ANNA LUIZA LACERDA SGUIZZATO

ASSESSING THE EFFECT OF rbST ON MAMMARY GLAND GROWTH AND REPRODUCTIVE CHARACTERISTICS OF HOLSTEIN × GYR PRE-PUBERTAL HEIFERS; MODELLING THE IMPACT OF MASTITIS ON MILK YIELD LOSSES

Thesis submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Marcos Inácio Marcondes

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Marcos Inácio Marcondes
Adviser

To my parents Sonia and Mauro, to my sister Anna Carolina, and to my husband Pedro for all the unconditional support, patience, love, and trust.

I dedicate.

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"Nothing contributes so much to tranquilize the mind as a steady purpose - a point on which the soul may fix its intellectual eye"

(Mary Shelley)

ABSTRACT

SGUIZZATO, Anna Luiza Lacerda, D.Sc., Universidade Federal de Viçosa, February, 2023. **Assessing the effect of rbST on mammary gland growth and reproductive characteristics Holstein × Gyr pre-pubertal heifers; Modelling the impact of mastitis on milk yield losses**. Adviser: Marcos Inácio Marcondes.

In this thesis, two chapters were based on a study with Holstein × Gyr pre-pubertal heifers and one on modeling mastitis data. The objective of the first chapter was to evaluate the effects of recombinant bovine somatotropin (rbST) on digestibility, performance, blood metabolites, mammary gland development, and carcass composition of high-performance pre-pubertal Holstein × Gyr heifers. It was observed that the rbST did not influence digestibility or performance parameters, increased IGF1 concentration and ribeye area, reduced parenchyma and fat pad pixels/mm², and decreased IGFBP3 expression in the mammary gland. In summary, rbST injections can be used to overcome the detrimental effects of high-performance diets on mammary gland growth and to improve lean carcass gain of pre-pubertal Holstein × Gyr heifers. For the second chapter, the objective was to assess the effects of rbST on reproductive traits, steroid concentration, and gene expression of cumulus-oocyte complex of high-performance pre-pubertal Holstein × Gyr heifers (same heifers and protocols mentioned in the first chapter). As expected, some heifers achieved puberty during the trial, approximately 36% of the assessed females. However, neither puberty attainment nor age, weight, or height at puberty were influenced by treatment. The reproductive condition did not affect ovary size, but there was an interaction effect of treatment and day, where heifers from the rbST treatment had greater ovary size on days 36, 43, 50, and 57. The rbST pubertal heifers had greater follicle sizes compared to the no rbST females on days 15 and 57, but no rbST heifers had larger follicles on days 71 and 85. Gene expression assessment resulted in a greater mRNA abundance of IGF1R on the cumulus-oocyte complex of heifers on no rbST treatment. To conclude, the evidence gathered in this study supports the hypothesis that the use of rbST would impact on reproductive characteristics of Holstein × Gyr heifers. The objective of the third chapter was to describe the impact of mastitis on milk production based on mastitis degree and moment of occurrence. Data from thirteen dairy farms were collected, generating a databank of 908,816 daily individual milk test records from 3,508 cows in different lactations, from January 2017 to December 2022. Mastitis drop and recovery were modeled

following three steps: removal of milk recorded at the diagnosis day of mastitis from the databank and fit of a Wood's Curve for each cow and lactation order; return of mastitis data to the database and estimation of the residual milk loss due to mastitis from 6 days before and after the mastitis event; use of a meta-analytical approach, including farm as a random control effect, to estimate residual milk loss. The estimations suggest that milk drop occurs three to four days prior to mastitis onset and can last until ten to twelve days from the diagnosis. Moreover, milk losses are greater than the values referenced in the literature and differ due to mastitis level. The novelty of our study indicates when and to what proportion mastitis drop and recovery occur and brings new perspectives to mastitis modeling.

Keywords: Crossbred. Somatotropin. IGFBP3. Reproduction. Modelling. Milk production. Mastitis.

RESUMO

SGUIZZATO, Anna Luiza Lacerda, D.Sc., Universidade Federal de Viçosa, fevereiro de 2023. **Assessing the effect of rbST on mammary gland growth and the onset of puberty of Holstein × Gyr pre-pubertal heifers; Modelling the impact of mastitis on milk yield losses.** Orientador: Marcos Inácio Marcondes.

Nesta tese, dois capítulos foram baseados em um estudo com novilhas pré-púberes Holandês × Gir e um na modelagem de dados de mastite. O objetivo do primeiro capítulo foi avaliar os efeitos da somatotropina recombinante bovina (rbST) sobre a digestibilidade, desempenho, metabólitos sanguíneos, desenvolvimento da glândula mamária e composição da carcaça de novilhas pré-púberes Holandês × Gir. Observou-se que o rbST não influenciou os parâmetros de digestibilidade e desempenho, aumentou a concentração de IGF1 e a área de olho de lombo, reduziu os pixels/mm² do parênquima e do fat pad e diminuiu a expressão de IGFBP3 na glândula mamária. Em suma, o rbST pode ser usado para evitar os efeitos prejudiciais de dietas de alto desempenho no crescimento da glândula mamária e para melhorar o ganho de carcaça de novilhas pré-púberes Holandês × Gir. Para o segundo capítulo, objetivou-se avaliar os efeitos do rbST nas características reprodutivas, na concentração de esteroides e na expressão gênica do complexo cumulus-oócito de novilhas pré-púberes Holandês × Gir (mesmas novilhas e protocolos mencionados no primeiro capítulo). Como esperado, algumas novilhas atingiram a puberdade, aproximadamente 36% das fêmeas avaliadas. No entanto, nem o momento da puberdade, nem a idade, o peso ou a altura na puberdade foram influenciados pelos tratamentos. A condição reprodutiva não afetou o tamanho dos ovários, mas houve interação entre o tratamento e o dia, onde as novilhas do tratamento rbST tiveram maior tamanho dos ovários nos dias 36, 43, 50 e 57. Novilhas rbST púberes tiveram maiores tamanhos de folículos comparadas às fêmeas sem rbST nos dias 15 e 57. A avaliação da expressão gênica resultou em maior abundância de mRNA de IGF1R no complexo cumulus-oócito de novilhas sem rbST. Concluindo, as evidências desse estudo apoiam a hipótese de que o uso do rbST impacta nas características reprodutivas de novilhas Holandês × Gir. O objetivo do terceiro capítulo foi descrever o impacto da mastite na produção de leite com base no grau e momento da mastite. Foram coletados dados de treze fazendas leiteiras, gerando um banco de dados de 908.816 registros individuais diários de amostras de leite de 3.508 vacas em diferentes lactações, de janeiro de 2017 a dezembro de 2022. A queda e recuperação da mastite foram modeladas seguindo três etapas:

remoção do registro de produção de leite no dia da mastite e ajuste da Curva de Wood para cada vaca e ordem de lactação; retorno dos dados de mastite ao banco de dados e estimativa da perda de leite residual devido à mastite, 6 dias antes e depois do evento de mastite; utilização de abordagem meta-analítica, incluindo as fazendas como covariáveis, para estimar a perda de leite residual. As estimativas sugerem que a queda do leite ocorre de três a quatro dias antes do início da mastite e pode durar de dez a doze dias a partir do diagnóstico. Além disso, as perdas de leite são superiores aos valores referenciados na literatura e diferem devido ao nível de mastite. Nosso estudo indica quando e em que proporção ocorre a queda e a recuperação da mastite e traz novas perspectivas para sua modelagem.

Palavras-chave: Meio-sangue. Somatotropina. IGFBP3. Reprodução. Modelagem. Produção de leite. Mastite.

SUMMARY

1. GENERAL INTRODUCTION

Dairy production faces many challenges every day, from basic shores to more complex interactions among animals, the environment, people, and market demands. It is a broad field for research with enough room to capture all kinds of enthusiasts. Thus, in this thesis, we investigated two distinguished research topics: “The effects of rbST on mammary gland growth and on reproductive characteristics of Holstein × Gyr pre-pubertal heifers” and “Modelled the impact of mastitis on milk yield losses”.

Addressing the first research topic we acknowledge that raising replacement heifers is an essential yet expensive practice on dairy farms. To reduce time in this unproductive phase, feeding diets to achieve increased daily gain rates during pre-puberty has become a widespread management strategy on farms to accelerate growth and hasten puberty (LE COZLER et al., 2008).

In Brazil, the majority of the dairy herd is composed of Holstein × Gyr animals, and despite recent efforts to improve energy and protein requirements (CASTRO et al., 2020; MARCONDES; SILVA, 2021), performance (CABRAL DA SILVA et al., 2020), growth (REIS et al., 2020), and reproduction (MACHADO et al., 2020), research is still needed to overcome production flaws such as late puberty occasioned by delayed reproductive characteristics (FERNANDEZ et al., 2020; SEVERINO-LENDECHY et al., 2020) and detrimental effects of high-performance diets on the mammary gland (ALBINO et al., 2017; WELLER et al., 2016).

Puberty is categorized as a critical period in heifer life encompassing the transition from an anovulatory state to the first ovulatory estrus (ATKINS; POHLER; SMITH, 2013; ESTILL, 2015), where the decisive event essential for puberty is the reversion of estradiol negative feedback to stimulatory GnRH release in the hypothalamus, which increases FSH and LH secretion (ATKINS; POHLER; SMITH, 2013; DUITTOZ et al., 2016; PERRY, 2016). Several factors, such as breed, age, and weight can influence puberty [ESTILL, 2014]. According to FERNANDEZ et al. (2020) and SEVERINO-LENDECHY et al. (2020), the average age at puberty of Holstein × Gyr heifers is 30 and 19 months of age for unsupplemented and supplemented heifers, respectively. Therefore, it is vital to search for strategies to overcome this breed’s late entrance into reproductive life. However, before achieving the onset of puberty, females also need to undergo extensive follicle proliferation, recruitment, and dominance, in addition to the growth of other reproductive tissues and their bodies (muscular and adipose tissue).

Regarding mammary gland growth during pre-puberty, feeding high gain diets can result in increased adipose tissue in the mammary gland of Holstein × Gyr heifers, even when an adequate level of metabolizable protein and energy is fed as a nutritional strategy to reduced damage on mammary growth (ALBINO et al., 2017; WELLER et al., 2016). Thus, knowing that the pre-pubertal mammary growth will affect the future production capacity of the cow, it is essential to guarantee adequate mammary development, associating nutritional strategies and hormonal pathways (BROWN et al., 2005; CAPUCO et al., 1995; SEJRSEN et al., 2000).

A technology long used in dairy production is the recombinant bovine somatotropin (rbST), the synthetical form of the growth hormone (GH) used to enhance milk production and positive correlated to mammary growth, lean carcass gain, and to beneficial effects on reproductive traits (BAUMAN, 1999). The GH is secreted from the anterior pituitary and regulates growth and metabolism, activating and promoting the transcription of GH-sensitive genes, such as the IGF1, an essential component of the IGF1/IGFBP3 (insulin-like growth factor binding protein 3) complex. The IGFBP3 is the major IGFBP found in bovine mammary cells and milk, which can inhibit IGF1 action (BAUMRUCKER; ERONDU, 2000). Additionally, in beef animals, the rbST, used for six months, effectively hastened the puberty of Angus × Hereford heifers (COOKE et al., 2013) . The GH was proved to act in reproductive tissues once mRNA GH receptors were found in corpus luteum, bovine follicles, granulosa and cumulus cells, and oocytes (IZADYAR et al., 1997; LUCY, 2000). Thus, using non-nutritional strategies, such as frequent application of rbST, may prevent future losses in productivity and promote adequate mammary gland development, animal performance, reproductive benefits, and hasten puberty.

Therefore, considering the literature background on reproductive improvements with the use of rbST and based on the limiting literature research focused on strategies to overcome the detrimental effects of high gain diets on the development of Holstein × Gyr heifers during pre-puberty, we hypothesized that rbST injections could improve the reproductive characteristics of pre-pubertal Holstein × Gyr heifers by improving oocyte quality, follicles growth, and increasing concentration of progesterone and estrogen, which are closely related to sexual maturation – puberty attainment. In addition, exogenous rbST could stimulate parenchymal tissue growth, decrease adipose tissue deposition in the mammary gland, improve protein deposition on carcass, and enhance the performance of pre-pubertal Holstein × Gyr heifers.

For our second research topic, we focused on a new modelling approach to identify when and to what proportion milk losses due to mastitis occur.

Mastitis is one of the most common causes of economic losses on dairy farms (SILVA et al., 2021), known to depress milk yield, reduce fertility, and increase culling rates (DAROS et al., 2019). Clinical cases of mastitis present visible signs of the infection, such as clots in the milk, discomfort while milking, swelling, fever, and loss of appetite (NIELSEN, 2009). This disease can also cause systemic reactions in the cow's body and a constant production loss for each one-unit increase in somatic cell score (RUEGG, 2017).

According to PUERTO et al. (2021), the highest reductions in positive clinical mastitic cows were observed during late- and mid-lactation (1,137 and 506 kg of milk, respectively), resulting in losses of $710 to $324 of cumulative milk value. van Soest et al. (2016) acknowledged that the average milk production loss of cows suffering from clinical mastitis was 336 kg per case per year, approximately €240 per lactating cow per year.

LESCOURRET; COULON (1994), NIELSEN, (2009) and RAJALA-SCHULTZ et al. (1999) observed that milk losses due to mastitis initiate two to four weeks before the diagnosis and could be influenced by previous production, lactation week, and parity. Additionally, NIELSEN (2009) estimated that, on mastitis day, primiparous cows could lose 5 kg of milk, while multiparous cows can have their production reduced from 1 to 8 kg. Moreover,) and LESCOURRET; COULON (1994; RAJALA-SCHULTZ et al., (1999) observed that milk recovery could occur over two or four weeks passed the disease identification, although milk yield becomes compromised for the entire lactation. However, despite the references suggesting daily and overall losses in lactation due to clinical mastitis cases, no recent study attempted to model daily milk losses before mastitis onset and the moment when it begins; similarly, more information is necessary regarding daily milk production reestablishment and recovery time after the mastitis identification.

Thus, considering the welfare impairment and the production losses that this disease can trigger in animals, it is of utmost importance to develop a new model with more robust data regarding mastitis incidence on dairy farms, which will estimate milk loss and the recovery time after the diagnosis of this disease.

Therefore, our overall objects were:

1st – To assess the effects of exogenous rbST on digestibility, growth, mammary gland development, and carcass composition of pre-pubertal Holstein × Gyr heifers fed high gain diets.

2nd – To assess the effects of exogenous rbST on reproductive characteristics, hormones concentration, and gene expression of the cumulus-oocyte complex.

3rd – To describe the impact of mastitis on milk production based on mastitis degree and moment of occurrence.

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2. CHAPTER ONE

IS BOVINE SOMATOTROPIN AN ALTERNATIVE STRATEGY TO OVERCOME THE DETRIMENTAL EFFECTS OF HIGH GAIN DIETS ON PRE-PUBERTAL HOLSTEIN × GYR HEIFERS?[1]

Effect of bovine somatotropin on Holstein × Gyr heifers

[1]This chapter is under revision at *Plos One Journal*.

