobase per Million mapped reads). The summarized transcriptome-mapped count data were tested for significant differences in transcript abundance between hypothalamus areas, using the edgeR (Empirical analysis of digital gene expression data in R) tool (McCARTHY; CHEN; SMYTH, 2012; ROBINSON; McCARTHY; SMYTH, 2010). Differences in expression between the areas of the hypothalamus were established by comparing each gene in each pool in each area.

The differential expression analysis of RNA-Seq generated values of log of fold change on base 2 (LogFC), *P*-values and adjusted *P*-values using Benjamini and Hochberg (1995) tests (BH Padj) to each gene. Were identified 12,881 common genes between areas, and from these were discarded those that had LogFC less than 1.2 and BH Padj higher than 0.05.

The remaining 331 genes were analyzed using the web-accessible program DAVID v.6.7 (Database for Annotation, Visualization and Integrated Discovery) and PANTHER (Protein ANalysis THrough Evolutionary Relationships) to identify the hypothalamic area with greater amount of genes related to the attainment of puberty.

DAVID were used to identify enriched biological themes, particularly gene ontology (GO) terms; discover enriched functional-related gene groups, and cluster redundant annotation terms (SHERMAN; LEMPICKI, 2009). The Panther is a classification system that classify proteins and their genes according to: molecular function (MF), biological process (BP) or pathway (MI et al., 2013). The terms generated by these 2 web-tools were visualized, filtered and correlated with the reproductive function based on GO, MF, BP associated with a literature review.

2.2.7 Real time PCR (qPCR) analyses

Seven genes were selected to verify RNA-Seq results by qPCR quantification. These genes were selected based on their contribution to reproductive process.

After the selection, primers for: cadherin, EGF LAG seven-pass G-type receptor 3 (CELSR3), corticotropin releasing hormone receptor 2 (CRHR2), EGF-like repeats and discoidin I-like domains 3 (EDIL3), growth hormone releasing hormone (GHRH), and Kruppel-like factor 9 (KLF9), Neuropeptide Y (NPY), NPY receptor-1 (NPY1R) target genes and the housekeeping gene 18S ribosomal RNA (RN18S1) gene, were designed for real-time PCR (qPCR) based on bovine GenBank sequences (Table 1), using the software Primer Blast do NCBI (YE, et al. 2012) and tested with the software OligoAnalyzer 3.1 (OWCZARZY, et al. 2008) to dimer and hairpin formation.

Relative gene expression quantification of CELSR3, CRHR2, EDIL3, GHRH, KLF9, NPY and NPY1R was performed using RN18S1 as a constitutive gene. The real-time PCR reactions was performed using 10 µL of SYBR Green master mix 2 X (Life Technologies, Brazil), 0.25 mM of each primer, hypothalamic cDNA samples, and water up to a final volume of 20 µL. Thermal cycling parameters were as follow: an initial denaturing step of 94°C for 10 min, followed by 44 cycles of denaturing (94°C for 15 s), annealing/elongation (60°C for 1 min), and a melting curve program (60-95°C with a heating rate of 0.3°C per cycle). All reactions were performed in duplicate wells.

The 7500® Real-Time PCR Systems (Life Technologies, Brazil), was used to perform the reactions. Amplification efficiency of all genes was verified by a dilution curve with a series of cDNA concentrations, and quantification was performed only when the efficiency

was not different than 100% (YUAN et al.; 2006). All analyses were done based on second derivative maximum method.

Table 1 – Oligonucleotide primer pairs designed for use in real-time polymerase chain reaction (qPCR) amplification

Genes*	Oligonucleotide primers: 5' → 3'	GenBank accession number**	Primer annealing temperature	PCR insert size (bp)
CELSR3	F-CCGGACTCCAGTTAGCATCC R-TGTTCTCCTTCACTCGCACC	NM_001205337.1	60°C	98
CRHR2	F-ACGAGCATCCACCACATCAG R-AGAAACCCTGGAACGACTGC	NM_001192545.1	60°C	179
EDIL3	F-GATGGCTTCACAGGTCCCAA R-TCTCCTCGGTACGCTTCACT	XM_002689374.3	60°C	140
GHRH	F-GGATTCCACGGTACGCAGAT R-GCTCCTTGCTCCTGGTTTCT	NM_178325.1	60°C	130
KLF9	F-GGAAACACGCCTCCGAAAAG R-AAGGGCCGTTCACCTGTATG	NM_001193214.1	60°C	115
NPY	F-ACCCCTCCAAGCCTGACAA R-TGCCTGGTGATGAGATTGATG	AY491054	60°C	100
NPY1R	F-ACAGGTCCAGTGAAGCCAAAA R-TGGTCCCAGTCAAACACAGTG	XM_580988	60°C	112
RN18S1	F-CGGCGACGACCCATTCGAAC R-GAATCGAACCCTGATTCCCCGTC	NR_036642.1	60°C	99

*Cadherin, EGF LAG seven-pass G-type receptor 3 (CELSR3); corticotropin releasing hormone receptor 2 (CRHR2); EGF-like repeats and discoidin I-like domains 3 (EDIL3); growth hormone releasing hormone (GHRH); Kruppel-like factor 9 (KLF9); neuropeptide Y (NPY); NPY receptor-1 (NPY1R); 18S ribosomal RNA (RN18S1).
**Primers were designed based on previously deposited bovine sequences.
Fonte: (DINIZ-MAGALHÃES, J., 2014).

2.2.8 Statistical analyses

All qPCR statistical analyses were conducted using SAS, version 9.1.2 for Windows (SAS Institute Inc., Cary, NC). Data were analyzed as a completely randomized design using the MIXED procedure of SAS, considering the fixed effect of area, and animal within area as a random effect. In all comparisons, significance was declared at $P \leq 0.05$, and tendencies at $P \leq 0.10$.

The data were analyzed for residual homogeneity of variance, and observations outside the range of ± 3 s*tudentized* wastes were considerate atypical observations and withdrawals of the analysis. Contrasts analysis was used to compare the effect of treatments and to estimate the ΔΔCt, and its standard error of the mean. In all comparisons, significance was declared at $P \leq 0.05$, and tendencies at $P \leq 0.10$.

2.3 RESULTS

To determine the hypothalamic areas importance to the gene expression related to the attainment of puberty the data were analyzed by RNA-Seq and qPCR, and the results are presented on separated sections.

2.3.1 Next generation RNA-sequencing (RNA-Seq)

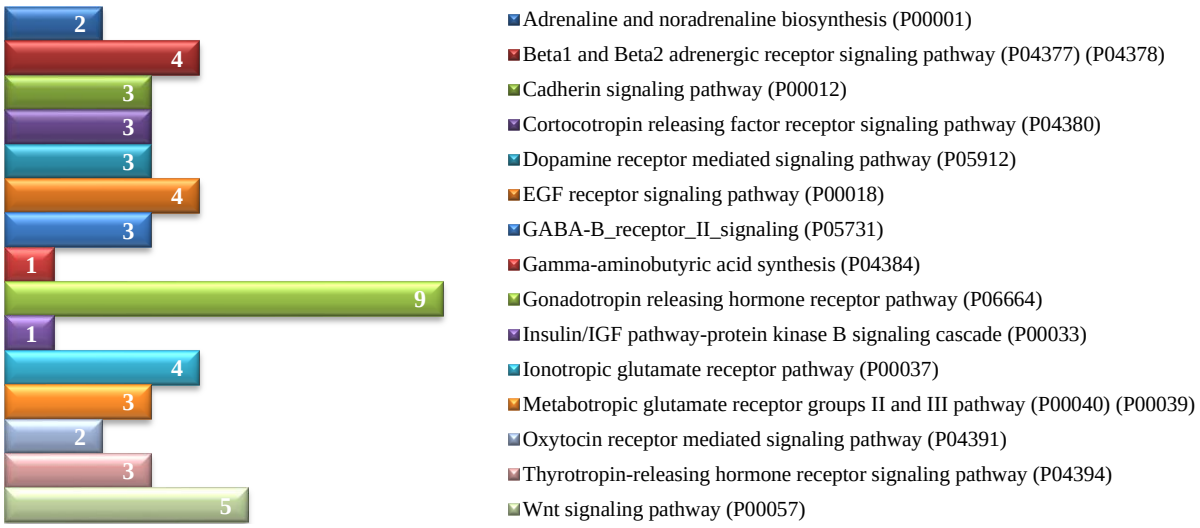
The next-generation RNA-sequencing is a technique to whole-transcriptome studies. In this experiment around 20 million reads were obtained in each sample, the data was filtered and generated around 18 million reads. Then the reads were mapped against a reference bovine (*Bos taurus*) genome and 16 million were identified, however, due to filtering of data just under a half of them were used in the differential expression analysis. This tool was used to identify the gene pathways and their contribution to the attainment of puberty according to their hypothalamic localization in peripuberal Nellore heifers. Differences in the gene expression were analyzed between preoptic and anterior hypothalamic areas using RNA-Seq.

When compared anterior and preoptic areas of hypothalamus, 12,881 common genes were founded. From these genes, 331 showed LogFC up to 1.2 and BH Padj lower than 0.05 and were considered differentially expressed. From the genes differentially expressed 139 had higher expression on anterior and the other 192 genes had higher expression on preoptic area.

The neuroendocrine activity of the hypothalamus-pituitary-gonadal axis is regulated by neurotransmitters that act on the stimulation or inhibition of GnRH neurons. While Neuropeptide Y and Gamma-AminoButyric Acid (GABA) are considered inhibitors, of the GnRH neurons activity, Kiss1 and glutamate are considered stimulators of the attainment of puberty. The influence of these classes of neurotransmitters on the hypothalamus areas was observed when the 331 genes were analyzed by PANTHER pathways. This analyze was based on receptor modulation, signaling pathways and modulation of biosynthetic pathways. On figure 1 are the numbers of genes according the pathway classification.