ABSTRACT

Feeding high gain diets and an inadequate energy and protein ratio during pre-puberty may lead to impaired growth and mammary gland development of heifers. Thus, frequent application of bovine somatotropin (**bST**) may prevent future losses in productivity, improve mammary development and animal performance. We aimed to evaluate the effects of bST on digestibility, performance, blood metabolites, mammary gland development, and carcass composition of high-performance pre-pubertal Holstein × Gyr heifers. Thirty-four Holstein × Gyr heifers with an average initial body weight of 218 ± 49 kg and 14 ± 4 months of age were submitted to an 84-day trial evaluating the effects of no bST or bST injections. Treatments were randomly assigned to each animal within one of the tree blocks. The bST did not influence digestibility or performance parameters. Regarding blood results, IGF1 concentration presented an interaction between treatment and day, where bST heifers had the highest IGF1 concentration. Heifers receiving bST also showed increased ribeye area; however, only an experimental day effect for backfat thickness was observed, with greater accumulation of carcass fat on day 84. Heifers receiving bST had lower pixels/mm² on parenchyma, characteristic of greater parenchymal tissue. Moreover, heifers on bST

treatment also had reduced pixels/mm², characteristic of reduced fat pad tissue. Lastly, bST injections did not influence liver and muscle gene expression, nor most genes evaluated in mammary gland tissue, except for *IGFBP3* expression, which was lower for bST heifers. In summary, we confirm the efficacy of bST injections to overcome the detrimental effects of high gain diets on mammary gland growth and to improve lean carcass gain of pre-pubertal Holstein × Gyr heifers.

INTRODUCTION

Raising replacement heifers is an essential yet expensive practice on dairy farms. To reduce time in this unproductive phase, feeding diets to achieve increased daily gain rates during pre-puberty has become a widespread management strategy on farms to accelerate growth and hasten puberty [1] and age at first calving, which enables a more rapid profit from heifers' milk production. However, feeding for high gain, along with an inadequate energy and protein ratio during pre-puberty, may lead to impaired growth and mammary gland development of heifers [2–4].

In Brazil, the majority of the dairy herd is composed of Holstein × Gyr animals, and despite recent efforts to improve energy and protein requirements [5,6], performance [7], growth [8], and reproduction [9], research is still needed to overcome production flaws such as late puberty [10,11] and detrimental effects of high gain diets on the mammary gland [4,12]. In addition, feeding high gain diets can result in a more significant deposition of subcutaneous backfat thickness in the carcass and increased adipose tissue in the mammary gland of Holstein × Gyr heifers, even when an adequate level of metabolizable protein and energy is fed as a nutritional strategy to reduced damage on mammary growth [4,12].

The mammary development begins in the fetus [13]. From birth to 2 or 3 months of age, the gland grows at similar rates as the body (isometric), and from around 3 months of age until puberty, or shortly thereafter, mammary growth is allometric [14], when the gland grows at a faster rate compared to the rest of the body. This phase is mainly characterized by restricted duct development and increased udder size by fat pad accumulation, which would later impact secretory tissue development [13,15,16]. Thus, knowing that the pre-pubertal mammary growth will affect the future production capacity of the cow, it is essential to guarantee adequate mammary development, associating nutritional strategies and hormonal pathways [15,17,18].

Previous studies have demonstrated a positive correlation between endogenous or exogenous growth hormone (GH), carcass leanness, and mammary growth [2,19]. GH is secreted from the anterior pituitary and regulates growth and metabolism, activating and promoting the transcription of GH-sensitive genes, such as the IGF1, an essential component of the IGF1/IGFBP3 (insulin-like growth factor binding protein 3) complex. The IGFBP3 is the major IGFBP found in bovine mammary cells and milk, which can inhibit IGF1 action [20]. However, depending on the stimulus, it can exert dependent or independent effects on cell growth, proliferation, and apoptosis [21]. Estrogen, along with the GH, is another hormone responsible for stimulating mammary epithelial cell proliferation [22], resulting in ductal elongation and bifurcation [23].

To our understanding, the sole use of nutritional strategies (as adequate metabolizable protein and energy ratio) is not sufficient to control the negative impacts (fat pad excessive accumulation on mammary stroma) of high gain diets on pre-pubertal Holstein × Gyr heifers [4,12]. Thus, using non-nutritional strategies, such as frequent application of bST, may prevent future losses in productivity and promote adequate mammary gland development and animal performance. The bovine somatotropin (**bST**) has the same biological functions as the GH in its natural form [24].

Furthermore, according to the literature, the bST can affect animal homeorhetic status, influence nutrient partitioning, and its use, and impact or not the digestive process [25]. Bovine somatotropin can also stimulate mammary growth by improving circulating levels of IGF1 during pre-puberty [15,19,26]. Additionally, it can increase N retention in growing dairy heifers [27] and reduce lipogenesis [25].

Therefore, based on this background and the limiting literature research focused on strategies to overcome the detrimental effects of high gain diets on the development of Holstein × Gyr heifers during pre-puberty, we hypothesized that exogenous bST could stimulate parenchymal tissue growth, decrease adipose tissue deposition in the mammary gland, improve protein deposition on carcass and enhance the performance of pre-pubertal Holstein × Gyr heifers. Therefore, we aimed to evaluate the effects of bST on digestibility, growth, blood metabolites, mammary gland development, and carcass composition of pre-pubertal Holstein × Gyr heifers fed high gain diets.

MATERIAL AND METHODS

Animals, experimental design, and feeding management

This study was carried out in strict accordance with the law nº. 11.794 of October 08th, 2008, Decree nº. 6899 of July 15th, 2009, and the rules issued by the Brazilian National Council for National Experimentation Control (CONCEA). It was approved by the Ethics Commission on the use of farm animals of Universidade Federal de Viçosa (CEUAP-UFV), protocol nº 0144/2019. Before treatment assignment, all heifers were allocated in *Brachiaria decumbens* paddocks for 24 days, managed in a rotation system. During this period, animals received supplemental feed (5% urea, 30% ground corn, 59% soybean meal, and 6% mineral) every two days in the amount of 700 g/d/heifer and underwent reproductive assessment to investigate the presence or not of a corpus

luteum, indicating the onset of puberty. Consequently, females presenting corpus luteum in one of the ovaries were removed from the trial; additionally, we collected blood from every heifer to evaluated progesterone levels, which accordingly to Roberts et al. [28], plasma concentration needs to be above 1ng/mL so heifers can be considered pubertal. Therefore, only pre-pubertal heifers were enrolled in this study. After this period, heifers were transferred to the feedlot and adapted to the experimental diet for 14 days. After that, the 34 heifers were weighed to initiate the trial.

Thirty-four pre-pubertal Holstein × Gyr heifers with an average initial body weight of 218 ± 49 kg and 14 ± 4 months of age were submitted to an 84-day trial (divided into three experimental periods of 28 days each) to evaluate the use of bST on digestibility, growth, blood metabolites, mammary development, and carcass traits. The 34 heifers were divided into three blocks according to their initial BW (B1 n = 12: 273.6 ± 19.2 kg; B2 n = 12: 214.4 ± 18.3 kg; B3 n = 10: 161 ± 19.7 kg). Moreover, two treatments (**no bST** injections – control or **bST** injections) were randomly assigned to the animals within each block. Thus, the 34 heifers were divided into two blocks of 12 animals each and one block of 10 animals, totaling 17 animals per treatment. Although we initiated the study with 34 heifers, one heifer (Block 2; bST treatment) suffering from a genetic hoof problem had to be removed from the trial at the end of the first period. Therefore, 33 heifers were evaluated in this trial.

Heifers were housed in group pens (six pens with six or five animals each – heifers on the same pen received the same treatment: no bST or bST) with free access to clean water and to a diet formulated to achieve an average daily gain of 1 kg, according to the NRC [29]. The experimental diet consisted of a 65:35 corn silage: concentrate ratio (Table 1). Diet was offered twice daily at 7 a.m. and 3 p.m., and group feed intakes were controlled to allow a 5% leftover (as-fed basis). In addition, every animal in the bST treatment received bST injections (a syringe of 2 g containing

500 mg of recombinant bovine somatotropin - BOOSTIN, Merc Animal Health) subcutaneously in the ischiorectal fossa every 14-day, as recommended by the company, beginning at day 3 and totaling seven administrations. Moreover, to mimic the stress suffered by these heifers, the no bST animals received the same volume of saline injections (sodium chloride, 0.9%), as a placebo, on the same days.

Table 1. Composition of ingredients and nutrients in the experimental diet (65:35 silage to concentrate proportion).

Item (g/kg of DM basis)	Roughage	Concentrate	Diet
Corn silage			510,25
Ground corn		325.43	89.41
Soybean meal		618.92	170.05
Limestone		31.60	8.68
Sodium bicarbonate		12.47	3.42
Magnesium oxide		6.24	1.71
Mineral mix[1]		5.34	2.09
Chemical composition[2]			
DM	295.22	859.33	492.66
OM	947.78	964.93	953.78
CP	622.75	300.42	509.93
EE	240.67	15.20	161.76
NDFap	425.96	127.31	321.43
NFC	435.48	521.98	465.76

[1]Composition: calcium, 40 g/kg; phosphorus, 25 g/kg; magnesium, 30 g/kg; sodium, 13 g/kg; potassium, 75 g/kg; sulphur, 10 g/kg; cobalt, 0.8 mg/kg; copper, 63 mg/kg; iodine, 2mg/kg; manganese 185 mg/kg; zinc, 222 mg/kg; selenium 2 mg/kg.

[2]DM = dry matter, OM = organic matter, CP = crude protein, EE = ether extract, NDF = neutral detergent fiber corrected to ashes and protein.

Digestibility trial, analyses, and calculations

Heifers underwent one digestibility trial at the end of the third experimental period, from day 73 to 80, where they received 10 g/d/heifer of chromium oxide and 15 g/d/heifer of titanium dioxide for six consecutive days (d73 to 79). The chromium oxide was orally infused to determine fecal excretion [30], and the titanium dioxide was offered mixed in the concentrate and divided into two meals to determine concentrate feed intake [31]. Fecal and urine sampling started after five days of marker provision. Three spot samples were collected for feces and urine, each of one on a different day, at 1200h (day 77), 1800h (day 78), and 0600h (day 79). On days 4 to 6, corn silage, concentrate, and refusal samples were also collected.

Feces and silage samples were dried in a ventilated oven at 55 ºC for 72h or until they were completely dry. Then, feces, silage, and concentrate samples were ground in a Willey mill using 2 and 1-mm sieves [32] to be later analyzed as composite samples. All 1-mm feces, silage, and concentrate samples were analyzed for DM (method 934.01), CP (method 990.13), ether extract (EE; method 2003.05), ash (method 942.05), according to AOAC [33], and NDF corrected for ash and protein contents, according to Detmann et al. [32]. The 2-mm samples were analyzed for undigestible NDF and used as an internal marker to estimate corn silage intake [34]. Moreover, feces were analyzed for chromium oxide and titanium dioxide [32] for estimating fecal excretion and concentrate feed intake, respectively. Digestible energy (DE) and ME were estimated according to the NRC [29], where DE (Mcal/kg) = (5.6 × dCP) + (9.4 × dEE) + (4.2 × dNDF) + (4.2 × dNFC); and ME (Mcal/kg) = 1.01 × DE - 0.45.

For spot urine samples (approximately 50 mL), we divided each sample collected at 1200h, 1800h, and 0600h into two samples. Ten mL of urine were diluted into 40 mL of sulfuric acid and stored at -20º C to prevent purine derivative degradation until we performed allantoin analyses

according to the technique described by Chen and Gomes [35]. The pure urine sample was used to determine uric acid, urea, and creatinine by the methods described by Kerscher and Ziegenhorn [36], Fujihara et al. [37], and Labtest Diagnóstica S.A, respectively. Total daily urinary excretion was estimated by the method proposed by Chizzotti et al. [38], using the daily creatine excretion for Holstein heifers: CE = 32.2 – (0.0109 × BW), where CE = creatinine excretion and BW = body weight. Purine derivatives excretion were estimated as the sum of daily allantoin and uric acid excretions. Crude microbial protein synthesis was estimated as a function of absorbed purines, calculated from purine derivatives [35].

As agreed among the authors in this study, it is important to state some concerns regarding purine derivatives. It is a useful technique, but it has intrinsic limitations. In their study, Hristov et al. [39] discussed about the unequal purine-to-total N ratios in protozoal and bacterial pools associated with the necessity to presume that dietary purines are completely degraded in the rumen [40,41]. Therefore, according to Hristov et al. [39], calculating absolute changes in microbial protein synthesis based on purine derivatives is not recommended. Nevertheless, it can be used in a controlled experimental trial (as in our study), where the differences in total purine derivatives excretion could indicate a discrepancy in microbial protein synthesis, finally generating concrete interpretations among treatments, as the interpretations observed in our study.

Growth, average daily gain, body condition score, and feed efficiency estimates

Heifers were weighed on an electronic scale before morning feeding for three consecutive days at the beginning and the end of the trial to evaluate their average daily gain. In addition, withers and hips heights were collected to assess growth on the same days. Intermediary weighting, measurements, and body condition scores were assessed at the beginning of every 28 days to follow animals' performance and adjust the diet according to their current weight.

Carcass ultrasound

Heifers were submitted to carcass ultrasound measurements on day 1 and every 28 days to evaluate the rib eye area and backfat thickness. Thus, with the aid of an ultrasound device, we collected images from the *gluteus medius* and *biceps femoris* muscles intercessions and the *longissimus dorsi* [42]. We used an 18-cm linear array ultrasound instrument (Aloka SSD-550V, Aloka Co.), operating at a frequency of 3.5 MHz. Muscle images were recorded and later analyzed using the BioSoft Toolbox® II for Beef software (Biotronics Ins.; [9]).

Mammary gland ultrasound

Mammary gland ultrasounds were also performed on day 1 and every 28 days. This procedure followed the technique used by Albino et al. [43]. With the aid of a real-time B-mode ultrasound machine equipped with a micro-convex transducer (Mindray DP2200) operating at a frequency of 6 MHz, images were taken of each mammary quarter. Heifers remained standing, and a commercial acoustic gel was applied on the udder quarter before the ultrasound exam. The lubricated micro-convex transducer was positioned at a 45º angle, followed by an image capture of each quarter. Then, we evaluated the pixel value using ImageJ software (NIH) in 8-bit format, collecting three random squares of 4 mm^2 for parenchyma and 16 mm^2 for fat pad areas. Next, the pixel value of each mammary quarter was obtained as the mean from the three squares randomly collected near to the parenchyma structures and mammary fat pad of each image. Finally, an average mammary gland pixel value was obtained for both the parenchyma and fat pad. Pixels values were evaluated according to the technique described by Esselburn et al. [44] and Albino et al. [45], where lower pixels values corresponded to more hypoechoic (black) areas, indicating the parenchyma, whereas higher pixels values corresponded to more hyperechoic (white) areas, indicating the fat pad.

Blood sampling and analyses

We collected blood samples to assess IGF1 and insulin levels on heifers submitted to treatments, on day 1 and every 28-d period, and to assess triiodothyronine (T_3), and thyroxine (T_4), only at the end of the third period, always before morning feeding. Samples were collected by coccygeal venipuncture with the aid of vacutainer tubes with a gel separator. Tubes were kept on ice until centrifugation (3,000 × *g* at 4 ºC for 20 min), then serum was pipetted into eppendorf tubes and stored at -20ºC until analyses. Analyses of insulin and IGF1 were performed using chemiluminescence immunoassay (Immulite 1000; Siemens Medical Solutions Diagnostics, Los Angeles, USA) [9].

Liver, muscle, and mammary gland biopsies procedures

To evaluate the effects of bST on the mammary gland, carcass, and liver, we performed biopsies in 18 randomly selected heifers (six of each block – three of each treatment: no bST and bST) on days 85 and 86, right after the last sampling of period three. The first two biopsies performed were on the carcass and liver (day 85). First, heifers were placed in a squeeze chute in a standing position to perform liver and muscle biopsies, followed by cleaning the biopsy site with ethyl alcohol and clipping the hair from a 15 cm² of the area where both incisions were made (liver and muscle). After that, we scrubbed the areas with povidone-iodine and administered anti-inflammatory intravenously via the jugular vein. Before incision, 2% lidocaine hydrochloride was applied subcutaneously for local anesthesia (ribs and *longissimus dorsi*). For the liver biopsy, a one-centimeter incision was made on a line from the tuber coxae to the shoulder point [12,46], with a scalpel blade followed by introducing a biopsy trocar until the liver was punctured. Then, a syringe was attached to the trocar, and liver tissue was suctioned [46]. For muscle biopsies, an incision of approximately three centimeters was made with a scalpel blade, and, with the aid of a hemostatic

forceps, a one-centimeter sample from the *longissimus dorsi* muscle was collected. After tissue collection, the muscle incision was closed following all aseptic techniques. Next, all samples were cleaned with saline solution (0.9% sodium chloride) and stored in liquid nitrogen until quantitative real-time PCR analysis (**qRT-PCR**).

Mammary gland biopsies occurred on day 86. Heifers fasted for 16 hours before the biopsy procedure. Then, each heifer was individually restrained in lateral recumbency and given a dose of general anesthetic (xylazine, 0.5 mL/100 kg of BW). The aseptic procedure followed the same steps described previously for liver and muscle biopsies. A two-centimeter incision was made on the left rear udder on the mid-parenchyma region. After collection, the mammary incision was closed following an aseptic technique, and heifers were released from the restraining position. After the biopsy, heifers were allocated in individual pens, received fresh feed and clean water, and were monitored for five days. Before storage, all samples were cleaned with saline solution (0.9% sodium chloride). Then, samples were kept in liquid nitrogen until qRT-PCR analysis.

Quantitative Real-Time PCR analyses

Total muscle RNA was isolated using the Trizol method (Invitrogen and treated with DNase using the RQ1 RNase-Free DNase (Promega. Total RNA was isolated using the PureLink RNA Mini Kit (Thermo Fisher) for liver and mammary gland samples. After that, we performed reverse transcription on muscle, liver, and mammary gland RNA samples using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher). Lastly, qRT-PCR was performed in duplicate using the GoTaq PCR Master Mix (Promega) in a QuantStudio 3 thermocycler (Applied Biosystems). Every technique was performed according to the manufacturer's instructions. The amplification efficiency of internal control and target genes was estimated using four dilutions of cDNA for each tissue evaluated. Amplification conditions for all systems consisted of an initial step at 95°C for 2

minutes, the second step of 40 cycles at 95°C for 15 s, and a final extension step at 60°C for one minute. After the amplification cycles, an additional gradient step from 60°C to 95°C was used to obtain a melting curve.

The ΔCt method was used to estimate the expression of each gene (target Ct – internal control Ct), where Ct represents the PCR cycle number of cDNA amplification above the threshold level. Moreover, gene expression differences were estimated using the $-2^{\Delta Ct}$ method [47]. Target genes evaluated in the present study were: *mTOR* and *AMPK* for muscle; *IGF1* and *GHR* for liver; and *IGF1*, *IGF1R*, *IGFBP3*, *FASN*, and *ESR1* for mammary gland. Primer pairs for internal control and target genes are presented in Table 2, according to their identification sequences from the GenBank database.

Table 2. Gene name, primer pair sequence, annealing temperature, and amplification efficiency of each target gene.

Genes[1]	Accession number[2]	Primer sequence (5´- 3´)	Amplicon, bp
IGF1	NM_001077828.1	Forward: AGCAGTCTTCCAACCCAATTA Reverse: ACAGGGCCAGATAGAAGAGA	103
GHR	NM_176608.1	Forward: CCTCAACTGGACTCTACTGAAC Reverse: CCAGGATTATCCATCCCATCTT	112
mTOR	XM_002694043.6	Forward: AAGGAGAAGGAACGGACA Reverse: CCAGCACACGAGGTAAATAG	
AMPK	NM_001109802.2	Forward: AGTTGCCTACCACCTCAT Reverse: GTGGTGATCGTCGAGAAAC	
IGF1R	NM_001244612.1	Forward: GTATGGAGGAGCCAAGCTAAA Reverse: GTCTTGGCCTGAACGTAGAA	123
IGFBP3	NM_174556.1	Forward: CTCCACTTCATGCCTTAGCA Reverse: GACAGGGCGTTCTTCTTCTT	120
FASN	XM_005220997.2	Forward: CAACAAAACTGGTGCTCACG Reverse: ATCAACTCTGAGGGGCTGAA	122
ER1	Connor et al., 2005[3]	Forward: TTGCTGGCTACTTCGTCTC Reverse: GGTGGATGTGGTCCTTCTC	148
RSP15A	NM_001037443.2	Forward: GGAGTGATCAGCCCTAGATTTG Reverse: AGCTGAGGTTGTCAGTACAATG	108
18S	NR_036642.1	Forward: GCCGCTAGAGGTGAAATTCT Reverse: TCGGAACTACGACGGTATCT	129
GAPDH	NM_001034034.2	Forward: GATGCTGGTGCTGAGTATGT Reverse: GCAGAAGGTGCAGAGATGAT	113

[1]Genes: insulin-like growth factor 1 (*IGF1*), growth hormone receptor (*GHR*), (*mTOR*), (*AMPK*), insulin-like growth factor 1 receptor (*IGF1R*), insulin-like growth factor-binding protein 3 (*IGFBP3*), fatty acid synthase (FASN), estrogen receptor alpha (*ER1*), ribosomal protein S15A (*RSP15A*), 18S ribosomal RNA (*18S*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*).

[2]GenBank.

[3]Connor et al. [48]

Statistical Analyses

All variables were analyzed using PROC GLIMMIX of SAS (Statistical Analysis System, 9.4 version) in a randomized complete block design, using initial BW as blocking criteria. Feed intake, digestibility, average daily gain, serum T_3, T_4, and gene data did not present repeated measures; thus, they were analyzed according to the following model:

$$Y_{ijk} = \mu + T_i + B_j + \varepsilon_{ijk} \quad (1)$$

μ = mean; T_i = fixed effect of treatment i; B_j = random effect of block j; and ε_{ijk} = random error with mean 0 and variance $\sigma 2$, variance among animal measurements.

Mammary gland and carcass ultrasound, body condition score, growth, and serum IGF1 and insulin data were evaluated along the experimental period (day 1, 28, 56, and 84); therefore, period measurements were included as repeated measures in the model as follows:

$$Y_{ijklm} = \mu + T_i + \delta_{ij} + B_k + P_l + (T \times P)_{il} + \varepsilon_{ijklm} \quad (2)$$

μ = mean; T_i = fixed effect of treatment i; δ_{ij} = random error with mean 0 and variance $\sigma 2$, the variance among animals within treatment equal to the covariance among repeated measures among animals; B_k = random effect of block k; P_l = fixed effect of period l; $(T \times P)_{il}$ = random effect of interaction between treatment i and period l; and ε_{ijklm} = random error with mean 0 and variance $\sigma 2$, variance among animal measurements. Measurements on day 1 were included as covariates for carcass characteristics, body condition score, and growth models.

Eight variance-covariance structures (AR, CS, FA, UN, TOEP, VC, ARH1, TOEPH) were tested and the one presenting the best fit based on the Akaike information criterion was used. Body condition score was used as a co-variable for itself, growth, and carcass ultrasound variables. In

addition, due to the unbalanced design (one heifer removed from the trial), degrees of freedom were corrected using the Kenward-Rodger approximation. The main effects of bST and days were discussed separately in the absence of interactions. For all analyses, significance was declared when $P \leq 0.05$.

RESULTS

Feed intake and digestibility of heifers

According to the digestibility trial conducted on days 73 to 80, the bST did not influence feed intake or digestibility ($P \geq 0.142$; Table 3), metabolizable energy, metabolizable protein, and their relations ($P \geq 0.216$), or microbial efficiency and nitrogen balance ($P \geq 0.103$).

Table 3. Feed intake and diet digestibility of Holstein x Gyr heifers submitted to control or bST treatment[1].

	Treatment		SEM	*P*-value[3]
	no bST	bST		Treatment
Feed intake[2]				
DM, kg/d	7.873	7.711	0.709	0.783
CP, kg/d	1.160	1.140	0.100	0.802
NDFap, kg/d	2.420	2.253	0.172	0.308
EE, kg/d	0.150	0.139	0.010	0.261
NFC, kg/d	3.932	3.539	0.293	0.221
OM, kg/d	7.511	7.357	0.676	0.783
DMI/BW, g/kg of BW	25.943	24,741	1.390	0.469
NDFap/BW, kg/kg of BW	7.536	6.8858	0.753	0.396
RDP intake, g/d	572.790	510.890	36.609	0.190

RUP intake, g/d	584.240	627.960	116.650	0.532
MEI, Mcal/kg	20.975	18.738	1.509	0.186
MPI, g/kg	835.550	830.090	82.181	0.926
MPI/BW, g/kg of BW	2.744	2.656	0.124	0.614
MEI/BW Mcal/kg of BW	66.137	61.046	4.446	0.216
MP:ME, g/Mcal	40.630	41.866	1.321	0.513
Digestibility[2]				
DM, g/kg	649.230	639.950	8.629	0.453
CP, g/kg	675.150	666.230	8.685	0.473
NDFap, g/kg	445.460	451.270	17.817	0.788
EE, g/kg	831.030	804.120	14.759	0.208
NFC, g/kg	849.310	834.930	6.7479	0.142
OM, g/kg	689.08	683.400	8.567	0.643
Nitrogen balance				
Microbial efficiency	109.170	101.950	13.235	0.465
Excreted, g/100 kg of BW	44.395	40.499	2.797	0.103
Retained, g/100 kg of BW	15.670	18.230	1.746	0.308

[1] Feed intake and digestibility were assessed during the last experimental period.

[2]NDFap = neutral detergent fiber free of ashes and protein, EE = ether extract, MEI = metabolizable energy intake, MPI = metabolizable protein intake.

[3] *P*-values indicate treatment effects ($P \leq 0.05$).

Growth, average daily gain, body condition score, and feed efficiency estimates

Heifers receiving bST injections had the same final body weight, average daily gain, and withers and rump height as heifers that did not receive this treatment ($P \geq 0.266$; Table 4), but animals grew during the experimental period, both for wither and rump height ($P = 0.001$; Fig 1). Regarding body condition score, it also increased along the days evaluated ($P = 0.042$; Fig 1), presenting a trend between treatment and experimental day interaction ($P = 0.081$). Moreover, there was no statistical difference in feed efficiency between treatments ($P = 0.574$).

Table 4. Performance of Holstein x Gyr heifers submitted to control or bST treatment.

	Treatment		Day			SEM	*P*-value[2]		
	no bST	bST	28	56	84		T	D	T × D
Performance[1]									
Initial BW, kg	209.86	229.66				12.360	0.266		
Final BW, kg	305.19	314.58				37.131	0.323		
ADG, kg/d	1.12	1.15				37.132	0.726		
WH, m	1.20	1.21	1.19[c]	1.20[b]	1.22[a]	0.035	0.436	0.001	0.922
RH, m	1.25	1.26	1.24[c]	1.25[b]	1.27[a]	0.037	0.750	0.001	0.291
BCS	3.23	3.17	3.15[c]	3.26[a]	3.19[b]	0.056	0.482	0.042	0.081
FE (kg ADG/kg DMI)	14.51	15.16				0.811	0.574		

[a-b] Means without common superscript letters in the same row between 2 treatments over 3 periods are significantly different ($P \leq 0.05$).

[1]BW = body weight, ADG = average daily gain, WH = withers height, RH = rump height, BCS = body condition score, and DMI = dry matter intake, FE = feed efficiency.

[2]*P*-values indicate treatment (T), experimental day (D), and treatment by experimental day interaction (T × D) effects.

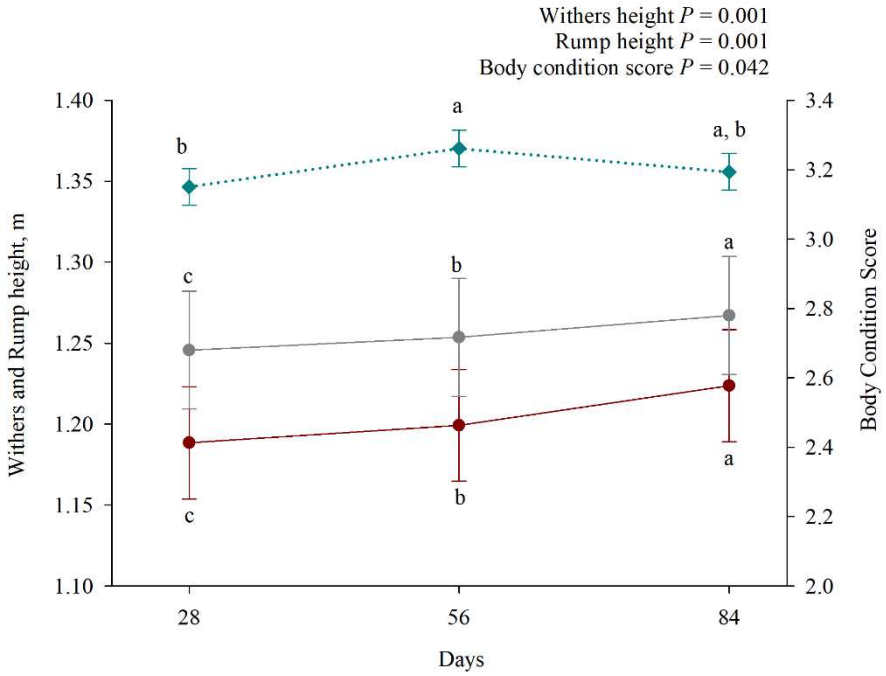

Fig 1. Experimental day effect on Holstein × Gyr heifers' withers and rump height and body condition score. Closed red circles represent withers height, closed grey circles - rump height, and blue diamond - body condition scores. Statistical differences were considered when *P* - value was ≤ 0.05.

Serum IGF1, insulin and thyroid hormones

We observed no interaction between the experimental day and treatment for insulin (*P* = 0.332; Table 5). However, there was an experimental day effect (*P* = 0.025), with increased insulin concentration on day 84 and a tendency to increase it on no bST treatment of heifers (*P* = 0.079). Regarding IGF1 concentration, we observed an interaction between treatment and experimental day (*P* = 0.005; Fig 2), where heifers on the bST treatment had the highest concentration of IGF1 regardless of the day. Heifers on the no bST treatment showed greater IGF1 serum concentrations on days 56 and 84, but their overall concentration was continuously below the bST treatment.

T_3 and T_4 serum concentrations were evaluated only on day 84. We observed a trend of increased T_3 serum concentration on bST animals ($P = 0.064$) and higher T_4 concentration for these same heifers ($P = 0.002$).

Table 5. Blood metabolites concentration of Holstein x Gyr heifers submitted to control or bST treatment.