After analyzes the genes were separated according to their PANTHER pathways (Tables 2 to 5). The most relevant pathways related with reproductive processes were used to identify candidate genes. The identified pathways were: gonadotropin releasing hormone receptor pathway (P06664; Table 2); ionotropic glutamate receptor pathway and metabotropic glutamate receptor groups II and III pathways (P00037; P00040; P00039; Table 3); GABA-B receptor II signaling (P05731; Table 4) and Gamma-aminobutyric acid synthesis (P04384; Table 4); and Cadherin/Wnt signaling pathway (P00057; P00012; Table 5).

Figure 2 – PANTHER pathways related to differentially expressed genes by comparing anterior x preoptic hypothalamic areas



Fonte: (DINIZ-MAGALHÃES, J., 2014).

The genes that regulate GnRH receptor pathway were differently expressed between hypothalamic areas (Table 2). The releasing hormones activities, as GnRH, are calcium (Ca^{2+})-dependent (PETERSEN O.; PETERSEN, C.; KASAI, 1994). The genes calcium channel, voltage-dependent, L type, alpha 1C subunit (CACNA1C), calcium channel, voltage-dependent, L type, alpha 1D subunit (CACNA1D), inositol 1,4,5-triphosphate receptor, type 3 (ITPR3), and mitogen-activated protein kinase kinase kinase 3 (MAP3K3) are part of Ca^{2+} metabolism and were more expressed on preoptic area, similarly with GNRH1 gene. While adenylate cyclase activating polypeptide 1 (pituitary) (ADCYAP1), glycoprotein hormones, alpha polypeptide (CGA), KiSS-1 metastasis-suppressor (KISS1), luteinizing hormone beta polypeptide (LHB), and neuropeptide VF precursor (NPVF) were more expressed on anterior hypothalamus.

From the results, it can be demonstrated that GnRH activity was higher on the preoptic area of the hypothalamus, and expression of genes likely regulated by the release of GnRH, such as CGA and LHB, had higher expression on the anterior hypothalamus. In fact, anterior hypothalamus and arcuate nucleus (ARC) are bridges between preoptic area and median eminence and some GnRH release-dependent genes are there transcripted.

Table 2 – Differences in gene expression between hypothalamic areas of genes related to gonadotropin releasing hormone receptor pathway (P06664) generated using PANTHER

Gene	**Gene name**	**Anterior[1]**	**POA[2]**	**logFC[3]**	**BH Padj[4]**
CACNA1C	calcium channel, voltage-dependent, L type, alpha 1C subunit	1.1	2.9	-1.3	0.02
CACNA1D	calcium channel, voltage-dependent, L type, alpha 1D subunit	1.1	4.0	-1.8	<0.01
GNRH1	gonadotropin-releasing hormone 1 (luteinizing-releasing hormone)	2.3	38.5	-3.9	<0.01
ITPR3	inositol 1,4,5-triphosphate receptor, type 3	2.5	15.4	-2.5	<0.01
MAP3K3	mitogen-activated protein kinase kinase kinase 3	3.2	8.3	-1.3	<0.01
ADCYAP1	adenylate cyclase activating polypeptide 1 (pituitary)	184.6	43.1	2.2	<0.01
CGA	glycoprotein hormones, alpha polypeptide	37.3	4.1	3.2	<0.01
KISS1	KiSS-1 metastasis-suppressor	7.8	3.0	1.6	0.03
LHB	luteinizing hormone beta polypeptide	7.0	1.7	2.1	<0.01
NPVF	neuropeptide VF precursor	5.1	0.2	4.5	<0.01

[1]Anterior hypothalamus; [2]POA: preoptic area; [3]Log of the fold change expression - If the LogFC was positive the gene was up-regulated on anterior area, if it was negative it was down-regulated; [4]BH Padj: P values adjusted by Benjamini and Hochberg tests.
Fonte: (DINIZ-MAGALHÃES, J., 2014).

GABA is the main inhibitory, and glutamate, its precursor, is the main excitatory neurotransmitter in the mammalian cortex (PETROFF, 2002). The genes that regulate glutamate receptors, GABA synthesis, and GABA receptor regulation were differently expressed between hypothalamic areas (Tables 3; 4). The genes: calcium channel, voltage-dependent, N type, alpha 1B subunit (CACNA1B); calcium channel, voltage-dependent, P/Q type, alpha 1A subunit (CACNA1A); glutamate receptor, ionotropic, N-methyl D-aspartate 2D (GRIN2D); and GNG8 guanine nucleotide binding protein, gamma 8 (GNG8) were more expressed on the preoptic area, following the pattern of expression of GnRH regulatory genes. The solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6 (SLC17A6) and betacellulin (BTC) had higher expression on the anterior hypothalamus. The SLC family acts on glutamate vesicular transport and BTC is one of the 7 EGFR ligands and is a mediator of LH action in the ovulatory follicles (PARK et al., 2004).

Cadherins perform important tasks in controlling the coordination of the morphological changes in steroid hormones (ROWLANDS et al., 2000). On table 5 are presented the genes regulators of the cadherin/wnt signaling pathway differently expressed between hypothalamic areas.

Table 3 – Differences in gene expression between hypothalamic areas of genes related to ionotropic glutamate receptor pathway and metabotropic glutamate receptor groups II and III pathways (P00037; P00040; P00039) generated using PANTHER

Gene	Gene name	Anterior[1]	POA[2]	logFC[3]	BH Padj[4]
CACNA1B	calcium channel, voltage-dependent, N type, alpha 1B subunit	1.9	7.2	-1.9	<0.01
CACNA1A	calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	6.4	16.6	-1.3	<0.01
GRIN2D	glutamate receptor, ionotropic, N-methyl D-aspartate 2D	0.6	1.6	-1.3	<0.02
SLC17A6	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6	158.3	38.4	2.2	<0.01

[1]Anterior hypothalamus; [2]POA: preoptic area; [3]Log of the fold change expression - If the LogFC was positive the gene was up-regulated on anterior area, if it was negative it was down-regulated; [4]BH Padj: P values adjusted by Benjamini and Hochberg tests.
Fonte: (DINIZ-MAGALHÃES, J., 2014).

Table 4 – Differences in gene expression between hypothalamic areas of genes related to GABA-B receptor II signaling (P05731) and Gamma-aminobutyric acid synthesis (P04384) generated using PANTHER

Gene	Gene name	Anterior[1]	POA[2]	logFC[3]	BH Padj[4]
CACNA1A	calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	6.4	16.6	-1.3	<0.01
CACNA1B	calcium channel, voltage-dependent, N type, alpha 1B subunit	1.9	7.2	-1.9	<0.01
GNG8	guanine nucleotide binding protein (G protein), gamma 8	1.5	4.6	-1.4	0.01
MAP3K3	mitogen-activated protein kinase kinase kinase 3	3.2	8.3	-1.3	<0.01
PIK3C2B	phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 beta	4.6	11.9	-1.3	<0.01
BTC	Betacellulin	9.8	4.5	1.2	<0.01
YSK4	YSK4 Sps1/Ste20-related kinase homolog (S. cerevisiae)	4.1	1.5	1.5	<0.01

[1]Anterior hypothalamus; [2]POA: preoptic area; [3]Log of the fold change expression - If the LogFC was positive the gene was up-regulated on anterior area, if it was negative it was down-regulated; [4]BH Padj: P values adjusted by Benjamini and Hochberg tests.
Fonte: (DINIZ-MAGALHÃES, J., 2014).

Table 5 – Differences in gene expression between hypothalamic areas of genes related to Cadherin/Wnt signaling pathway (P00057; P00012) generated using PANTHER

Gene	Gene name	Anterior[1]	POA[2]	logFC[3]	BH Padj[4]
CDH9	cadherin 9, type 2 (T1-cadherin)	4.0	10.3	-1.2	<0.01
CELSR3	cadherin, EGF LAG seven-pass G-type receptor 3	2.4	8.0	-1.6	<0.01
GNG8	guanine nucleotide binding protein (G protein), gamma 8	1.5	4.6	-1.4	0.01
ITPR3	inositol 1,4,5-triphosphate receptor, type 3	2.5	15.4	-2.5	<0.01
TCF7L2	transcription factor 7-like 2	5.9	2.3	1.4	0.04

[1]Anterior hypothalamus; [2]POA: preoptic area; [3]Log of the fold change expression - If the LogFC was positive the gene was up-regulated on anterior area, if it was negative it was down-regulated; [4]BH Padj: P values adjusted by Benjamini and Hochberg tests.
Fonte: (DINIZ-MAGALHÃES, J., 2014).

The DAVID enrichment was used to determine the gene ontology (GO) of the 331 previous genes selected. Over-representation of GO terms was determinate based on Fisher's exact test and filtered with a false discovery rate (FDR) of < 10% (Table 6). Sixty five genes were classified as regulators of biological process, 9 with hormone activity, and 17 with receptor binding.

Table 6 – Gene Ontology (GO) associated with 331 genes that are differentially expressed between hypothalamic areas

GO ID	GO Description	Number of genes[1]	FDR[2]
GO:0005576	extracellular region	33	<0.01
GO:0065007	biological regulation	70	<0.01
GO:0050789	regulation of biological process	65	<0.01
GO:0005179	hormone activity	9	0.01
GO:0050794	regulation of cellular process	58	0.02
GO:0005102	receptor binding	17	0.09

[1]number of genes according to Gene Ontology term; [2]FDR: false discovery rate
Fonte: (DINIZ-MAGALHÃES, J., 2014).

2.3.2 Candidate genes expression

Seven genes (CELSR3, CRHR2, EDIL3, GHRH, KLF9, NPY and NPY1R) were selected based on their contribution to the reproductive process and documented role in regulation of puberty.

2.3.2.1 Next-generation RNA-sequencing (RNA-Seq)

On table 7 were described the RNA-Seq results of genes selected to qPCR validation. These genes were selected based on their contribution to reproductive process, not just the RNA-Seq results.