	Treatment		**Day**					***P*-value[2]**		
	no bST	**bST**	**1**	**28**	**56**	**84**	**SEM**	**T**	**D**	**T × D**
Blood metabolites[1]										
Insulin, µUI/dL	0.26	0.17	0.14^{c}	0.25^{ab}	0.18^{bc}	0.29^{a}	0.036	0.079	0.025	0.332
IGF1, ng/mL	161.87	244.88	126.04^{b}	220.73^{a}	227.17^{a}	239.57^{a}	32.997	0.001	0.001	0.005
T_3, ng/mL	1.10	1.28					0.065	0.064		
T_4, ng/mL	4.49	5.76					0.386	0.002		

[a-c] Means without common superscript letters in the same row between 2 treatments over 3 periods are significantly different ($P \leq 0.05$).

[1]T_3 = triiodothyronine, T_4 = thyroxine.

[2]*P*-values indicate treatment (T), experimental day (D), and treatment by experimental day interaction (T × D) effects.

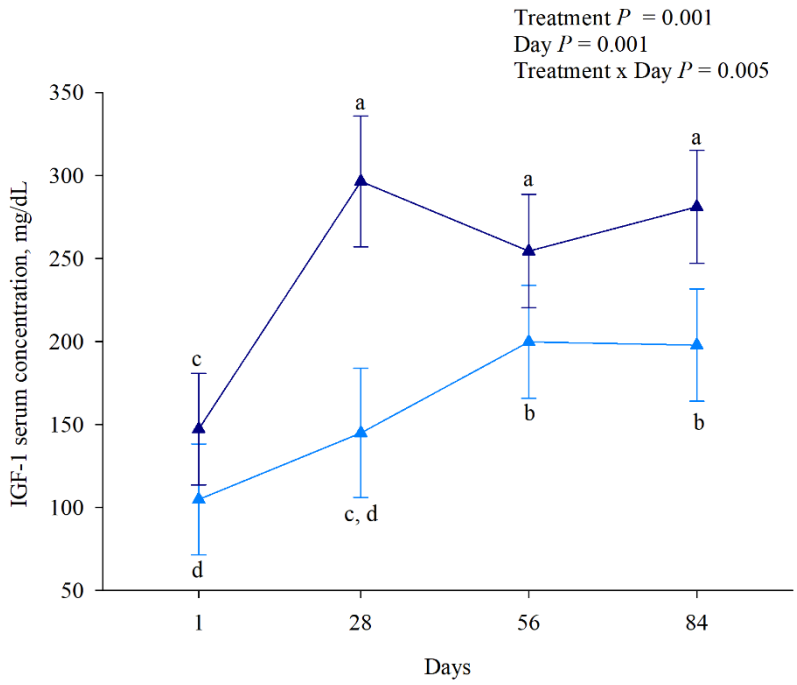

Fig 2. Treatment and day interaction on IGF1 serum concentration of Holstein × Gyr Heifers. Dark blue triangles represent bST treatment, and light blue triangles represent no bST treatment. Differences were considered when *P* - value was ≤ 0.05.

Carcass ultrasound

Heifers receiving the bST injections showed an increased ribeye area compared to the no bST treatment, around 25% greater deposition of lean tissue ($P = 0.001$; Fig 3). This increase in the rib eye area (cm^2) was also observed along the days evaluated, with the greatest area observed on experimental day 84 (40.76; $P = 0.001$). On the other hand, we observed only an experimental day effect for backfat thickness (cm^2), with a more significant accumulation of carcass fat on experimental day 84 ($P = 0.001$). No interactions were observed between treatment and experimental day ($P \geq 0.453$).

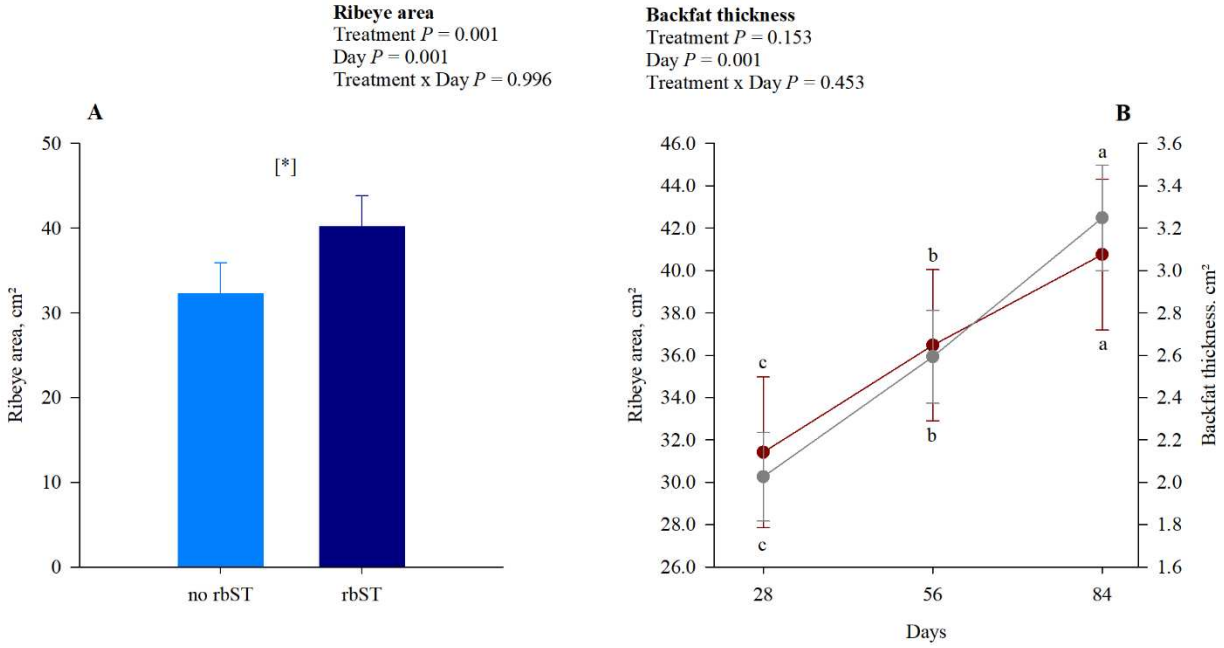

Fig 3. Representation of carcass ultrasound results. A - Carcass ribeye area between treatments. B - Carcass ribeye area and backfat thickness among days. Red circles represent the ribeye area, and grey circles represent backfat thickness. Statistical differences were considered when *P*-value was ≤ 0.05.

Mammary gland ultrasound

Heifers receiving bST injections had lower pixels/mm² on parenchyma when compared with no bST treatment ($P = 0.003$), meaning a less brightening color, such as black, which is characteristic of parenchymal tissue. According to Esselburn et al. [44], a more hypoechoic (black) area is indicative of parenchyma, whereas more hyperechoic (white) areas are the fat pad. In addition, we also observed an experimental day effect on mammary parenchyma, with the lowest pixels/mm² on day 1 (59.79; $P = 0.001$), which increased on the first 28 experimental days and remained constant until the end of the trial (Fig 4). Regarding fat pad tissue, heifers on bST treatment also had reduced pixels/mm² ($P = 0.031$), regardless of time. Animals presented the

lowest fad pad pixels/mm² on experimental day 1, followed by experimental day 84, and the highest values were observed on experimental days 28 and 56. Moreover, no interactions were observed between treatment and experimental day for parenchyma and fat pad (P = 0.773 and P = 0.154, respectively).

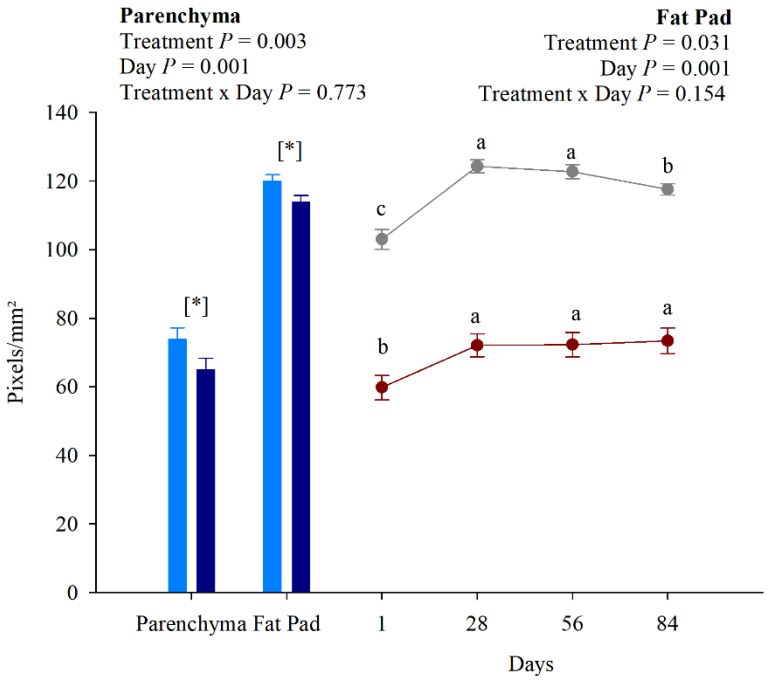

Fig 4. Representation of mammary gland ultrasound results. Light blue columns represent no bST treatment, and dark blue columns represent bST treatment in parenchyma and fat pad areas. Red circles represent parenchyma, and grey circles represent fat pad areas among the experimental days. Statistical differences were considered when $P \leq 0.05$.

Liver, muscle, and mammary gland genes expressions

The application of bST did not influence liver and muscle gene expression ($P \geq 0.323$ and $P \geq 0.442$, respectively; Table 6). In addition, bST injections did not affect most genes evaluated in mammary gland tissue (*IGF1*, *IGF1R*, *FAS*, *ER1*; $P \geq 0.207$), except for *IGFBP3* expression, which

was greater for no bST treatment when compared to heifers receiving bST injections (0.28 vs. 0.15; $P = 0.023$).

Table 6. Liver, muscle, and mammary gland gene expression of Holstein x Gyr heifers.

	Treatment			*P*-value[2]
Genes[1]	**no bST**	**bST**	**SEM**	**Treatment**
Liver				
IGF1	0.144	0.136	0.017	0.739
GHR	0.518	0.439	0.055	0.323
Muscle				
mTOR	0.0001	0.0001	0.00001	0.442
AMPK	0.0002	0.0002	0.00005	0.803
Mammary gland				
IGF1	0.017	0.009	0.004	0.207
IGF1R	0.019	0.027	0.006	0.337
IGFBP3	0.276	0.151	0.035	0.023
FASN	0.005	0.006	0.0016	0.706
ER1	0.059	0.048	0.015	0.605

[1]Insulin-like growth factor 1 (*IGF1*), growth hormone receptor (*GHR*), (*mTOR*), (*AMPK*), insulin-like growth factor 1 receptor (*IGF1R*), insulin-like growth factor-binding protein 3 (*IGFBP3*), fatty acid synthase (FASN), estrogen receptor alpha (*ER1)*.

[2]*P*-values indicate treatment effects ($P \leq 0.05$).

DISCUSSION

The use of bST is a strategy used on farms to improve animals' performance, milk production, and mammary development. Nevertheless, very few studies have evaluated bST effects on Holstein × Gyr heifers, a crossbred of substantial economic impact on the dairy system of tropical countries. Thus, we observed the necessity of understanding the effects of this non-nutritional strategy on pre-pubertal Holstein × Gyr heifers. To confirm the veracity of pre-pubertal status of heifers used in

this trial, data in S1 Table presents progesterone levels on the first day of the trial, which were lower than 1ng/mL, and reproductive assessment results.

The bST is known to have an homeorhetic effect on animals, which can alter nutrient partitioning and redirect its use after intestinal absorption. However, the bST effect on the digestive process is minimal [25,27]. Our results follow Eisemann et al. [49] and Crooker et al. [27], who evaluated the effects of bST on dietary intake and digestibility of Hereford and Holstein heifers, respectively. They observed no statistical difference in the nutrient intake or digestibility with the use of bST injections. Additionally, these authors reported a significant decrease in urinary nitrogen excretion, which was not observed in our study. Likewise, Nascimento et al. [50] did not observe differences in nutrient intake. Still, heifers receiving bST injections every 14 days had greater crude protein and organic matter apparent digestibility than control or single-dose treatment. Contradicting our findings and the ones reported in the literature, Gandra et al. [51] observed increased dry matter intake as a percentage of BW of Holstein heifers receiving bST injections.

Although voluntary feed intake increases in lactating cows due to enhanced energetic demand for milk production [25,52], the same biological response is not observed in growing animals. In our study, heifers had a dry matter intake (7.85 kg of dry matter) close to the feed intake predicted by NRC [29] and Silva et al. [53] for heifers with an average body weight of 314 kg (7.20 kg of dry matter). Moreover, evaluating the performance according to the new NASEM [54] software, we observed that the initial diet formulated for heifers to achieve 1 kg/d actually predicted 1.2 kg/d of energy allowed for growth (frame and reserves). Thus, the results in our study regarding average daily gain and, consequently, final body weight indicate an improvement in prediction equations used in the 8th version of NASEM [54].

Lima et al. [55], also evaluating the effects of bST on Holstein × Gyr heifers, observed no somatotropin effects on average daily gain and final BW. However, in another study, Fudimoto et al. [56] reported an increased final BW of Holstein × Gyr heifers treated with bST every 30 days. Moreover, there is a considerable difference when comparing our results with studies conducted with pure Holstein heifers, as in Radcliff et al. [19], who reported increased average daily gain and higher withers height of pubertal Holstein heifers receiving bST injections. Gandra et al. [51] also showed that bST injections could improve thoracic perimeter, length, rump width, and BW, but bST only affected average daily gain for 60 days; and Moallem et al. [57] confirmed that bST injections enhanced Holstein heifers performance from 90 to 314 days of age, increasing BW and hip height, but no effect was observed on heifers from 314 until 644 days of age. In our study, no effect of bST on withers and rump height was observed. Albeit the proven bST effect on promoting lipolysis and improving animals' lean carcass gain, mainly characterized by the local action of IGF1, none of these outcomes were observed in our study [19,25,58].

To better understand the limiting response of bST on animals, we can relate it to the saturation of GH receptors on these heifers. Campos [59] evaluated increasing doses of bST on milk production of crossbred Holstein × Gyr cows; however, no differences between doses were observed. The author described the lack of results as a saturation effect of GH receptors on crossbred animals due to their reduced genetic merit for milk production compared to the Holstein breed. According to Bauman [25], animals with reduced genetic merit have decreased circulating endogenous GH levels. Therefore, in Campos [59] study, Holstein × Gyr cows would need a lower level or exogenous bST to achieve their maximum production, consequently caused by the saturation of GH receptors. Despite the distinct physiological phase (growing animals vs. lactating cows), we speculate that the same explanation [59] can be attributed to our study. Therefore, the

Holstein × Gyr heifers evaluated in our study could present saturation of GH receptors, leading to the limiting performance response. However, in this case, it would not be attributed to lower genetic merit but to an increased feeding level. It is well known that under high feeding levels, GH concentration is reduced as much as its receptors [60-62]. Thus, GH receptors' saturation may have occurred primarily due to the increased feeding level heifers were subjected, causing the somatotropic axis uncoupling. Thus, this primary response did not allow the exogenous administration of bST to exert its full effect on animals' performance, occasioning the minor effects of bST on the heifer's growth and development.

The limiting response of bST on growth and development may also be assimilated with the ones obtained with serum concentration of blood parameters. The insulin, a pancreatic hormone highly correlated with feeding level, acts to promote the storage of metabolites in peripheral tissues such as skeletal muscles and adipose tissue. However, its role in hepatic tissues for the ruminant species seems less important once glucose uptake by the liver is minimal in this specie [63]. Therefore, in our study, the increase in serum insulin over the days could be attributed to the daily feed intake of animals, which grew according to the heifers' body weight. However, since we did not perform a digestibility trial every 28 d, we cannot confidently explain the higher insulin serum concentration on day 28 of the experimental period, although it is possibly a response to the greater feed intake of nutrients at that specific sampling period.

Regarding the tendency of increased insulin serum concentration observed on no bST heifers, our results go against the literature, which suggests a possible interaction between this hormone and the bST, wherein the exogenous or endogenous growth hormone would allow an insulinemic state in periparturient dairy cows, with a less pronounced effect on growing heifers [64-66]. Additionally, Hall et al. [67] observed increased insulin serum concentrations in heifers receiving

bST injections. The bST may not have caused an insulin-resistant state on our heifers, but it could have impaired or reduced insulin secretion on bST animals once feed intake variables remained the same between treatments.

Growth hormone and IGF1 concentrations at birth are considerably lower than at puberty. The coupling of the somatotropic axis during puberty promotes an increase in GH release from the pituitary gland and greater expression of its receptors, resulting in higher production and secretion of IGF1 [68]. Moreover, some authors [26,69,70] reported an increased IGF1 serum concentration in heifers treated with exogenous somatotropin. Therefore, our results concerning IGF1 serum concentration are in accordance with literature findings, confirming a higher concentration of this hormone on bST-treated heifers and according to the proximity of days towards puberty.

Since thyroid hormones are known to be related to metabolic activities, homeostasis processes, and animal responses to nutritional level, reproductive and immunological status [71], they can act synergistically with somatotropin enhancing growth [72]. According to Root et al. [72], the GH influences the peripheral metabolism of thyroid hormones, enhancing T_4 degradation and its conversion to T_3. This pattern of T_3 serum concentration agrees with the tendency observed on bST heifers. However, to our knowledge, no studies suggest an explanation for the increased T_4 levels in bST-treated heifers; neither it was possible to associate T_4 results with an increased metabolic rate of these animals. Therefore, despite the limiting results on average daily gain or feed efficiency, the greater serum concentration of IGF1, T_3 and T_4 likely acted to improve carcass muscle deposition and mammary growth on bST heifers.

The 25% increase in the ribeye area of bST heifers can be linked to greater IGF1 concentrations observed for these animals. Growth hormone improves liver production and release of IGF1 in the blood; then, this insulin growth factor will act on target tissues, in this case, on the

carcass, stimulating lean gain and protein accretion [25,73]. Radcliff et al. [19] observed improved carcass weight and protein deposition in heifers receiving bST injections. On the other hand, bST treatment was inefficient in reducing carcass adipose tissue deposition, as observed by Moallem et al. [74]. In our present study, we noticed around 60% increase in backfat thickness area along the days, without difference between treatments, which agrees with body condition score results, although there was a greater body condition score on day 56 of the experimental period.

Pre-pubertal Holstein × Gyr heifers can present higher fat deposition on the carcass when fed high gain diets [4], which can explain the 60% increase in backfat thickness. Nevertheless, our results indicate that, to control their homeorhetic status, heifers prioritized their metabolism for lean carcass and mainly mammary gland growth (discussion in the next section), which agrees with the accelerated growth rate of these tissues during this physiological stage, pre-puberty [14], despite reducing backfat thickness deposition. Therefore, based on previous studies, our assumption that exogenous GH would improve lean gain was confirmed, suggesting the efficacy of bST injections to improve carcass growth in Holstein × Gyr heifers.

Focusing on the mammary gland, the effects of somatotropin on its growth during pre-puberty are extensively discussed in the literature, as much as the essential actions of estrogen [62,75]. The pituitary somatotropin promotes mammary growth mediating the activation of the GH-IGF axis, where part of GH signaling occurs through the mammary stromal cells and via paracrine production of IGF1 [76,77]. However, when feeding diets for elevated growth rates (1 kg/day), GH levels decrease, uncoupling the GH-IGF axis [15]. Thus, the exogenous administration of bST can improve mammary gland growth when the serum concentration of GH is reduced by increasing the percentage of protein, the amount of RNA, and total parenchyma on the mammary gland of pre-pubertal heifers [19,76].

The technique study by Albino et al. [43] and validated with Holstein × Gyr heifers allowed us to observe a more remarkable parenchymal growth on bST-treated heifers through mammary gland ultrasound. The lower pixels/mm^2 found in the parenchyma of bST animals indicates enhanced ductal growth compared to heifers with no bST treatment. According to Albino et al. [43], the ultrasound images do not distinguish between epithelial duct tissue and duct lumens of parenchyma; however, it assures a difference in tissue growth. Sejrsen et al. [78] and Radcliff et al. [19] also observed differences in the amount of parenchymal content of the mammary gland, either by protein percentage or by RNA and total parenchyma. Moreover, we also observed an increase in parenchyma pixels/mm^2 from day 1 to 28, so, considering that the parenchyma is an epithelial tissue surrounded by intra and inter lobular stroma, this result suggests the elongation and growth of ductal tissue which penetrates the mammary stroma (Fig 5).

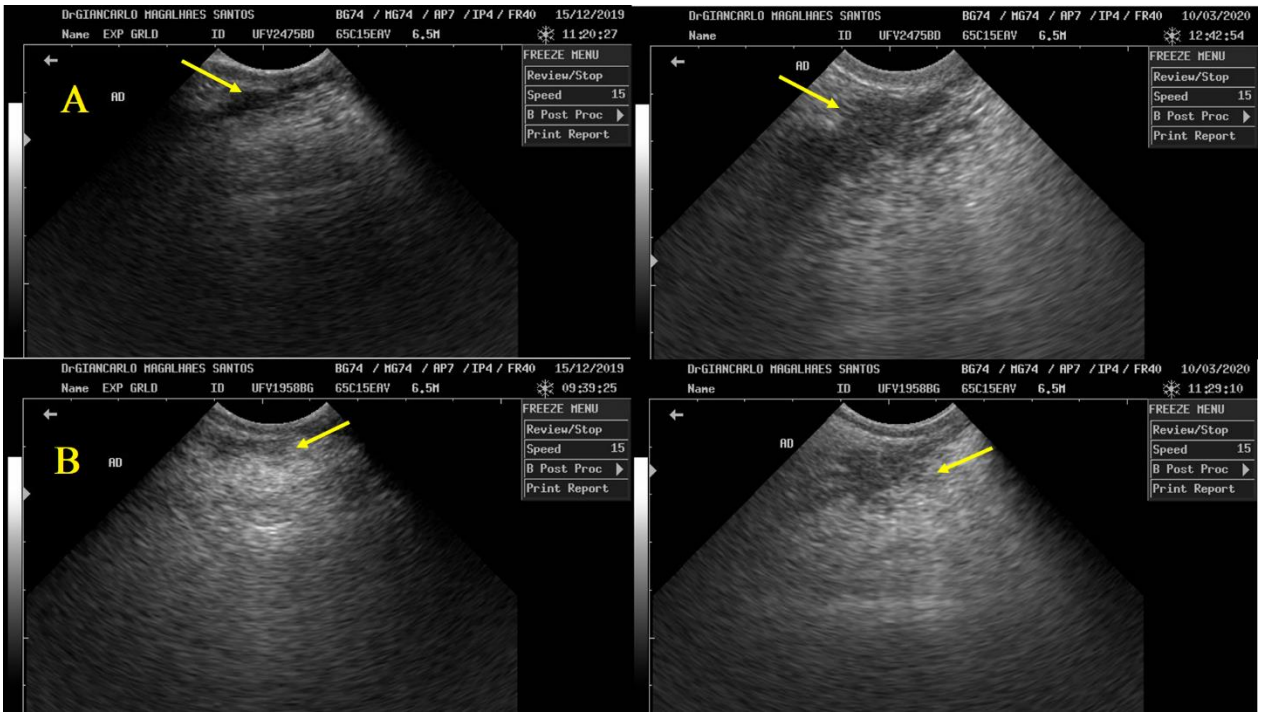

Fig 5. Increase in the mammary parenchyma tissue of Holstein x Gyr heifers (indicated by yellow arrows). A - Heifer in the bST treatment, days 1 and 84. B - Heifer in no bST treatment, days 1 and 84.

Regarding fat pad tissue, we suspect that the sharp increase observed in the first 28 days of the trial occurred due to some residual compensatory gain. Before the beginning of the experimental period, heifers were not fed to achieve high growth rates, they were fed on a supplemented pasture system for one month - daily free access to a well-managed *Brachiaria decumbens* and approximately 1 kg of concentrate supplemented three times a week. Therefore, their feed intake was likely limited, as well as nutrient partitioning and availability to mammary development. However, with a balanced diet and exogenous growth hormone provision, the mammary gland had the ideal nutritional and hormonal environment to develop, as indicated by greater circulating levels of IGF1 and increased parenchymal tissue.

The mammary fad pad has an essential role in the proliferation of parenchyma and synthesizes growth factors that have mitogenic actions [79]. However, it can also be detrimental to mammary development, especially in Holstein × Gyr heifers [4], which do not seem to respond to increased feeding levels as pure Holsteins heifers, even when adequate MP:ME ratios are fed [45]. Therefore, to our understanding, the bST treatment stimulated parenchyma tissue growth by enhancing serum concentration of IGF1 and promoting duct elongation towards mammary stroma, which minimized the detrimental effects of high gain diets on the mammary gland of pre-pubertal Holstein × Gyr heifers.

Gene expression analyses can also explain some of the responses discussed previously. The absence of changes in liver *GHR* expression due to bST treatment follows Radcliff et al. [26] findings. According to these authors, bST injections do not alter the *GHR* mRNA expression of

non-producing animals as they do on lactating cows. This response could be attributed to distinct metabolic or endocrine changes driven by animals' homeorhetic status, frequency of bST injections, or hepatic GH bindings peak around puberty [26]. In addition, we can also suggest a decreased GHR for Holstein × Gyr heifers due to their inferior genetic merit for milk production compared to purebred Holstein heifers [25,59]. Moreover, in our study, bST injections increased only serum IGF1 concentration but not its expression in the liver. Although Radcliff et al. [79] did not observe differences in *GHR* expression on bST-injected heifers, they found an increased expression of IGF1 mRNA. Therefore, considering that bST administration exclusively improved serum IGF1 concentration, we assumed that the extra IGF1 produced by the liver acted on target tissues, such as muscle and mammary gland, enhancing their growth rates.

The *mTOR* regulates cellular machinery by exporting growth-promoting mRNA and enhancing protein synthesis [80]. In rats, Hayashi and Proud [81] confirmed the process of protein synthesis from *GH* action towards the *mTOR* signaling pathway, which occurs possibly through activation of the PI 3-kinase and the *PKB/Akt*. Moreover, the *AMPK* activation suppresses the *mTOR* pathway during low energetic status reducing protein synthesis [82]. In a recent study evaluating the effects of bST on beef heifers, Hergenreder et al. [83] observed an increase in *AMPKα* expression, which was related to reduced marbling score and greater fiber cross-sectional area of bST-treated heifers. Despite the outcomes presented in the literature, bST treatment did not affect *mTOR* or *AMPK* expression. Thus, considering that *mTOR* activity hardly changes, we acknowledge the limitation of the analysis performed and suggest investigating the activity of *mTOR* to better understand the metabolism within lean tissue deposition in heifers treated with bST.

Regarding the genes assessed in the mammary gland tissue, the bST treatment impacted their expression to a lower extent than we expected. Plath-Gabler et al. [84] observed considerable expression of *IGF1* on the mammary gland of virgin heifers (18 months of age), indicating an intense proliferative role for this hormone at this phase. Albino et al. [4] and Weller et al. [12] observed an enhanced expression of *IGF1R* and *FASN*, respectively, in Holstein × Gyr heifers fed high gain diets compared to maintenance animals. Moreover, according to Weller et al. [12], the expression of *FASN*, one of the genes involved in adipose tissue synthesis, can be controlled by dietary and hormonal characteristics. Nevertheless, none of these genes had altered expression according to our treatments.

The major response for mammary gene expression was observed for *IGFBP3*, which was lower for heifers receiving bST injections. Lew et al. [85] evaluated the gene expression profile of Holstein heifers' mammary gland while animals were fed different diets and received bST injections. They observed an altered expression of fifty-three tissue-developing genes, up-regulating thirty-four proliferative and two anti-proliferative genes, only due to bST treatment. Moreover, among the seventeen genes downregulated by bST treatment, six were classified as anti-proliferative, such as the *IGFBP3*. The *IGFBP3* is the major IGFBP found in bovine mammary cells and milk, which can interfere with *IGF1* action, as demonstrated by its exogenous application [20]. However, Leibowitz and Cohick [21] observed that *IGFBP3* could exert dependent or independent effects on cell growth, proliferation, and apoptosis. Thus, dependent on the stimulus, the ternary complex formed among the *IGF*, *IGFBP3*, and an acid-labile unit can hinder *IGF* translocation to the target tissue, impairing tissue growth or development [12].

Similar to our finding, Berry et al. [70] also evaluated the bST effects on heifers' mammary gland and observed a reduction in *IGFBP3* expression in their mammary parenchyma. In addition,

Berry et al. [70] also found an enhanced serum IGF1 concentration in heifers receiving exogenous somatotropin. However, the increased serum IGF1 was associated with greater *IGF1* mRNA in the mammary fat pat due to estrogen applications. As suggested by these authors, *IGF1* mRNA expression may be more accentuated in mammary stromal tissue, which can explain the absence of statistical difference obtained in our study for *IGF1* response because our samples consisted of a minimum mammary fat pad. The limiting response in *ER1* could partially be explained by the same reason, as according to Connor et al. [86], *ER1* can be found on both epithelial cells and the mammary fat pad of pre-pubertal heifers.

Considering our overall findings, we associated the reduction in *IGFBP3* expression with the increased IGF1 serum concentration, which resulted in greater parenchyma ductal growth represented by the reduced pixel value in the mammary ultrasound of bST-treated heifers.

CONCLUSIONS

The administration of bovine somatotropin in pre-pubertal Holstein × Gyr heifers fed for high daily gain rates does not improve growth parameters, feed efficiency, or final body weight. However, bST injections can increase the IGF1 serum concentration and, as a result, we can expect improved metabolism, mammary parenchyma growth, and lean carcass gain of heifers. In summary, we confirm the efficacy of bST injections to overcome the detrimental effects of high gain diets on mammary gland growth and to improve lean carcass gain of pre-pubertal Holstein × Gyr heifers.

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3. CHAPTER TWO

EXOGENOUS RECOMBINANT SOMATOTROPIN AND ITS INFLUENCE ON REPRODUCTIVE CHARACTERISTICS OF HOLSTEIN × GYR HEIFERS[2]

Effects of bovine somatotropin on reproduction

[2] This manuscript was written to be submitted to *Plos One Journal*.