The epidermal growth factors (EGF) mediate the stimulation of GnRH neurons hypothalamus during female sexual maturation, and the genes CELSR3, CRHR2, and EDIL3 are regulated by EGF. The CRHR2 gene also modulates the steroid secretion on adrenal cortex. KLF9 is a zinc finger transcription factor that modulates the activity of progesterone receptor (PGR). The GHRH releases GH1, and its gene expression is negatively regulated by GH1, which stimulates the somatostatin and IGF-1 secretion. NPY and its receptor are potential inhibitors of GnRH synthesis.

Table 7 – RNA-Seq results of genes selected to qPCR analyses

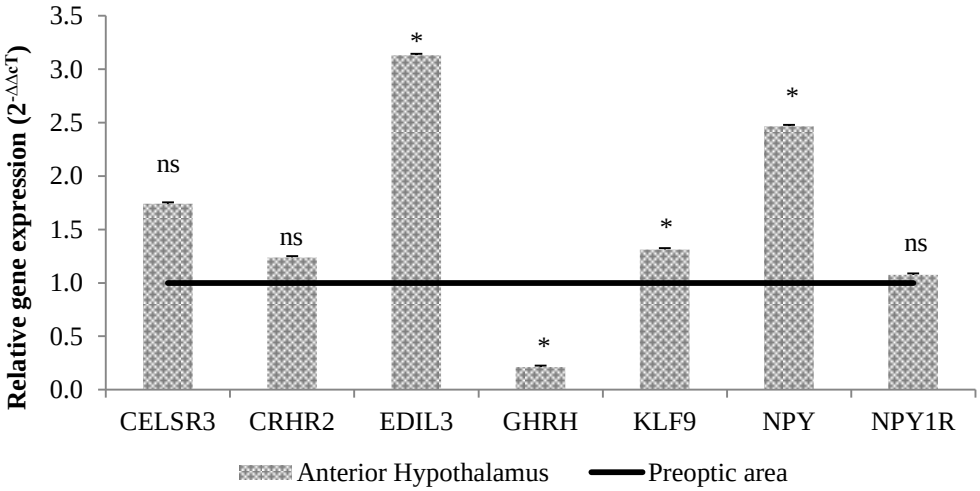
Gene	Gene name	Anterior[1]	POA[2]	logFC[3]	BH Padj[4]
CELSR3	Cadherin, EGF LAG seven-pass G-type receptor 3	2.42	7.97	-1.6	<0.01
CRHR2	corticotropin releasing hormone receptor 2	1.75	7.24	-2.0	<0.01
EDIL3	EGF-like repeats and discoidin I-like domains 3	4.58	6.49	-0.5	0.53
KLF9	Kruppel-like factor 9	4.25	5.83	-0.4	0.51
GHRH	growth hormone releasing hormone	20.89	0.98	4.6	<0.01
NPY	neuropeptide Y	40.04	19.30	1.1	0.12
NPY1R	NPY receptor-1	15.59	12.51	0.4	0.36

[1]Anterior hypothalamus; [2]POA: preoptic area; [3] Log of the fold change expression; [4]BH Padj: P values adjusted by Benjamini and Hochberg tests.
Fonte: (DINIZ-MAGALHÃES, J., 2014).

2.3.2.2 Relative quantification of gene expression by qPCR

The relative expression (RE) of genes was different between hypothalamic areas. The RE on anterior and preoptic hypothalamus was adjusted using ΔΔCt method, considering the RE on the preoptic area as a fixed expression. The anterior hypothalamus had 3 genes had higher expression on the anterior area: EDIL3 (RE = 3.1; $P < 0.01$), KLF9 (RE = 1.3; $P = 0.04$) and NPY (RE = 2.5; P = 0.04), and one gene had lower expression GHRH (RE = 4.7; P < 0.01), when compared with the preoptic area. The CELSR3 (RE = 1.7; P = 0.09) gene expression tended to be higher on anterior hypothalamus. Expression of CRHR2 (RE = 1.2; P = 0.56) and NPY1R (RE = 1.1; P = 0.65) was not different between the areas of the hypothalamus (Figure 3).

Figure 3 – Comparative relative gene expression between anterior (bars) and preoptic (line) hypothalamic areas



Relative gene expression (RE) of CELSR3, CRHR2, EDIL3, GHRH, KLF9, NPY and NPY1R on anterior and preoptic hypothalamic areas in Nellore heifers. The RE on anterior and preoptic hypothalamus was adjusted using ΔΔCt method, considering the RE on the preoptic area as a fixed expression (line). RE on anterior hypothalamus was presented as a bar graph. *$P < 0.05$; ns: not significant.
Fonte: (DINIZ-MAGALHÃES, J., 2014).

2.4 DISCUSSION

The hypothalamus is a central nervous system (CNS) neuroendocrine gland extending from the optic chiasm to the mammillary bodies, and occupies a small portion of the third ventricle. It is a center for controlling the internal environment and comprises numerous paired nuclei that integrate physiological signals of the body. The hypothalamus is divided in regions in the rostrocaudal direction (preoptic, anterior, tuberal, and mammillary; AIRES, 2008).

Two important hypothalamic nuclei are related to reproduction: arcuate nucleus (ARC) that controls basal secretion of LH and FSH; and preoptic nucleus that controls preovulatory surge of LH and FSH. In our study the hypothalamus was isolated based on anatomically structures and the ARC was collected as part of anterior hypothalamus sample. Puberty occurs after the first ovulation, and the comprehension of the pathways genetic regulation related with the attainment of puberty were studied in both hypothalamic areas.

Puberty can be defined as the ability to accomplish reproduction successfully. The regulation of the timing of puberty is directly related to the ability of presynaptic neurons to provide information to GnRH neurons (SENGER, 2012). A great number of genes were expressed in both hypothalamic areas and were identified as regulators of the GnRH neurons function, or other reproductive modulators, related to the attainment of puberty. The study of release hormones activity is a way to evaluate this adjustment.

According to the results of this experiment we could observe the presence of genes that are involved in calcium (Ca^{2+}) metabolism, since GnRH release is Ca^{2+}-dependent (PETERSEN O.; PETERSEN, C.; KASAI, 1994). The GnRH induces intracellular Ca^{2+} mobilization immediately followed by extracellular Ca^{2+} influx. Extracellular Ca^{2+} enters the cell through voltage-gated calcium channels in the plasma membrane, while inositol 1,4,5-triphosphate (IP3) releases Ca^{2+} from intracellular stores (KRAUS; NAOR; SEGER, 2001). Both intracellular and extracellular Ca^{2+} were shown to be involved in the secretion of LH and FSH (STOJILKOVIC et al., 1994; STOJILKOVIC; CATT, 1995). However, the IP3-released calcium seems to be critical for gonadotropin secretion, whereas Ca^{2+} influx through the plasma membrane is required mainly for the renewal of internal stores (TSE et al., 1997).

In addition, Ca^{2+} has been shown to play a role in GnRH receptor signaling toward MAPKs (KRAUS; NAOR; SEGER, 2001). Haisenleder et al. (1998) determined *in vitro* the role of MAPK activation on gonadotrope by gene expression; they evaluated MAPK

activation according to GnRH pulse frequency, and observed the maximal increase (3-fold vs. controls) after 120-min pulses. The calcium channel subunits, ITPR3 and MAP3K3 genes were highly expressed in the preoptic hypothalamic area, as the GnRH1 gene, which indicates possible local regulation.

This study is based on the attainment of puberty that is clearly influenced by the action of the GnRH on synthesis and secretion of gonadotropins, which demonstrates the importance of understanding the transcription of genes regulated by it. The CGA was extensively studied on gonadotrope cells and pituitary (WINDLE; WEINER; MELLON, 1990; BRINKMEIER et al., 1998; BURRIN et al., 1998; SEASHOLTZ et al., 2009), but its hypothalamic localization in heifers was not clear. When we observed the CGA expression between areas, we could see that the CGA gene was expressed in preoptic and anterior area, with a higher expression in anterior hypothalamus. The CGA gene is associated with the biosynthesis of gonadotropin α-subunits (LH and FSH) and of the thyroid-stimulating hormone (PIERCE; PARSONS, 1981).

Genes that regulates GABA and glutamate were founded in both hypothalamic areas. They can be related with the modulation of GnRH and estradiol hormone. Fortes and Reverter (2010) indicated a link between estrogen pathways and GABA and glutamate signaling, they found a relationship between estradiol receptor and GABA receptor (GABRA1) which might influence puberty, by modifying the input on GnRH neurons. GABA can inhibits GnRH secretion indirectly, via effects exerted on neurons connected to the GnRH neuronal network, but can also stimulate GnRH neurons directly through activation of GABAA receptors (MOENTER; DeFAZIO, 2005; OJEDA et al., 2010).

The EGFR signaling is another pathway that has been described as modulator of sexual maturation, and can be associated with cadherin/wnt signaling. These signaling pathways are related to genes that regulate the signal transcription at glial cells in the hypothalamus, and were founded in both studied areas. Schneider and Wolf (2008) described the mechanisms taking place in the hypothalamus during female sexual maturation and associated the regulation of puberty in female mice to glial cells. One of these mechanisms involves receptors of epidermal growth factors (EGFR), which mediates the stimulation of GnRH neurons by alpha transforming growth factor (TGFα). Hypothalamic astrocytes express increasing amounts EGFR during sexual maturation, with further increases preceding the first preovulatory surge of gonadotropins (MA et al., 1994).

Cell-cell adhesions are mediated via cadherins and their associated catenins (BARTH; NÄTHKE; NELSON, 1997). β-catenin signaling is crucial for normal mammary development during the sexual maturation and involves the wnt signaling activation (DESSAUGE; FINOT;

WIART, 2009). Bekirov et al. (2002) observed cadherin mRNA within hypothalamic nuclei, but they were not studied the CELSR3 gene. The contribution of wnt signaling to attainment of puberty was not yet clear.