ABSTRACT

It is vital to search for strategies to improve the reproductive characteristics of pre-pubertal Holstein × Gyr heifers that could impact the onset of puberty. Thus, our objective was to assess the effects of exogenous bovine somatotropin on reproductive traits, steroid concentration, and gene expression of the cumulus-oocyte complex of high-performance pre-pubertal Holstein × Gyr heifers. Thirty-four Holstein × Gyr heifers with an average initial body weight of 218 ± 49 kg and 14 ± 4 months of age were submitted to a 95-day trial evaluating the effects of no rbST or rbST injections. Treatments were randomly assigned to each animal within one of the tree blocks. As expected, some heifers achieved puberty during the trial (~ 36% of the animals). However, neither puberty attainment nor age, weight, or height at puberty were influenced by treatment. The reproductive condition did not affect ovary size, but there was an interaction effect of treatment and experimental day, where heifers from the rbST treatment had greater ovary size on experimental days 36, 43, 50, and 57. The rbST pubertal heifers had greater follicle sizes compared to the no rbST females on experimental days 15 (15.61 mm vs. 12.61 mm) and 57 (19.04 mm vs. 13.04 mm), but no rbST heifers had larger follicles on experimental days 71 (14.68 mm vs. 11.03 mm) and 85 (15.87 mm vs. 10.53 mm). Moreover, on experimental days 71 and 85, non-pubertal

heifers from rbST treatment had greater follicle size than the pubertal ones. Gene expression assessment resulted in a greater mRNA abundance of IGF1R on the cumulus-oocyte complex of heifers on no rbST treatment. To conclude, the evidence gathered in our study supports our hypothesis that the use of rbST would impact the reproductive characteristics of Holstein × Gyr heifers.

INTRODUCTION

Puberty is a critical period in the heifer's life, encompassing the transition from an anovulatory state to the first ovulatory estrus [1,2]. It results from a series of events that require the maturation of the hypothalamic-pituitary-ovarian axis [3]. According to the model developed by Day et al. [4], the endocrine control of puberty occurs as follows: before puberty, there is a decline in the concentration of estradiol receptors in the hypothalamus and pituitary gland. This decline reduces estradiol negative feedback on luteinizing hormone (LH), which can increase the pulsatile secretion of LH (increase LH concentration and the frequency of its pulses and reduce LH pulse amplitude). Simultaneously, the increased secretion of LH improves estradiol production by the ovarian follicles, which will eventually stimulate the first preovulatory surge of LH, causing the first ovulation, or puberty. In addition, follicle-stimulating hormone (FSH) is present during this process, but its level does not fluctuate as LH in the peripubertal period, suggesting that its role in puberty might be a permissive one [2].

Factors such as breed, age, and weight can influence puberty [2]. Nogueira [5] stated that for Zebu females to calve around two years of age, there must be a combination of improved post-weaning nutrition and selection for early puberty. According to Fernandez et al. [6] and Severino-Lendechy et al. [7], the average age at puberty of Holstein × Gyr heifers is 30 and 19 months of

age for unsupplemented and supplemented heifers, respectively. Therefore, searching for strategies to overcome this breed's late entrance into reproductive life is vital. However, before reaching puberty, females also need to undergo extensive follicle proliferation, recruitment, and dominance, in addition to the growth of other reproductive tissues and their bodies (muscular and adipose tissue).

The recombinant bovine somatotropin (rbST) is a technology long used in the dairy industry. This is the synthetical form of the growth hormone (GH), used to enhance milk production and lean carcass gain [8], besides its beneficial effect on reproductive traits. In beef animals, the use of rbST for six months (12 shots, each of 250 mg of sometribove zinc) effectively hastened the puberty of Angus × Hereford heifers (initial BW = 219 ± 2 kg; initial age = 208 ± 2 d) fed meadow foxtail and alfalfa hay [9]. Gong et al. [10] observed that rbST injections increase the number of antral follicles (≥ 2mm) in cycling Hereford × Friesian heifers. Moreover, the GH was proved to act in reproductive tissues once mRNA GH receptors (GHR) were found in the corpus luteum, bovine ovary, follicles, granulosa, cumulus cells, and oocytes [11–14]; meanwhile, it additionally participated in nuclear oocyte maturation [15].

Despite the extensive knowledge about GH functions, there are no reports regarding its benefits towards the improvement of reproductive traits in prepubertal Holstein × Gyr heifers. Thus, because of the significant productive and economic importance of this crossbred, particularly in tropical countries, it becomes imperative to search the effects of GH's exogenous administration on reproductive traits, focusing on ovaries and follicle growth and oocyte characteristics.

Therefore , we hypothesized that exogenous administration of rbST could affect the number and size of follicles, size of ovaries, improve oocyte quality, and increase the concentration of steroid hormones (progesterone and estrogen) in pre-pubertal Holstein × Gyr heifers. Thus, we

aimed to assess the effects of exogenous bovine somatotropin on reproductive traits, steroid concentration, and gene expression of cumulus-oocyte complex of high-performance pre-pubertal Holstein × Gyr heifers.

MATERIAL AND METHODS

This study was carried out in strict accordance with the law nº. 11.794 of October 08th, 2008, Decree nº. 6899 of July 15th, 2009, and the rules issued by the Brazilian National Council for National Experimentation Control (CONCEA). It was approved by the Ethics Commission on the use of farm animals of Universidade Federal de Viçosa (CEUAP-UFV), protocol nº 0144/2019.

Preconditioning, heifers, experimental design, housing, and feeding

Before treatment assignment, all heifers were allocated to Brachiaria decumbens paddocks for 24 days, managed in a rotation system where they changed to the next paddock every two days. During this period, animals received supplemental feed (5% urea, 30% ground corn, 59% soybean meal, and 6% mineral) every two days at 700 g/d/heifer. After that, heifers were transferred to the feedlot and adapted to the experimental diet for 14 days. Then, the 34 heifers were weighed to initiate the trial. During these periods, all heifers underwent reproductive assessment by ultrasound every 14 days (while in paddocks) and every 7 days (while on the experimental diet) to investigate the presence or absence of a corpus luteum. Heifers presenting corpus luteum in one of the ovaries were removed from the trial. Therefore, only pre-pubertal heifers were enrolled in this study. A summary of the preconditioning and experimental period can be found in Fig 1.

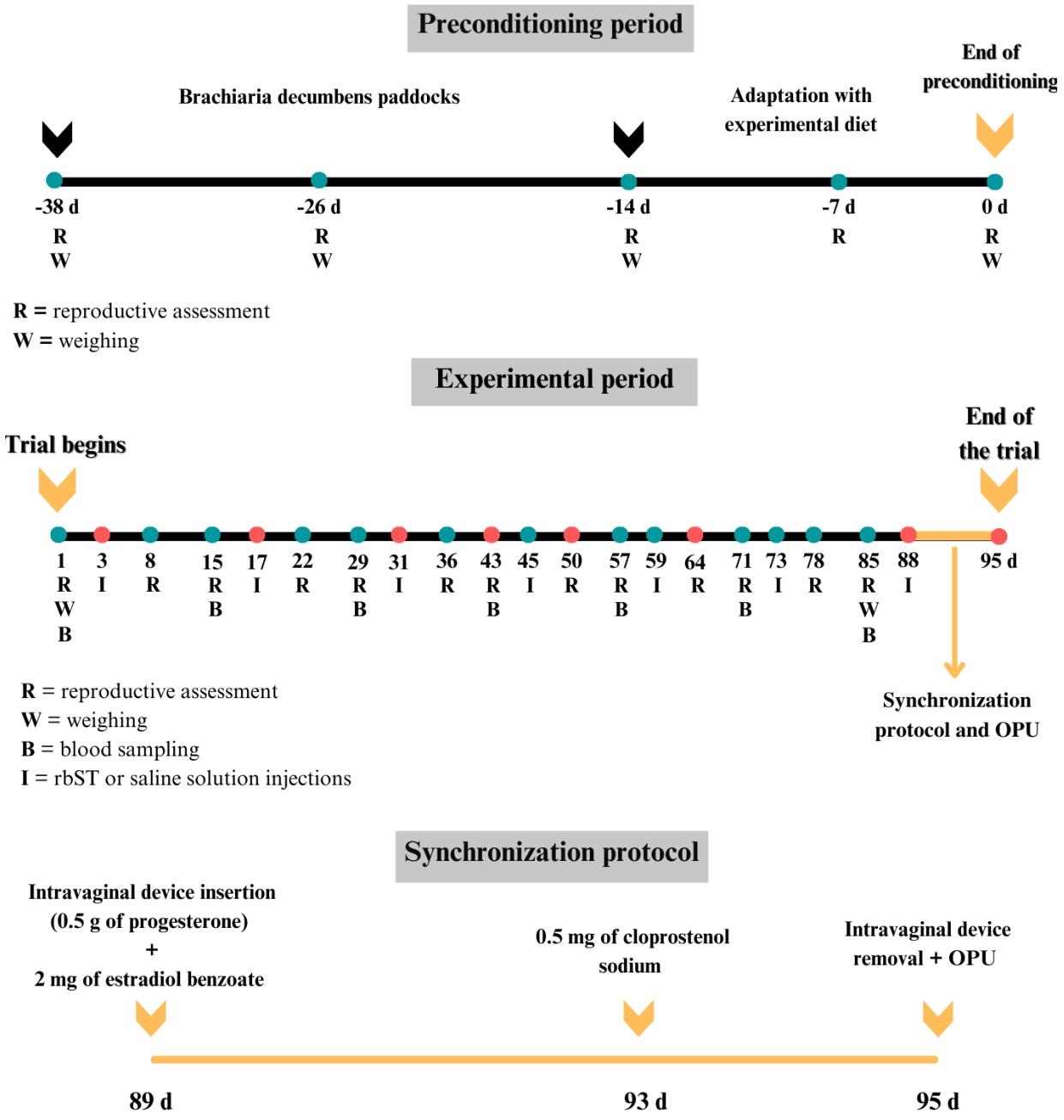


Fig 1. Summary of preconditioning and experimental periods including sampling moments and synchronization protocol.

Thirty-four Holstein × Gyr heifers with average initial body weight (iBW) of 218 ± 49 kg and 14 ± 4 months of age were submitted to a 95-day trial to evaluate the use of rbST on reproductive

characteristics, blood hormones and metabolites concentration, and gene expression of the cumulus-oocyte complex. The 34 heifers were divided into three blocks according to their iBW (B1 n = 12: 273.6 ± 19.2 kg; B2 n = 12: 214.4 ± 18.3 kg; B3 n = 12: 161 ± 19.7 kg). Thus, the 34 heifers were divided into two blocks of 12 animals, each, and one block of 10 animals . Heifers within each block were randomly assigned to two treatments (rbST injections or no rbST injections – control), totaling 17 animals per treatment. Briefly, heifers in the rbST treatment received intramuscularly (I.M.) 500 mg of recombinant bovine somatotropin (Boostin®, Merc Animal Health) every 14 days, starting on experimental day 3 and totaling seven injections. Moreover, to mimic the stress suffered by these heifers, the no rbST animals received the same volume of saline injections (sodium chloride, 0.9%) as a placebo on the same experimental days. Although we initiated the study with 34 heifers, one heifer suffered from lameness and had to be removed from the trial at the end of the first period. Therefore, 33 heifers were evaluated in this study.

Heifers were housed in group pens (six or five animals each, depending on the block) with free access to clean water and a diet formulated to achieve an average daily gain of 1 kg, according to the NRC [16]. The experimental diet consisted of a 65:35 corn silage: concentrate ratio (Table 1). Diet was offered twice daily at 7 a.m. and 3 p.m. and the daily intake of each group, housed in one of the six pens, was controlled to allow a 5% leftover (as-fed basis).

Table 1. Composition of ingredients and nutrients in the experimental diet (65:35 silage to concentrate proportion).

Item (g/kg of DM basis)	*Roughage*	*Concentrate*	*Diet*
Corn silage			510.25
Ground corn		325.43	89.41
Soybean meal		618.92	170.05
Limestone		31.60	8.68

Sodium bicarbonate		12.47	3.42
Magnesium oxide		6.24	1.71
Mineral mix[1]		5.34	2.09
Chemical composition[2]			
DM	295.22	859.33	492.66
OM	947.78	964.93	953.78
CP	62.28	300.42	145.63
EE	24.07	15.20	20.96
NDFap	425.96	127.31	321.43
NFC	435.48	521.98	465.76

[1]Composition: calcium, 40 g/kg; phosphorus, 25 g/kg; magnesium, 30 g/kg; sodium, 13 g/kg; potassium, 75 g/kg; sulphur, 10 g/kg; cobalt, 0.8 mg/kg; copper, 63 mg/kg; iodine, 2mg/kg; manganese 185 mg/kg; zinc, 222 mg/kg; selenium 2 mg/kg.

[2]DM = dry matter; OM = organic matter; CP = crude protein; EE = ether extract; NDFap = neutral detergent fiber free of ashes and protein; NFC = non-fiber carbohydrates.

Blood sampling

Blood samples were collected to assess progesterone (P_4) concentration on experimental day 1 and every 14 days until day 85 and estrogen (E_2) concentrations were measured from the blood samples collected on day 85. All blood samples were collected by coccygeal venipuncture using vacutainer tubes with a gel separator. Tubes were kept on ice until centrifugation (3,000 × *g* at 4 ºC for 20 minutes), then plasma was pipetted into Eppendorf tubes and stored at - 20 ºC until analyses. All analyses were performed by a commercial laboratory using the Chemiluminescence method with respective laboratory kits for Attelica IM Analyzer – Simens.

Reproductive assessment

Reproductive assessments were performed on experimental day 1 and every 7 days until day 85. Heifers were subject to a transrectal ultrasound examination with a Mindray DP 2200-Vet ultrasound and a linear transducer setup at 7.5 MHz. An experienced veterinarian positioned the

rectal transducer to count the number of antral follicles (> 2 mm) on each ovary. Subsequently, the largest follicle on each ovary was identified and measured by freezing the image at the apparent maximal follicle area. Two perpendicular measurements (height and width) were taken to estimate follicle diameter using the caliper option of the ultrasound. The same methodology was used to estimate ovarian diameter during the ultrasound examination. Details of this technique can be found in Gomez-León et al. [17] and Fernandez et al. [6].

Follicular aspiration

On experimental day 88, after finishing all data collection, we gave heifers one last rbST injection to prepare animals for follicular aspiration and ovum pickup (OPU). We only assessed block 1 and block 2 heifers (n = 23) due to adequate size to perform the OPU collection. Heifers from block 3 (n = 10) were too small and could have been injured if the OPU was performed. The OPU was scheduled to be performed seven days after the rbST shot so that somatotropin would be at its peak of action [18].

On experimental day 89, heifers were submitted to a 6-day synchronization protocol (Fig 1). First, an intravaginal device containing 0.5 g of progesterone was inserted in each heifer (Primer®, Agener União), followed by I.M. administration of 2 mg of estradiol benzoate (RIC-BE®, Agener União). Four days after the beginning of the protocol, all heifers received an I.M. administration of 0.5 mg of cloprostenol sodium (Estron®, Agener União). Lastly, the intravaginal progesterone devices were removed on day 6 of the protocol, followed by immediate OPU.

Before the OPU procedure, follicles in each ovary were counted and measured by ultrasound as described in the "Reproductive assessment" section. Subsequently, the OPU procedure was performed according to Machado et al. [19]. An ultrasound device (B-mode) equipped with a micro-convex transducer working at a frequency of 6.5 MHz (DP2200, Mindray, China) coupled

to a transvaginal OPU guide (WTA) was used. A 20 G needle and a 1.2 m follicular aspiration system were added to this system (WTA, Cravinhos, SP, Brazil). Only follicles equal to or smaller than 8 mm were aspirated. For each heifer, the recovered fluid was collected into a 50 mL vial containing 10 mL of saline and sodium heparin (0.9% and 10 IU/mL, respectively) and preserved at 35 – 36 ºC. After aspiration, the cumulus-oocyte complex (COC) was kept in a saline solution to be evaluated according to Leibfried and First [20] characterization of bovine follicular oocytes. Lastly, a pool of four COC, from numerical scores 1 and 2 and 3 and 4 [20], of each heifer was rapidly stored in liquid nitrogen until qRT-PCR analysis.