To validate the expression by RNA-Seq of the genes found in each area of the hypothalamus, the relative expression of the previously selected genes was analyzed using the qPCR. The genes were chosen based on their functions: the genes CELSR3, CRHR2, and EDIL3 are regulated by EGFR, while KLF9 modulates the activity of progesterone receptor (PABONA, et al. 2012); NPY and its receptor are puberty inhibitors, and NPY stimulates the secretion GHRH (ZIEBA, et al. 2003).

The qPCR quantification demonstrated a higher expression of NPY on the anterior hypothalamus, similarly to the RNA-Seq results, although the difference between the areas detected by RNA-Seq was no significant. The hypothalamic NPY is synthesized primarily in ARC neurons, and acts in adjacent hypothalamic areas such as the paraventricular nucleus (PVN), dorsomedial nucleus (DMN), and the medial preoptic area (MORRIS, 1989). This fact can explain the higher expression on anterior hypothalamus. There is a contradictory result between RNA-Seq and qPCR analyzes for GHRH, but GHRH neurons are present on ARC (HASHIZUME, et al. 2005), validating the RNA-Seq results.

2.5 CONCLUSIONS

The expression of genes related to the attainment of puberty was different according to the studied areas. Both the anterior and preoptic hypothalamic areas should be evaluated separately when the objective is to identify changes in hypothalamic gene expression.

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3 II CHAPTER – PROGESTERONE AND ESTRADIOL EFFECTS ON HYPOTHALAMIC GENES RELATED TO THE ATTAINMENT OF PUBERTY IN NELLORE HEIFERS

3.1 INTRODUCTION

Several studies demonstrated that the use of progesterone may advance puberty in heifers (GONZALEZ-PADILLA, 1975; ANDERSON, et al. 1996; CLARO JR, et al. 2010). Claro Jr et al. (2010) observed an increase of 20% in the estrus rate in heifers with progesterone device at 45 days when compared to the control group. These same heifers showed an increase of 11% in pregnancy rate. However, the molecular mechanism of progesterone action in the puberty control is not completely elucidate.

Peripheral concentration and the pulses frequency of luteinizing hormone (LH) increases at the puberty in heifers (DAY, et al. 1987; KINDER; DAY; KITTOK, 1987). Anderson et al. (1996) supposes that exogenous progesterone reduces the number of hypothalamic estradiol receptors, resulting in increased frequency of LH pulses after the end of treatment with P4. Day et al. (1987) observed that the cytosolic concentration of estradiol receptors on the anterior and medial basal hypothalamus and anterior pituitary reduced linearly as puberty approached.

The estradiol regulation is another relevant element on control of the first ovulation. The follicular growth increases levels of circulating estradiol and the activation of β receptors on hypothalamus that induces the release of GnRH and, consequently, the release of gonadotropins (DAY; ANDERSON, 1998).

Follicular aspiration removes the preovulatory follicles and thus eliminates the action of estradiol on ovulation, allowing to evaluation reportedly to the effect of exogenous progesterone on the molecular mechanisms of controling the onset of puberty. Therefore, our objective was to study the effect of estradiol and of progesterone on the expression of genes related to the attainment of puberty in different hypothalamic areas, and thus contribute to the elucidation of the molecular mechanisms that regulate the onset of puberty.

3.2 MATERIALS AND METHODS

All experimental procedures are in agreement with the Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching (Consortium, 1999), and approved by the Animal Bioethics Committee of the Universidade de São Paulo (protocol number 2475/2011).

3.2.1 Experimental Site

The experiment was conducted from September to November of 2011, at the Beef Cattle Research Laboratory (LPGC) of the Universidade de São Paulo, located in Pirassununga (São Paulo state, southeast of Brazil, 21°59'46'' S, 47°25'33'' O, 627 m above sea level). According to the Köppen classification, in Pirassununga the climate is Cwa type, characterized by hot and wet summer and dry winter with mean temperatures of 23°C and ~ 1100 mm of annual rainfall. The rain season lasts from October to March and the dry season from April to September.

3.2.2 Animals description, reproductive evaluation, experimental treatments and slaughter selection

Ninety weaned Nellore heifers were weighed and body condition score (BCS) was evaluated. They were fed ad libitum once a day, with concentrate diet and corn silage, formulated to promote average daily gains of 0.9 kg/day. After four months of supplementation the heifers reached 295 ± 11 kg and BCS 5.9 ± 0.7 in a 1 to 9 scale. Six days before the beginning of the experimental period, heifers were submitted to transrectal ultrasonography (USG) to evaluate the reproductive status. The heifers had dominant follicles with 10 ± 1.2 mm of diameter and were considered able to the experiment. Heifers with *corpora lutea* were removed from the experiment.

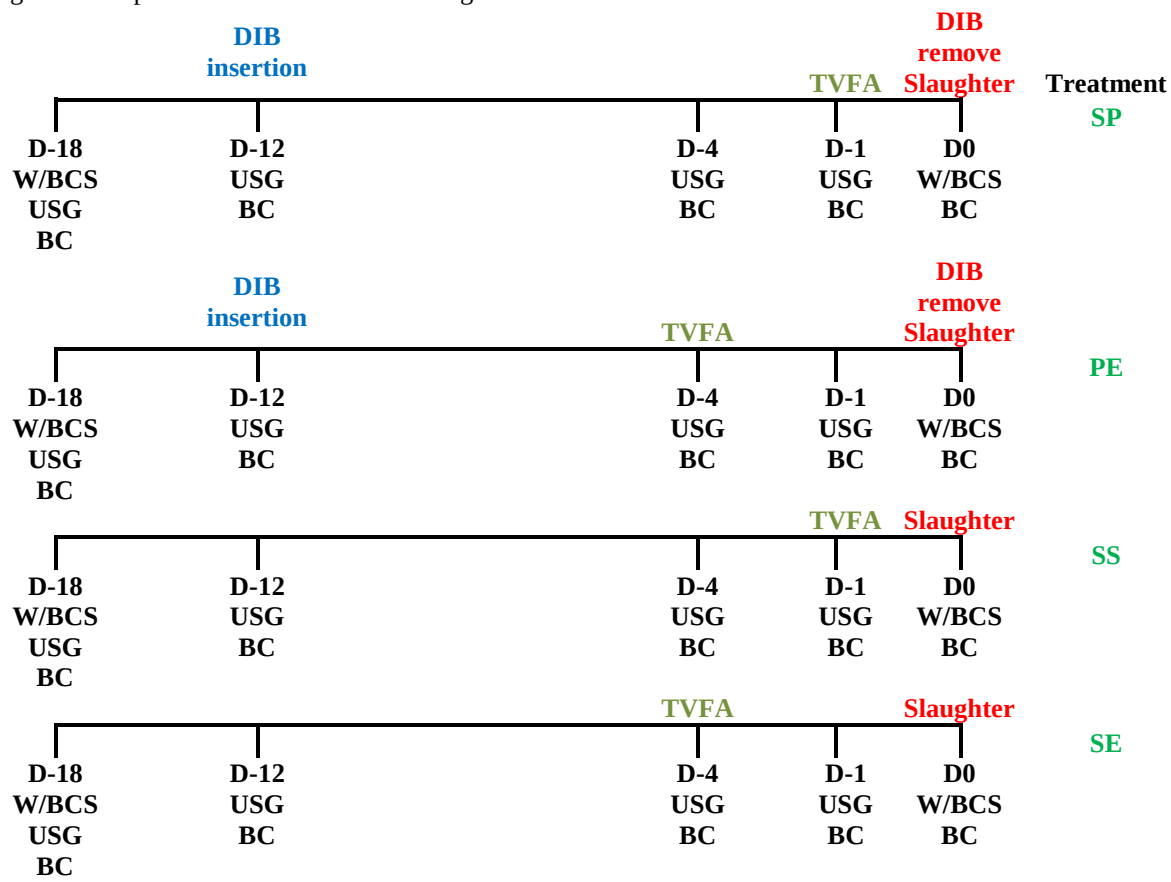
Sixty non-pubertal heifers, between 13 and 14 months of age, were divided into four treatments (fifteen per treatment): progesterone (P4) device without endogenous estradiol (**SP**), P4 device with endogenous estradiol (**PE**), without P4 device and without endogenous estradiol (**SS**), and without P4 device and with endogenous estradiol (**SE**). The treatments were described on figure 4. Although just thirty five heifers were slaughtered (D0) at the end of the hormonal treatments and had the hypothalamus harvest and processed for analysis. The remaining heifers were used as puberty controls, and received a progesterone device to verify if they could attain puberty with progesterone stimulation in these corporal conditions.

A new USG evaluation were proceeded six days before insertion of bovine intravaginal device (DIB®, MSD Animal Health Brazil), considerate the day -18 (D-18). Another USG evaluation was performed in all heifers on D-12, just before the device insertion in the heifers of SP and PE treatments. To improve or decline endogenous estradiol were used the transvaginal follicular aspiration (TVFA). Heifers in PE and SE treatment had the follicles up to 3 mm aspired on D-4, to stimulate a new follicular wave and a pic of estradiol at slaughter (Figure 4). Already in the heifers from treatment without estradiol, our objective was decline the action of endogenous estradiol at slaughter, so they were aspirated on D-1 (SP and SS). All treatments were synchronized to slaughter and hypothalamus collections occur simultaneously.

3.2.3 Hormonal quantification

Bovine intravaginal device (DIB®, MSD Animal Health Brazil) contained 1.0 g of progesterone were insert on heifers of SP and PE treatments and removed on D0, just before the slaughter (Figure 4). The blood was collected by jugular venipuncture on heparinized vacuum tubes on days -18, -12, -4, -1 and 0 (immediately before device remove, Figure 4). The cold blood was centrifuged at 1.500 x g for 15 min and 4 ºC. The plasma was pipetted with an automatic pipette and frozen at -20 ºC until hormonal analyses. Progesterone concentration was determinate by radioimmunoassay using the kit Coat-A-Count (Diagnostic Product Corporation, USA) on Endocrinology Laboratory of Professor Guilherme de Paula Nogueira (FMV-UNESP-Araçatuba, SP, Brazil).

Figure 4 – Experimental treatments and slaughter



W: Weighing; USG: Ultrasonography; TVFA: Transvaginal Follicular Aspiration; BC: blood collection; Treatments: SP – progesterone without estradiol; PE: progesterone with estradiol; SS: without progesterone without estradiol (control); SE: without progesterone with estradiol.
Fonte: (DINIZ-MAGALHÃES, J., 2014).

3.2.4 Hypothalamus collection

Tree heifers groups were formed according to their body weight and the slaughter occurred on 3 different days to reduce the harvest time of the hypothalamus. Thirty five peripubertal heifers, between 13-14 months of age, were slaughtered at the experimental *abbatoir* of the Universidade de São Paulo and the hypothalamus was harvested and processed for analysis.

To avoid hypothalamus damage, captive bolt stunning was used to render animals insensible with a single shot on the spinal cord. After stunning, the jugular vein was clean severed to allow bleeding, the head was removed, and then the brain. To locate the hypothalamus structures from diencephalon were used as delimiters: dorsally to the median eminence, caudally to optic chiasm, frontally to mammillary body and ventrally to third

ventricle. The preoptic area and anterior hypothalamus were harvested, Glass, Amann and Nett (1984) description.

Samples from preoptic area and anterior hypothalamus were collected, frozen in liquid nitrogen, and stored at -80°C for subsequent RNA extraction.

3.2.5 Sample preparation

Hypothalamus samples were submitted to total RNA extraction using TRIzol (Life Technologies, Brazil) reagent protocol based on Chomczynski and Sacchi (1987). These samples were used to next-generation RNA-sequencing (RNA-Seq) and gene expression quantification by real time PCR (qPCR). The RNA concentration was quantified on NanoDrop 2000 (Thermo Scientific, USA) and some pools of samples were used to verify the RNA integrity on Bioanalyser 2100 (Agilent Technologies).

To RNA-Seq analyses twelve pools, 3 per treatment, of each hypothalamus area were formed by mixing the RNA sample of 2 or 3 heifers according to plasma progesterone concentration, slaughter date, body weight, BCS and follicle diameter before slaughter. RNA concentration of each sample was normalized before pooling.

To conduct the qPCR analyses the RNA samples of the 35 heifers, according to these areas and treatments, were submitted to cDNA synthesis. Before synthesis, 2.75 µg of total RNA was treated with DNase I (Life Technologies, Brazil), to avoid genomic contamination. The cDNA syntheses were did with 2.0 µg of treated RNA in a reverse transcriptase reaction using Superscript II cDNA synthesis kit (Life Technologies, Brazil).

3.2.6 Next-generation sequencing (RNA-Seq)

A comprehensive study about the effects of progesterone administration and endogenous estrogen on genes related to the attainment of puberty on anterior and preoptic hypothalamic areas was conducted through RNA-Seq. The objective was identify possible candidate genes and correlate they to puberty physiology. The sample preparation and

bioinformatics analyses were performed on the Genomic Facility, located at ESALQ-USP in Piracicaba city, Brazil.

The methodology is based on whole-genome sequencing using RNA as template, eliminating the use of probes. Expression analysis of large or highly repetitive genomes, such as that of bovine genome, requires paired-end sequencing reads of 100 or 150 bp to accurately map the reads to the genome. To RNA sample prepare were used the TruSeqTM RNA Sample Prep Kit v2 Set A (48rxn) (Illumina, USA). Then the samples were clustering on flow cells using the TruSeq PE Cluster Kit v3 cBot HS, and sequenced using TruSeq SBS Kit v3-HS (Illumina, USA). The sequencing was performed using the HiSeq 1000 (Illumina, USA).

3.2.7 Bioinformatics analyses

Around 20 million sequence mRNA reads were obtained in each sample, and then 16 million were mapped against a bovine (*Bos taurus*) reference genome. Indexing of RNA-Seq libraries with 12 bp barcodes allows for sequencing of multiple samples in the same sequencing reaction. The indexed reads need to be demultiplexed in order to assign each read to the corresponding sample. These indexes were generated a in a Fastq format which is a text-based format for storing a nucleotide sequence and its corresponding quality scores. The quality of the obtained raw Fastq files was checked and adjusted with a tool to quality control for high throughput sequence data the FastQC. Then the reads were direct mapped against a reference bovine (*Bos taurus*) genome, using Bowtie2, a tool for aligning sequencing reads to long reference sequence (LANGMEAD; SALZBERG, 2012). The count data needed to be normalized according to the paired-end exon model FPKM (Fragments Per Kilobase per Million mapped reads). The summarized transcriptome-mapped count data were tested for significant differences in transcript abundance between hypothalamus areas, using the edgeR (Empirical analysis of digital gene expression data in R) tool (McCARTHY; CHEN; SMYTH, 2012; ROBINSON; McCARTHY; SMYTH, 2010). Differences in expression between exogenous progesterone and endogenous estradiol and the hormonal effect in each area of the hypothalamus were established by comparing each gene in each pool according to treatment.

To determine the estradiol effect SE and PE samples were analyzed against the SS and SP samples, while to determine progesterone effect PE and SP samples were analyzed against

the SS and SE samples in each area. The differential expression analysis of RNA-Seq generated values of log of fold change on base 2 (LogFC), *P*-values and adjusted *P*-values using Benjamini and Hochberg (1995) tests (BH Padj) to each gene. On anterior hypothalamus were identified 12,755 genes and on 13,119 genes on preoptic area, and from these were discarded of analyses those that had LogFC less than 1.3 and BH Padj higher than 0.10. Following the described criteria 268 genes on anterior and 70 genes on preoptic area were selected to continue the analyses.

The selected genes were analyzed separated according to hypothalamic area using the web-accessible program DAVID v.6.7 (Database for Annotation, Visualization and Integrated Discovery), and PANTHER (Protein ANalysis THrough Evolutionary Relationships) to identify the genes altered by progesterone or estradiol looking for possible genes related to the attainment of puberty.

DAVID were used to identify enriched biological themes, particularly gene ontology (GO) terms; discover enriched functional-related gene groups, and cluster redundant annotation terms (SHERMAN; LEMPICKI, 2009). The PANTHER is a classification system that classify proteins and their genes according to: molecular function (MF), biological process (BP) or pathway (MI et al., 2013). The terms generated by these 2 web-tools were visualized, filtered and correlated with the reproductive function based on GO, MF, BP associated with a literature review.

3.2.8 Real time PCR (qPCR) analyses

Nine genes were selected to verify RNA-Seq results by qPCR quantification. These genes were selected based on their RNA-Seq results and their contribution to reproductive process.

After the selection, primers for: ADAM metallopeptidase domain 11 (ADAM11), cadherin, EGF LAG seven-pass G-type receptor 3 (CELSR3), corticotropin releasing hormone receptor 2 (CRHR2), EGF-like repeats and discoidin I-like domains 3 (EDIL3), growth hormone releasing hormone (GHRH), and Kruppel-like factor 9 (KLF9), Neuropeptide Y (NPY), NPY receptor-1 (NPY1R), oxytocin, prepropeptide (OXT) target genes and the housekeeping gene 18S ribosomal RNA (RN18S1) gene, were designed for real-time PCR (qPCR) based on bovine GenBank sequences (Table 8), using the software

Primer Blast do NCBI (YE, et al. 2012) and tested with the software OligoAnalyzer 3.1 (OWCZARZY, et al. 2008) to dimer and hairpin formation.

Relative gene expression quantification of ADAM11, CELSR3, CRHR2, EDIL3, GHRH, KLF9, NPY, NPY1R, and OXT was performed using RN18S1 as a constitutive gene. The real-time PCR reactions was performed using 10 µL of SYBR Green master mix 2 X (Life Technologies, Brazil), 0.25 mM of each primer, hypothalamic cDNA samples, and water up to a final volume of 20 µL. Thermal cycling parameters were as follow: an initial denaturing step of 94°C for 10 min, followed by 44 cycles of denaturing (94°C for 15 s), annealing/elongation (60 or 65 °C for 1 min), and a melting curve program (60-95°C with a heating rate of 0.3°C per cycle). All reactions were performed in duplicate wells.

The 7500® Real-Time PCR Systems (Life Technologies, Brazil), was used to perform the reactions. Amplification efficiency of all genes was verified by a dilution curve with a series of cDNA concentrations, and quantification was performed only when the efficiency was not different than 100% (YUAN et al.; 2006). All analyses were done based on second derivative maximum method.

3.2.9 Statistical analyses

All qPCR statistical analyses were conducted using SAS, version 9.1.2 for Windows (SAS Institute Inc., Cary, NC). Data were analyzed as a completely randomized design using the MIXED procedure of SAS, considering the fixed effect of area, and animal within area as a random effect.

The data were analyzed for residual homogeneity of variance, and observations outside the range of ± 3 s*tudentized* wastes were considerate atypical observations and withdrawals of the analysis. Contrasts analysis was used to compare the effect of treatments and to estimate the ΔΔCt, and its standard error of the mean. In all comparisons, significance was declared at $P \leq 0.05$, and tendencies at $P \leq 0.10$.