Gene expression of the cumulus-oocyte complex

Only COC classified according to numerical scores 1 and 2 underwent RNA extraction and cDNA synthesis. The Cells-to-cDNA™ kit (Ambion™ – Thermo Fisher) was used according to the manufacturer's recommendation, and cDNA concentration was quantified using 1 µL of sample in a NanoVue Plus spectrophotometer. Samples were diluted to 50 ng/µL concentration and then stored at – 20 ºC until quantitative real-time PCR (qRT-PCR) analysis.

Table 2. Classification schemes for oocyte investment[1].

Description of category	***Numerical score***
Complete, cumulus oophorus present: more than three layers thick; compact[2].	1
Partial, cumulus present: either not surrounding the oocyte or less than three layers thick; compact.	2
Expanded, cumulus present: cellular investment shows expansion[3]; cumulus cells appear in scattered clumps in the matrix.	3
Nude, cellular investment is not present; the oocyte is only enclosed by zona pellucida.	4

[1]Adapted from Leibfried and First [20].
[2]Compact indicates a cumulus oophorus that tightly adheres to the zona and where cells of the investment are in tight association.
[3]Expanded denotes a cumulus oophorus where cells lose the tight association and are held together by a "stick" matrix.

Quantitative real-time PCR was performed in duplicate using the GoTaq PCR Master Mix (Promega), following the manufacturer's instructions in an ABI Prism 7300 Sequence Detection System thermocycler (Applied Biosystems, Waltham, MA). The amplification efficiency of housekeeping and target genes was estimated using four dilutions of cDNA for each tissue evaluated. Amplification conditions for all systems consisted of an initial step at 95°C for 2 minutes, the second step of 40 cycles at 95°C for 15 seconds, and a final extension step at 60°C for one minute. After the amplification cycles, an additional gradient step from 60°C to 95°C was used to obtain a melting curve.

The ΔCt method was used to estimate the expression of each gene (target Ct – internal control Ct), where Ct represents the PCR cycle number of cDNA amplification above the threshold level [21]. Target genes evaluated in the COC were: IGF1R, IGF2R, LHR, and FSHR; nevertheless, we were able to obtain the expression of IFG1R and FSHR solely – the other genes did not have replications on their CT cycles. In addition, internal control gene efficiency varied among samples, so we considered the geometric mean of three housekeeping genes (GAPDH, HPRT1, and ACTB) to estimate target gene expression by the ΔCt method. Primer pairs for internal control and target genes are presented in Table 3 according to their identification sequences from the GenBank database.

Table 3. Gene name, primer pair sequence, annealing temperature, and amplification efficiency of each target gene.

Genes[1]	***Primary function***	***Accession number[2]***	***Primer sequence (5′- 3′)***	***Amplicon, bp***
IGF1R	Important at the antral stage of follicle development, involved in the regulation of follicle growth and stimulation of somatic cell proliferation [22].	NM_001244612.1	Forward: GTATGGAGGAGCCAAGCTAAA Reverse: GTCTTGGCCTGAACGTAGAA	123
FSHR	Necessary for the selection and growth of ovarian follicles, but also to produce estrogens from androgen substrates [23].	NM_174061.1	Forward: CATGCTCATCTTCACCGACTT Reverse: GACCAGGAGGATCTTTGACTTG	112
GAPDH	Internal control gene – generally involved in energy metabolism.	NM_001034034.2	Forward: GATGCTGGTGCTGAGTATGT Reverse: GCAGAAGGTGCAGAGATGAT	113
HPRT1	Internal control gene - involved in hypoxanthine phosphoribosyltransferase 1, producing an enzyme that allows cells to recycle purines	NM_001034035.2	Forward: GTGGGATATGCCCTTGACTATAA Reverse: GGACTCTCATCTTAGGCTTTGT	104
ACTB	Internal control gene – involved in cell motility, structure, and integrity.	NM_173979.3	Forward: ACTCCTGCTTGCTGATCCACATCT Reverse: AAGATCAAGATCATCGCGCCTCCA	

[1]Genes: growth hormone receptor (*GHR*), insulin-like growth factor 1 receptor (*IGF1R*), insulin-like growth factor 2 receptor (*IGF2R*), follicle-stimulating hormone receptor (*FSHR*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), hypoxanthine phosphoribosyltransferase 1 (HPRT1), β-actin (ACTB). [2]GenBank.

Statistical analysis

All variables were analyzed using PROC GLIMMIX of SAS (Statistical Analysis System, 9.4 version) in a random block design, using initial BW as blocking criteria (random effect). Because some heifers achieved puberty during the trial, we chose to separately assess data from pubertal and non-pubertal heifers (reproductive status) once it could bring a more detailed response of the analyzed variables.

Estrogen concentration, COC phenotypical classification, and gene expression did not present repeated measures; thus, they were analyzed according to the following model:

$Y_{ijk} = \mu + T_i + B_j + \varepsilon_{ijk}$

μ = mean; T_i = fixed effect of treatment i; B_j = random effect of block j; and ε_{ijk} = random error with mean 0 and variance σ^2, variance among animal measurements.

Plasma progesterone and estrogen and phenotypical classification of oocytes did not follow the normal distribution; thus, data were assessed using Lognormal distribution (normal residues).

Plasma progesterone, the number of follicles, and the average size of follicles and ovaries were evaluated repeatedly during the experimental period. Therefore, period measurements (referred to as the experimental day of data collection) were included as repeated measures in the model as follows:

$Y_{ijklm} = \mu + T_i + \delta_{ij} + B_k + P_l + (T \times P)_{il} + \varepsilon_{ijklm}$

μ = mean; T_i = fixed effect of treatment i; δ_{ij} = random error with mean 0 and variance σ^2, the variance among animals within treatment equal to the covariance among repeated measures among animals; B_k = random effect of block k; P_l = fixed effect of period l; $(T \times P)_{il}$ = random effect of interaction between treatment i and period l; and ε_{ijklm} = random error with mean 0 and variance σ^2, variance among animal measurements.

Eight variance-covariance structures (AR1, CS, FA, UN, TOEP, VC, ARH1, TOEPH) were tested and the one presenting the best fit based on the Akaike information criterion was used. In addition, due to the unbalanced design, degrees of freedom were corrected using the Kenward-Rodger approximation. The main effects of rbST, reproductive status, and days were discussed separately in the absence of interactions. For all parameters and gene expression analyses, significance was declared when $P \leq 0.05$.

RESULTS

This study aimed to understand how the exogenous rbST would interfere with reproductive traits, steroid concentration, and gene expression of the cumulus-oocyte complex of high-performance pre-pubertal Holstein × Gyr heifers. Nonetheless, as expected, not all heifers achieved puberty during the trial. Thus, conscious of the impact that the pubertal phase can have on reproductive characteristics, we choose to analyze, present, and discuss the outcomes considering treatments, days of data sampling, and reproductive conditions (non-pubertal or pubertal). Moreover, we would like to highlight that pubertal heifers were considered as the ones that entered puberty at some point during the experimental period, but they did not reach puberty at the same time.

Heifers' growth at puberty

To identify puberty, we considered the first physiological estrus as the presence of a viable *corpus luteum* properly identified and evaluated by an experienced veterinarian. There was no difference in the initial or final body weight of non-pubertal (212.6 ± 16 kg; 303.5 ± 33 kg) and pubertal (232.7 ± 22 kg; 320.41 ± 34 kg) heifers ($P \geq 0.188$; Table 4). As well as for their average daily gain (non-pubertal = 1.08 ± 0.06 kg and pubertal = 1.21 ± 0.07 kg; $P = 0.179$).

Approximately 36% of the assessed females achieved puberty (41.2% no rbST and 31.3% rbST; Table 4), without statistical difference between treatments ($P = 0.450$). Moreover, age, weight, and height at puberty were similar ($P \geq 0.187$). Heifers receiving rbST attained puberty at, on average, 18 months of age, weighing 329 kg, and withers and rump height of 126 and 132 cm, respectively. The most discrepant difference was regarding age, where heifers receiving rbST injections reduced age at puberty by two months. However, this difference was not significant ($P = 0.319$).

Table 4. Characteristics of Holstein Gyr heifers entering puberty.

Items	**Treatments**			***P-value*[1]**
	no bST	**rbST**	***SEM***	**Treatment**
Nº of heifers	17	16		
N° of heifers entering puberty	7 (41.2%)	5 (31.3%)	0.141	0.450
Age at puberty (months)	20.0	18.1	1.705	0.319
Weight at puberty (kg)	324	329	11.419	0.754
Withers height (cm)	124	126	0.016	0.475
Rump height (cm)	129	132	0.015	0.187

[1]*P*-values indicate treatment effects ($P < 0.05$).

Plasma progesterone and estrogen concentration

Regarding progesterone results, there was no difference between treatments ($P = 0.243$; Fig 2). We also considered evaluating only heifers that presented *corpus luteum* on the same day when progesterone levels were above 1 ng/mL (inferior limit indicating puberty) [2], but no difference between treatments was observed ($P = 0.071$). However, we observed an interaction between experimental day and reproductive condition ($P < 0.001$) where heifers that became pubertal presented the highest progesterone concentration on experimental day 85,

indicating that, on this day, all heifers in this group were pubertal. These females had greater progesterone concentrations beginning on experimental day 43, yet plasma concentrations were still below the threshold. Moreover, estrogen concentration was assessed only on experimental day 85, as mentioned previously, and its concentration was similar between treatments (no rbST = 25.28 ± 0.07 pg/mL and rbST = 23.62 ± 0.07 pg/mL; $P = 0.483$) and reproductive conditions (non-pubertal = 23.58 ± 0.06 pg/mL and pubertal = 25.32 ± 0.08 pg/mL; $P = 0.466$). No interactions were observed for estrogen concentration ($P = 0.119$).

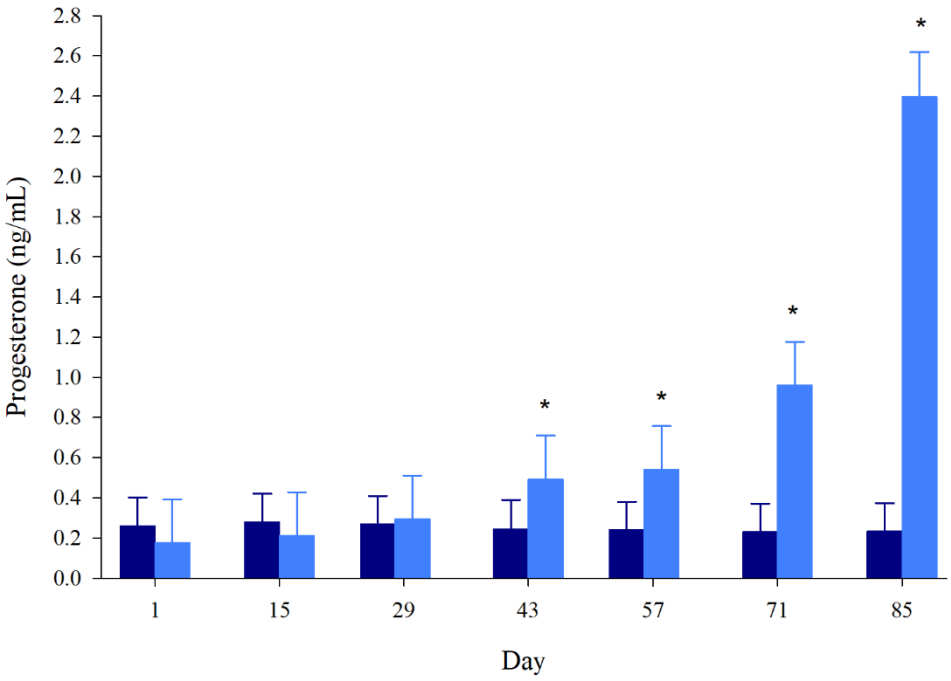


Fig 2. Means of interaction between experimental day and reproductive condition (non-pubertal and pubertal) effect on plasma progesterone concentration (ng/mL) of Holstein × Gyr heifers (n = 33). Dark blue columns represent non-pubertal heifers and light blue columns represent pubertal heifers. Error bars represent SEM and asterisks indicate reproductive condition differences on each experimental day. Differences were considered when $P < 0.05$.

Reproductive measurements

We observed no treatment effects on the number of follicles ($P = 0.471$) but an interaction between experimental day and reproductive condition ($P = 0.045$). Fig 3 illustrates the variation in follicle quantity with a greater number of follicles on experimental day 78 for non-pubertal heifers compared to the pubertal ones. Moreover, heifers that entered puberty had the lowest number of follicles on experimental day 43 and, on average, a reduced number of follicles along the days evaluated.

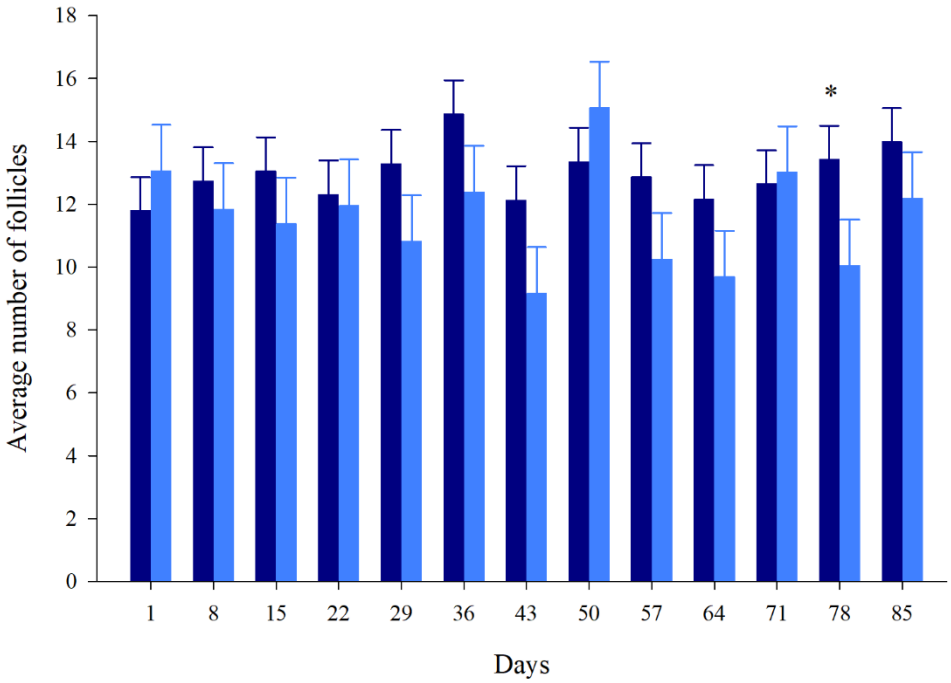


Fig 3. Means of interaction between experimental day and reproductive condition (non-pubertal and pubertal) effect on the average number of follicles measured every seven days during the reproductive assessment of Holstein × Gyr heifers (n = 33). Dark blue columns represent non-pubertal heifers and light blue columns represent pubertal heifers. Error bars represent SEM and asterisks indicate the differences in reproductive condition. Differences were considered when $P < 0.05$.

The reproductive condition did not affect ovary size (P = 0.063), but there was an interaction effect of treatment and experimental day (P = 0.009), where heifers from the rbST treatment had greater ovary size on experimental days 36, 43, 50, and 57 (Fig 4).