Table 8 – Oligonucleotide primer pairs designed for use in real-time polymerase chain reaction (qPCR) amplification

Genes*	Oligonucleotide primers: 5' → 3'	GenBank accession number**	Primer annealing temperature	PCR insert size (bp)
ADAM11	F-ATGCACTCTGACTCACGACG R-GGTTAGGTGGACACTGGCTC	XM_002696078.2	60°C	152
CELSR3	F-CCGGACTCCAGTTAGCATCC R-TGTTCTCCTTCACTCGCACC	NM_001205337.1	60°C	98
CRHR2	F-ACGAGCATCCACCACATCAG R-AGAAACCCTGGAACGACTGC	NM_001192545.1	60°C	179
EDIL3	F-GATGGCTTCACAGGTCCCAA R-TCTCCTCGGTACGCTTCACT	XM_002689374.3	60°C	140
GHRH	F-GGATTCCACGGTACGCAGAT R-GCTCCTTGCTCCTGGTTTCT	NM_178325.1	60°C	130
KLF9	F-GGAAACACGCCTCCGAAAAG R-AAGGGCCGTTCACCTGTATG	NM_001193214.1	60°C	115
NPY	F-ACCCCTCCAAGCCTGACAA R-TGCCTGGTGATGAGATTGATG	AY491054	60°C	100
NPY1R	F-ACAGGTCCAGTGAAGCCAAAA R-TGGTCCCAGTCAAACACAGTG	XM_580988	60°C	112
OXT	F-ACCATGGCAGGTTCCAGCCTCG R-CAGGGGAGACACGTGCGCAC	NM_176855.1	65°C	134
RN18S1	F-CGGCGACGACCCATTCGAAC R-GAATCGAACCCTGATTCCCCGTC	NR_036642.1	60°C	99

* ADAM metallopeptidase domain 11 (ADAM11); cadherin, EGF LAG seven-pass G-type receptor 3 (CELSR3); corticotropin releasing hormone receptor 2 (CRHR2); EGF-like repeats and discoidin I-like domains 3 (EDIL3); growth hormone releasing hormone (GHRH); Kruppel-like factor 9 (KLF9); neuropeptide Y (NPY); NPY receptor-1 (NPY1R); oxytocin, prepropeptide (OXT).
**Primers were designed based on previously deposited bovine sequences.
Fonte: (DINIZ-MAGALHÃES, J., 2014).

3.1 RESULTS

To determine the effects of estradiol and progesterone on both hypothalamic areas the gene expression were analyzed by RNA-Seq and qPCR, and the results are presented on separated sections.

3.1.1 Next generation RNA-sequencing

The next-generation RNA-sequencing is a technique to whole-transcriptome studies. In this experiment were obtained around 20 million reads in each sample, the data was filtered and generated around 18 million reads. Then the reads were mapped against a reference bovine (*Bos taurus*) genome and 16 million reads were identified. However, due to filtering of data just under a half of them were used in the differential expression analysis. This tool was used to identify the gene pathways and their contribution to the attainment of puberty according to estradiol and progesterone regulation and their hypothalamic localization in peripuberal Nellore heifers.

The estradiol and progesterone effects were analyzed in both hypothalamic areas. There was no effect of estradiol on gene expression on the preoptic area of the hypothalamus. Neither effect of progesterone on gene expression on the anterior hypothalamus. The regulatory mechanisms involving progesterone and estradiol can be area-specifics.

3.1.1.1 Estradiol effect on anterior hypothalamus

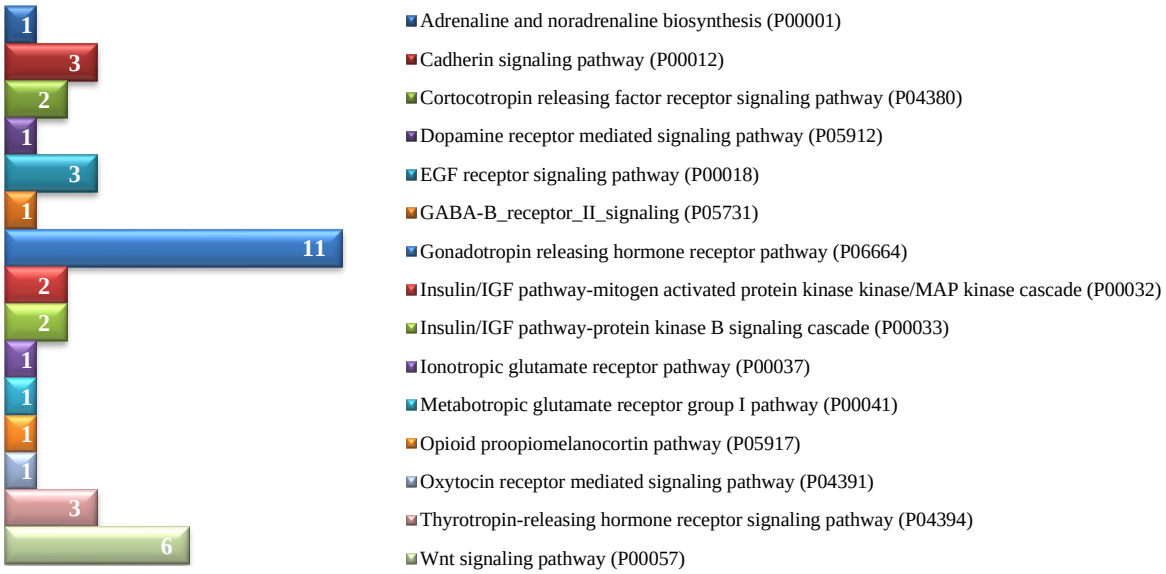
On anterior hypothalamus 12,755 genes were identified. From these genes, 268 were affected by estradiol (LogFC $\geq$ 1.3 and BH Padj $<$ 0.10). From 268 genes differentially expressed, 234 had higher expression on estradiol presence and the other 34 genes had lower expression.

The control of the hypothalamus-pituitary-gonadal (HPG) axis is regulated by neurotransmitters that act on the stimulation or inhibition of GnRH neurons. The estradiol

influence on these classes of neurotransmitters on the anterior hypothalamus was observed when the 268 genes were analyzed by PANTHER pathways. The PANTHER pathways specify the relationships between the interacting molecules as signaling, biosynthesis and receptor modulation. On figure 5 are the counts of genes according to pathway classification.

After analyzes, the genes were separated according to their PANTHER pathways (Tables 9 to 11). The most relevant pathways related with reproductive processes were used to identify candidate genes. The identified pathways were: gonadotropin releasing hormone receptor pathway (P06664; Table 9); EGF receptor signaling pathway (P00018; Table 10) and opioid proopiomelanocortin pathway (P05917; Table 11).

Figure 5 – PANTHER pathways related to differentially expressed genes affected by estradiol on anterior hypothalamic area



Fonte: (DINIZ-MAGALHÃES, J., 2014).

The neurotransmitters that control the energy intake can suggest the nutrient sufficiency and insufficiency to the hypothalamus, and is one mechanism that can regulate the attainment of puberty. Hypothalamic NPY/agouti-related protein (AgRP) neurons, and proopiomelanocortin (POMC) neurons are considered major pathways of nutritional signals (CROWN; CLIFTON; STEINER (2007). These genes had lower expression on estradiol presence, confirming that they are down-regulated by estradiol in peripubertal heifers (Tables 9 and 11).

In the ewe, subpopulations of neurons in the ARC and preoptic area are thought to control estradiol's positive and negative feedback on GnRH neurons that contains kisspeptins receptors (SMITH, 2009). The present study, estradiol reduced the expression of kisspeptin1 (KISS1) gene, suggesting a negative feedback control (Table 9).

Table 9 – Differences in gene expression affected by estradiol presence related to gonadotropin releasing hormone receptor pathway (P06664) generated using PANTHER on anterior hypothalamic area

Gene	Gene Name	Estradiol+[1]		Estradiol-[2]		LogFC[5]	BH Padj[6]
		P4+[3]	P4-[4]	P4+	P4-		
AGRP	agouti related protein homolog (mouse)	8.0	7.0	32.2	24.4	-1.9	0.02
CGA	glycoprotein hormones, alpha polypeptide	15.5	6.0	110.4	17.3	-2.6	0.03
KISS1	KiSS-1 metastasis-suppressor	5.5	1.8	15.6	8.3	-1.7	0.05
NMU	neuromedin U	2.9	2.6	5.6	7.7	-1.3	0.08
NPY	neuropeptide Y	15.7	26.9	79.5	38.1	-1.4	0.08
PRL	Prolactina	3.8	28.2	44.5	200.9	-3.1	0.01
ADCYAP1R1	adenylate cyclase activating polypeptide 1 (pituitary) receptor type I; GHRH receptor	3.4	5.2	1.7	0.9	1.6	0.02
GNAQ	guanine nucleotide binding protein (G protein), q polypeptide	3.6	2.7	1.1	0.5	1.8	0.02
IRS2	insulin receptor substrate 2	2.4	1.7	1.2	0.3	1.3	0.10
KAT2B	K(lysine) acetyltransferase 2B	148.7	118.0	67.0	34.1	1.3	<0.01
NFATC3	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	3.1	2.3	1.6	0.3	1.3	0.07
PXN	Paxillin	2.9	2.6	1.4	0.4	1.5	0.03
SOS1	son of sevenless homolog 1 (Drosophila)	2.0	3.6	1.4	0.7	1.4	0.05
TCF7L1	similar to HMG-box transcription factor TCF-3	2.2	3.7	1.4	0.5	1.5	0.03
ZEB1	zinc finger E-box binding homeobox 1	62.4	37.2	18.2	8.5	1.8	<0.01

[1]Estradiol (E2) presence; [2]E2 absence; [3]Progesterone (P4) presence; [4]P4 absence; [5]Log of the fold change expression; [6]BH Padj: P values adjusted by Benjamini and Hochberg tests.
Fonte: (DINIZ-MAGALHÃES, J., 2014).

Another pathway affected by estradiol is the epidermal growth factors (EGF) family. The EGFs mediate the stimulation of hypothalamic GnRH neurons during female sexual maturation, and the genes EGF-like repeats and discoidin I-like domains 3 (EDIL3), GRB2-associated binding protein 2 (GAB2), multiple EGF-like-domains 9 (MEGF9), and neurofibromin 1 (NF1) are part of the EGF receptor pathway and had higher expression on estradiol presence (Table 3).

Table 10 – Differences in gene expression affected by estradiol presence related to EGF receptor signaling pathway (P00018) generated using PANTHER on anterior hypothalamic area

Gene	Gene Name	Estradiol+[1]		Estradiol-[2]		LogFC[5]	BH Padj[6]
		P4+[3]	P4-[4]	P4+	P4-		
EDIL3	EGF-like repeats and discoidin I-like domains 3	7.3	6.8	2.9	1.3	1.6	0.01
GAB2	GRB2-associated binding protein 2	28.3	20.7	10.1	4.8	1.6	<0.01
MEGF9	multiple EGF-like-domains 9	4.1	2.9	1.6	0.9	1.3	0.04
NF1	neurofibromin 1	10.5	7.2	4.4	1.8	1.4	0.02

[1]Estradiol (E2) presence; [2]E2 absence; [3]Progesterone (P4) presence; [4]P4 absence; [5]Log of the fold change expression; [6]BH Padj: P values adjusted by Benjamini and Hochberg tests.
Fonte: (DINIZ-MAGALHÃES, J., 2014).

Table 11 – Differences in gene expression affected by estradiol presence related to Opioid proopiomelanocortin pathway (P05917) generated using PANTHER on anterior hypothalamic area

Gene	Gene Name	Estradiol+[1]		Estradiol-[2]		LogFC[5]	BH Padj[6]
		P4+[3]	P4-[4]	P4+	P4-		
PMCH	pro-melanin-concentrating hormone	352.7	249.8	891.0	878.4	-1.7	<0.01
POMC	Proopiomelanocortin	93.9	120.8	286.4	379.1	-1.7	<0.01

[1]Estradiol (E2) presence; [2]E2 absence; [3]Progesterone (P4) presence; [4]P4 absence; [5]Log of the fold change expression; [6]BH Padj: P values adjusted by Benjamini and Hochberg tests.
Fonte: (DINIZ-MAGALHÃES, J., 2014).

The DAVID enrichment was used to determine the gene ontology (GO) of the 268 previous genes selected on the anterior hypothalamic area. Over-representation of GO terms was determinated based on Fisher's exact test, and filtered with a false discovery rate (FDR) of < 10% (Table 12). Fifty genes were classified as regulators of biological process and 45 as regulators of cellular process (Table 12).

Table 12 – Gene Ontology (GO) associated with the 286 genes that are differentially expressed on anterior hypothalamus

GO ID	GO Description	Number of genes[1]	FDR[2]
GO:0065007	biological regulation	53	<0.01
GO:0050789	regulation of biological process	50	<0.01
GO:0050794	regulation of cellular process	45	0.02

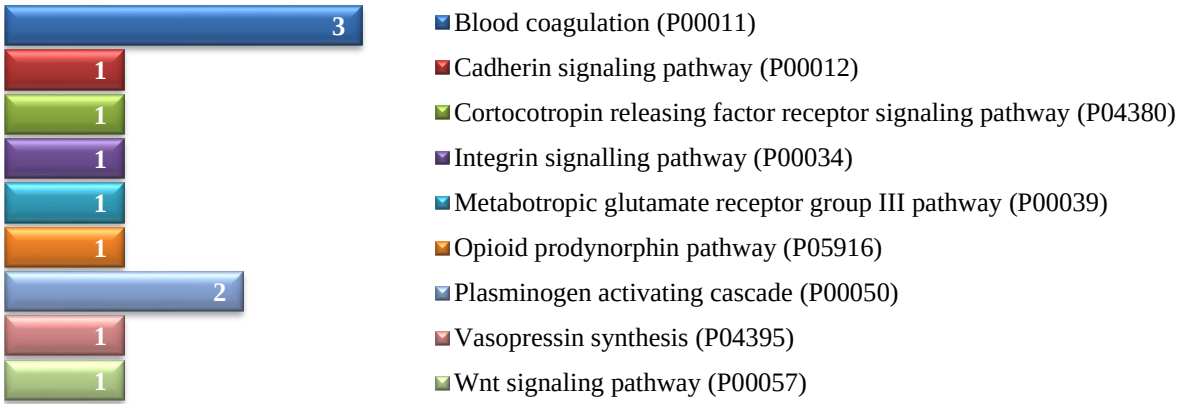
[1]number of genes according to Gene Ontology term; [2]FDR: false discovery rate
Fonte: (DINIZ-MAGALHÃES, J., 2014).

3.1.1.2 Progesterone effect on preoptic area

On the preoptic area 13,119 genes were identified. From these genes, 70 were affected by progesterone (LogFC ≥ 1.3 and BH Padj < 0.10). From the 70 genes differentially expressed, 21 had higher expression on progesterone presence and the other 49 genes had lower expression.

The influence of the classes of neurotransmitters and coagulation factors on the preoptic area was observed. The influence of these classes of neurotransmitters and coagulation factors was observed when the 70 genes were analyzed by PANTHER pathways. On figure 6 are the numbers of genes according to the pathway classification.

Figure 6 – PANTHER pathways related to differentially expressed genes affected by progesterone on preoptic hypothalamic area



Fonte: (DINIZ-MAGALHÃES, J., 2014).

After analyzes, the genes were separated according to their function based on the PANTHER pathways results (Table 13). The most relevant pathways related with reproductive processes affected by progesterone were used to identify candidate genes. The PANTHER pathways: cadherin signaling pathway (P00012), cortocotropin releasing factor receptor signaling pathway (P04380), metabotropic glutamate receptor group III pathway (P00039), opioid prodynorphin pathway (P05916), and wnt signaling pathway (P00057) are related with reproductive processes and were used to select the genes (Figure 6).

Two ADAM metallopeptidases, ADAM11 and ADAM33, were less expressed on progesterone presence, as CELSR3. The ADAMs are suggested to play a pivotal role in the

activation of EGFR (BLOBEL, 2005). The placental growth factor (PGF) is a GnRH inhibitor and had higher expression in progesterone presence (MILENKOVIĆ et al. (1994).

Table 13 – Differences in gene expression affected by progesterone presence related to reproduction generated using PANTHER on preoptic area

Gene	Gene Name	Progesterone+[1]		Progesterone-[2]		LogFC[5]	BH Padj[6]
		E2+[3]	E2-[4]	E2+	E2-		
ADAM11	ADAM metallopeptidase domain 11	0.9	0.6	3.1	3.9	-2.1	0.02
ADAM33	ADAM metallopeptidase domain 33	1.8	1.6	5.3	6.4	-1.7	0.02
CELSR3	cadherin, EGF LAG seven-pass G-type receptor 3	4.7	3.3	13.9	10.0	-1.5	<0.01
OXT	oxytocin, prepropeptide	5015.9	4189.1	839.8	2429.5	1.6	0.06
PGF	placental growth factor	61.1	57.9	16.6	27.7	1.5	<0.01
SLC17A7	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member	2.3	82.2	0.7	1.6	5.4	<0.01

[1]Progesterone (P4) presence; [2]P4 absence; [3]Estradiol (E2) presence; [4]E2 absence; [5]LogFC: Log of the fold change expression; [6]BH Padj: P values adjusted by Benjamini and Hochberg tests.
Fonte: (DINIZ-MAGALHÃES, J., 2014).

The DAVID enrichment was used to determine the gene ontology (GO) of the 70 previous genes selected on the preoptic area. Over-representation of GO terms was determinated based on Fisher's exact test and filtered with FDR of < 10% (Table 14). All clustered genes were classified as regulators of extracellular region.

Table 14 – Gene Ontology (GO) associated with the 70 genes that are differentially expressed on preoptic area

GO ID	GO Description	Number of genes[1]	FDR[2]
GO:0005576	extracellular region	15	<0.01
GO:0044421	extracellular region part	8	0.06

[1]number of genes according to Gene Ontology term; [2]FDR: false discovery rate
Fonte: (DINIZ-MAGALHÃES, J., 2014).

3.1.2 Candidate genes expression

To select the genes to validation by qPCR, after PANTHER and DAVID analyses. Participation on reproductive process was the main factor used.

3.1.2.1 Next-generation RNA-sequencing (RNA-Seq)

The RNA-Seq results for these genes were presented according to the area and the progesterone and estradiol effect on tables 15 and 16. The epidermal growth factors (EGF) mediate the stimulation of hypothalamic GnRH neurons during female sexual maturation, and the genes CELSR3, CRHR2, and EDIL3 are regulated by EGF (SINGH; HARRIS, 2005). The CRHR2 gene also modulates the steroid secretion on adrenal cortex (HARRIS, 2010). KLF9 is a zinc finger transcription factor that modulates the activity of progesterone receptor (PGR; PABONA, et al. 2012). The GHRH releases GH1, and its gene expression is negatively regulated by GH1, which stimulates the somatostatin and IGF-1 secretion. NPY and its receptor are potential inhibitors of GnRH synthesis (GONZALES et al., 2003).

3.1.2.1.1 Estradiol effect on anterior hypothalamus

Considering a BH Padj < 0,10, the genes EDIL3 and KLF9 were more expressed on estradiol presence, and GHRH and NPY were less expressed on anterior hypothalamus (Table 15).

Table 15 – RNA-Seq results of genes on anterior hypothalamus submmited to qPCR analyses

Gene	Estradiol+[1]		Estradiol-[2]		logFC[5]	BH Padj[6]
	P4-[3]	P4+[4]	P4-	P4+		
CELSR3	9.0	9.2	7.3	3.5	0.7	0.31
CRHR2	4.0	6.8	6.2	4.0	0.0	0.99
EDIL3	22.0	20.4	8.6	4.0	1.6	0.01
GHRH	34.0	26.3	109.2	81.2	-1.6	0.09
KLF9	21.1	19.3	7.7	2.9	1.8	<0.01
NPY	47.1	80.6	238.4	114.4	-1.4	0.08
NPY1R	39.5	49.5	51.0	47.1	-0.2	0.70

[1]Estradiol (E2) presence; [2]E2 absence; [3]Progesterone (P4) presence; [4]P4 absence; [5]LogFC: Log of the fold change expression; [6]BH Padj: P values adjusted by Benjamini and Hochberg tests.
Fonte: (DINIZ-MAGALHÃES, J., 2014).

3.1.2.1.2 Progesterone effect on preoptic area

Considering a BH Padj < 0,10, the gene OXT were more expressed on progesterone presence, and GHRH and ADAM1, CELSR3 and CRHR2 were less expressed on preoptic hypothalamic area (Table 16).

Table 16 – RNA-Seq results of genes on preoptic area submmited to qPCR analyses

Gene	Progesterone+[1]		Progesterone-[4]		logFC[5]	BH Padj[6]
	E2-[1]	E2-[3]	E2-	E2-		
ADAM11	2.8	1.9	11.6	9.3	-2.1	0.02
CELSR3	14.1	10.0	30.0	41.6	-1.5	<0.01
CRHR2	13.2	7.0	47.4	19.3	-1.7	0.01
EDIL3	14.8	7.7	38.4	16.9	-1.2	0.32
GHRH	4.6	1.8	3.2	2.2	0.3	0.98
KLF9	18.8	12.5	17.5	21.2	-0.2	0.96
NPY	29.5	110.5	27.7	63.9	0.8	0.84
NPY1R	22.3	48.9	36.0	43.0	0.0	1.00
OXT	15047.7	12567.2	7288.4	2519.4	1.6	0.06

[1]Progesterone (P4) presence; [2]P4 absence; [3]Estradiol (E2) presence; [4]E2 absence; [5]Log of the fold change expression; [6]BH Padj: P values adjusted by Benjamini and Hochberg tests.
Fonte: (DINIZ-MAGALHÃES, J., 2014).

3.1.2.2 Relative gene expression by qPCR

Nine genes: ADAM11, CELSR3, CRHR2, EDIL3, GHRH, KLF9, NPY NPY1R and OXT, were selected based on their contribution to the reproductive process and documented role in regulation of puberty.

3.1.2.2.1 Estradiol effect on anterior hypothalamus

The relative expression of genes was calculated based on the ΔΔCt method and the values adjusted ($2^{(-\Delta\Delta Ct)}$) to generate the data. The estradiol effect was analyzed on anterior hypothalamus. Just NPY1R was affected by an interaction between estradiol and progesterone ($P = 0.01$), but this data was justified by the progesterone presence. The other genes CELSR3, CRHR2, EDIL3, GHRH, KLF9 and NPY were not changed by estradiol or progesterone (Table 17). The genes ADAM11 and OXT were not analyzed by qPCR.

Table 17 – Relative gene expression of CELSR3, CRHR2, EDIL3, GHRH, KLF9, NPY, and NPY1R affected by endogenous estradiol and exogenous progesterone on anterior hypothalamus

Gene	Estradiol+[1]		Estradiol-[2]		SEM	Pvalue[5]		
	P4-[3]	P4+[4]	P4-	P4+		E2	P4	E2*P4
CELSR3	1.0	1.0	0.7	0.9	0.01	0.39	0.61	0.69
CRHR2	1.0	0.6	0.5	0.7	0.03	0.61	0.92	0.42
EDIL3	1.0	1.0	1.2	1.0	0.01	0.56	0.58	0.70
GHRH	1.0	1.2	1.3	0.7	0.03	0.73	0.69	0.42
KLF9	1.0	0.8	1.2	0.8	0.00	0.64	0.15	0.72
NPY	1.0	2.0	1.6	0.5	0.06	0.49	0.76	0.19
NPY1R	1.0	1.7[a]	1.5	0.9[b]	0.00	0.50	0.99	0.01

[1]Estradiol (E2) presence; [2]E2 absence; [3]Progesterone (P4) presence; [4]P4 absence; [5]Pvalues: E2 – estradiol effect; P4 – progesterone effect; E2*P4 – interaction value.
Fonte: (DINIZ-MAGALHÃES, J., 2014).

3.1.2.2.2 Progesterone effect on preoptic area

The relative expression of genes was calculated likewise on anterior hypothalamus, but the treatments effect was expressed based on the progesterone presence (SP and PE) or absence (SS and SE) and the estradiol influence on preoptic area. The genes GHRH (P = 0.04) and NPY (P = 0.01) were affected by an interaction between estradiol and progesterone. And the gene ADAM11, were affected just by the estradiol (P = 0.03). Expression of the other genes CELSR3, CRHR2, EDIL3, KLF9, NPY1R, and OXT were not changed by estradiol or progesterone ($P < 0.05$; Table 18) on the preoptic area.

Table 18 – Relative gene expression of ADAM11, CELSR3, CRHR2, EDIL3, GHRH, KLF9, NPY, NPY1R, and OXT affected by exogenous progesterone and endogenous estradiol on preoptic area

Gene	Progesterone+		Progesterone-		SEM	Pvalue		
	E2-	E2+	E2-	E2+		E	P	E*P
ADAM11	0.3	0.4[A]	1.0	0.8[A]	0.02	0.03	0.97	0.60
CELSR3	0.5	0.4	1.0	0.5	0.04	0.35	0.46	0.77
CRHR2	0.3	0.4	1.0	0.3	0.01	0.46	0.41	0.24
EDIL3	0.8	0.6	1.0	0.7	0.01	0.34	0.16	1.00
GHRH	2.7	1.9	1.0	6.5	0.03	0.82	0.17	0.04
KLF9	1.0	0.8	1.0	1.1	0.01	0.42	0.61	0.35
NPY	3.2	0.8	1.0	3.9	0.04	0.72	0.97	0.01
NPY1R	0.9	0.8	1.0	0.8	0.01	0.86	0.45	0.87
OXT	0.3	0.4	1.0	0.2	0.06	0.60	0.27	0.15

[1]Progesterone (P4) presence; [2]P4 absence; [3]Estradiol (E2) presence; [4]E2 absence5Pvalues: E2 – estradiol effect; P4 – progesterone effect; E2*P4 – interaction value.
Fonte: (DINIZ-MAGALHÃES, J., 2014).

3.2 DISCUSSION

The Nellore cattle is characterized by a slow sexual maturation, estradiol and progesterone are involved in regulating the onset of puberty by modulating the expression of several genes in the hypothalamus. Aiming to interfere on the timing of first ovulation, hormonal protocols were developed. Administration of exogenous progesterone or progestins in the prepubertal period acts as an inducer of puberty on the hypothalamic-pituitary-gonadal axis, improving reproductive rates. Changes in gene expression were observed on hypothalamic specific areas according to the hormonal treatment established.

The endogenous estradiol affected the genes related to the attainment of puberty in peripubertal heifers on the anterior hypothalamus and progesterone altered genes on the preoptic area. In this experiment, the neuropeptides that signalize the metabolic status to hypothalamus were affected by endogenous estradiol presence or absence.

The heifers in this experiment were supplemented after weaning and were from a herd with precocious Nellore heifers. In a recent review, Amstalden et al. (2011) summarized the neuroendocrine pathways to characterized the acceleration of puberty. They highlighted the signals of nutrient sufficiency that hastens the peripubertal activation of GnRH neurons, by reducing inhibitory signals (NPY) and enhancing stimulatory signals (kisspeptin, POMC-derived peptides), therefore leading to increased frequency of episodic release of GnRH and early onset of puberty (AMSTALDEN, et al. 2011). With few exceptions, neuropeptides that stimulate ingestion inhibits the reproduction process (SCHNEIDER, 2004), as is the case of AGRP and NPY. As observed in this study by RNA-Seq analyzes NPY, AGRP, KISS1, POMC, and PMHC had less expression in estradiol presence on the anterior hypothalamus. This is a contradictory result because both inhibitor (gene) and stimulators (gene) mechanisms of GnRH neurons were suppressed by estradiol. In the ARC, estrogen inhibits the Kiss1 expression in rodents, leading to reduced GnRH secretion, consistent with negative feedback regulation, similarly to the observed in this experiment (SINCHAK; WAGNER, 2012).

At the time of puberty, positive feedback loop between estradiol, GnRH, and LH/FSH is also established, possibly through kisspeptin-secreting neurons in the ARC or the POA regions (DAY; ANDERSON, 1998). Mouse kisspeptin neurons of the ARC produce an additional peptide, the neurokinin B (TAC3; NAVARRO, et al. 2009), which is encoded by a gene, recently shown to be required for puberty to occur (TOPALOGLU, et al. 2009). These observations indicate that the excitatory regulation of GnRH secretion is provided by neurons

that use glutamate, kisspeptin (OJEDA, et al. 2010), and perhaps neurokinin B (TOPALOGLU, et al. 2009).

The estradiol presence also stimulated the expression of KLF9, which seems to be a regulator of progesterone receptor (PGR). Loss of KLF9 function attenuated PGR expression in the women endometrium (PABONA, et al. 2012). The reduction of KLF9 expression, coincidently with reductions in PGR, and wnts, can contribute to a regulation in reproductive processes, but this function in hypothalamus regulation and attainment of puberty is not clear.

In our model, the exogenous progesterone altered just 6 genes that can be related to the reproductive process on the preoptic area. Among them are the 2 members of ADAM metallopeptidases. The ADAM play a role in bovine preovulatory follicles during the LH surge (LI, et al., 2009; BLISS, et al. 2010). The ADAMs 10 and 17 were described as regulators of LH function control on follicular cells (LI, et al., 2009). Other members of ADAM family (ADAM 11 and 33) were more expressed in the estradiol presence on the preoptic area, suggesting that ADAMs 11 and 33 can regulate the GnRH release and control LH surge.

3.3 CONCLUSIONS

The expression of genes related to the attainment of puberty was altered by estradiol on the hypothalamus, and was area specific. The estradiol presence modulates genes related to metabolic control on anterior hypothalamus, affecting direct or indirectly the GnRH neurons. The EGF control is regulated by estradiol and progesterone, and this occurs in different hypothalamic areas.

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4 CONCLUSIONS

The expression of genes related to the attainment of puberty was altered by estradiol on the hypothalamus, and was area specific.

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