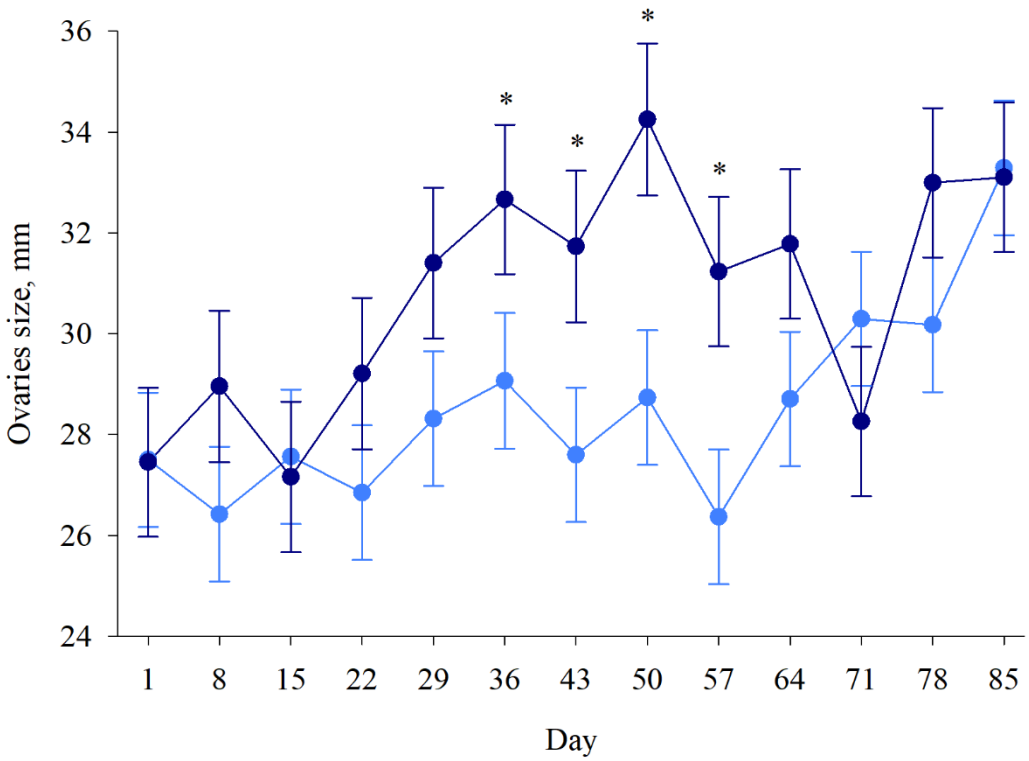


Fig 4. Means of interaction between treatment (no rbST and rbST) and experimental day effect on ovaries size measured every seven days during the reproductive assessment of Holstein × Gyr heifers (n = 33). Light blue circles represent the ovaries of no rbST treatment and dark

blue circles represent the ovaries of rbST treatment. Error bars represent SEM and asterisks indicate treatment differences on each experimental day. Differences were considered when P < 0.05.

For the follicle sizes, we noted a triple interaction among treatment, experimental days, and reproductive conditions (P = 0.027). Thus, to explain this complex interaction, we

presented the results divided into two figures, both representing treatment and experimental day interaction in each reproductive condition (Fig 5).

The rbST pubertal heifers had greater follicle sizes ($P \leq 0.010$) compared to the no rbST females on experimental days 15 (15.61 mm vs. 12.61 mm) and 57 (19.04 mm vs. 13.04 mm), but no rbST heifers had larger follicles ($P \leq 0.040$) on experimental days 71 (14.68 mm vs. 11.03 mm) and 85 (15.87 mm vs. 10.53 mm). In addition to the differences stated, heifers on the same treatment but on distinct reproductive conditions had significant changes in follicle size ($P \leq 0.039$), which on average corresponded to a larger size of follicles for pubertal heifers on experimental days 8, 15, 29, 50, and 57, independently of the treatment. However, on experimental days 71 and 85, non-pubertal heifers from rbST treatment had greater follicle size than the pubertal ones.

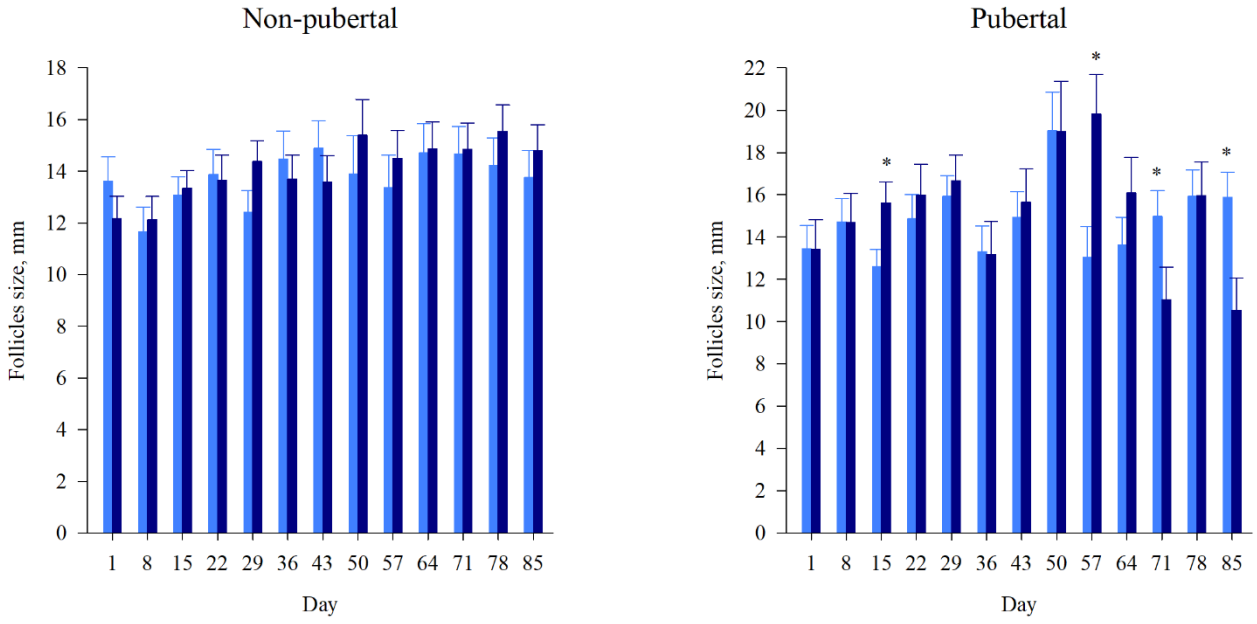
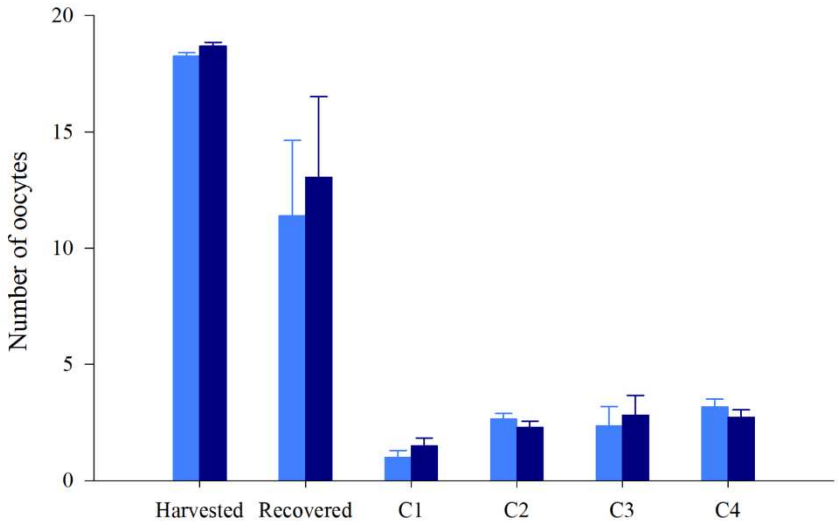


Fig 5. Means of interaction between treatment (no rbST and rbST), experimental day, and reproductive condition (non-pubertal and pubertal) effect on follicles size measured

every seven days during the reproductive assessment of Holstein × Gyr heifers (n = 33). Light blue bars

represent the follicles of no rbST treatment and dark blue bars represent the follicles of rbST treatment. Error bars represent SEM and asterisks indicate treatment differences on each experimental day for a specific treatment (no rbST or rbST). Differences were considered when $P < 0.05$.

There was no interaction, treatment, or condition effect for phenotypical characteristics ($P > 0.05$; Fig 6). The number of oocytes harvested and recovered during the ovum pickup procedure was similar between treatments ($P = 0.917$ and $P = 0.731$). Also, there was no difference in the classification of oocytes according to the cumulus-oocyte complex structure ($P \geq 0.517$).



Oocytes	no rbST	rbST	*P* - value
Harvested	18.27	18.69	0.917
Recovered	7.98	9.14	0.731
C1	1.01	1.52	0.517
C2	2.65	2.30	0.641
C3	2.37	2.81	0.621
C4	3.18	2.74	0.745

Fig 6. Number of oocytes harvested and recovered and phenotypical assessment of cumulus-oocyte complex of Holstein × Gyr heifers (n = 33). Light blue columns represent no rbST treatment, and dark blue columns represent rbST treatment. C1: complete, cumulus oophorus present: more than three layers thick; compact[2]; C2: partial, cumulus present: either not surrounding the oocyte or less than three layers thick; compact; C3: expanded, cumulus

present: cellular investment shows expansion[3]; cumulus cells appearing in scattered clumps in the matrix; C4: Nude, cellular investment not present; oocyte only enclosed by zona pellucida. Error bars represent SEM. Differences were considered when $P < 0.05$.

Gene expression

We did not observe interaction or reproductive condition effects on the expression of IGF1R or FSHR ($P \geq 0.226$). Regarding treatment (no rbST and rbST) effects, FSHR presented similar mRNA abundance (0.732 vs. 0.5011, respectively; $P = 0.3748$). Nevertheless, the mRNA abundance of IGF1R on the cumulus-oocyte complex of heifers on no rbST treatment was higher than the rbST one (0.313 vs. 0.149, respectively; $P = 0.019$; Fig 7).

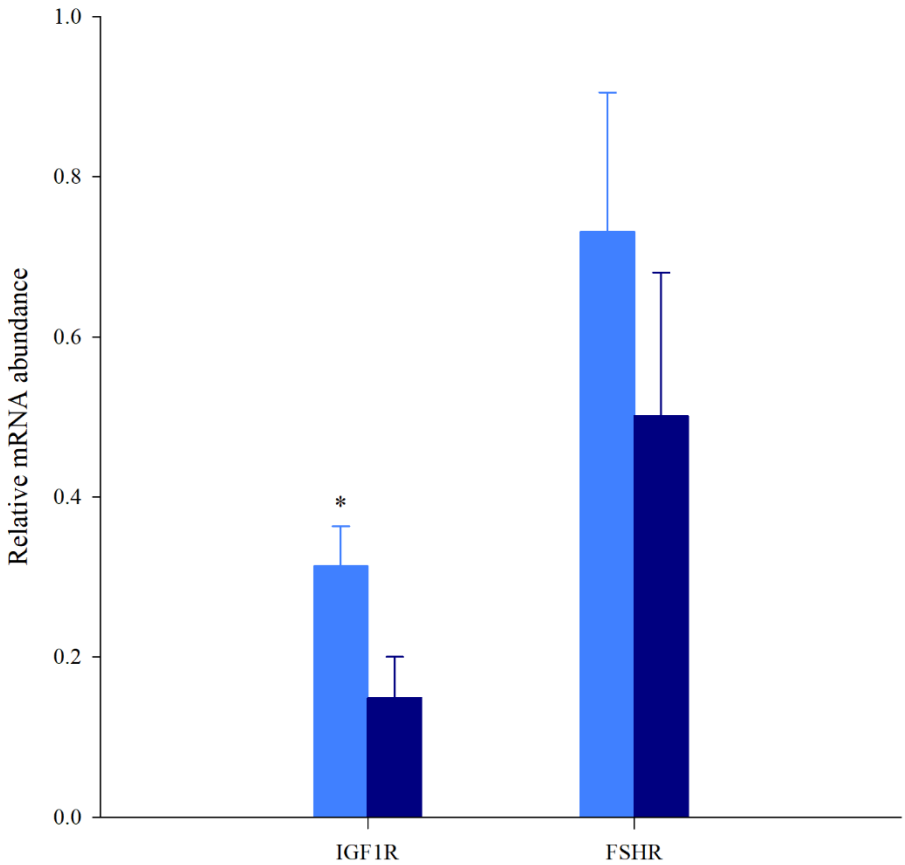


Fig 7. Relative mRNA abundance of genes in the cumulus-oocyte complex of Holstein × Gyr heifers (n = 33). Light blue bars represent no rbST treatment and dark blue bars represent

rbST treatment. Error bars represent SEM and asterisks indicate treatment differences or a specific treatment (no rbST or rbST). Differences were considered when $P < 0.05$.

DISCUSSION

Heifers' growth at puberty

The growth hormone is synthesized by the pituitary gland, which acts in various tissues in the animal body, as explained by the somatomedin hypothesis [24]. Additionally, the GH is involved in animal growth, mammary gland development, lactation, and reproduction [8]. Some authors reported that mutation of the GH gene or depletion of its receptor (GHR) could delay puberty and induce subfertility, but GH exogenous application could increase the number of ovarian follicles [25].

According to Sartori et al. [26], Zebu heifers achieve puberty at a later age than *Bos taurus* breeds, approximately 25 months of age, which could result from discontinuous genetic selection for reproductive characteristics. Fernandez et al. [6] observed that Holstein × Gyr heifers had their first estrus at 29 months of age, weighing 354 kg. These animals were kept in a pasture area of *Panicum maximum* cv. Tanzania and fed sorghum silage once a day as a complement to the diet at specific times of the year. Moreover, Schmidt [27] confirmed that Holstein × Gyr heifers of two main genetic groups (3/4 and 5/8) attained puberty at 15 and 16 months, weighing 337 and 340 kg, respectively. Lastly, Fonseca et al. [28] evaluated age at puberty and reproductive characteristics of F1 Holstein × Gyr heifers with different residual feed intakes and observed an average age at puberty of 11 months with 321 kg of body weight. According to Fonseca et al. [28], the lower age at puberty obtained was related to increased daily gain rates during the post-weaning period, resulting in the maturation of the neuroendocrine system, which hastens the release of higher LH pulses during pre-puberty.

We observed some heifers entering puberty, but not due to the exogenous GH treatment. It was possibly an endocrine response to the adequate diet, phenotypical and genetic characteristics of heifers, and adequate environment. This corroborates with the results obtained by Radcliff et al. [29], who evaluated the effects of diet and rbST on Holstein heifers; however, in their study, heifers receiving rbST injections every 14 days, regardless of the diet, had greater BW and withers height at puberty, what was not observed within our results (Table 4).

In our study, even heifers that achieved 326.5 kg of BW (average weight observed at puberty) did not attain the onset of puberty (Fig 7). In pure-breed animals, puberty is expected at 50 to 55% [30] of mature body weight. According to Busanello et al. [30], Holstein × Gyr cows present mature body weight of 607 kg, and although our heifers attained puberty, on average, with 54% of mature body weight, there are limited references indicating the adequate percentage of mature body weight at puberty for this crossbred, due to the numerous possibilities for blood composition. Moreover, the literature recognizes that weight does not act as a single variable affecting the onset of puberty. Nogueira [4] and Sartori et al. [26] stated that age, body composition (especially the percentage of body fat), nutrition, and genetics (puberty high heritability for Zebu breeds) essentially contribute to puberty achievement.

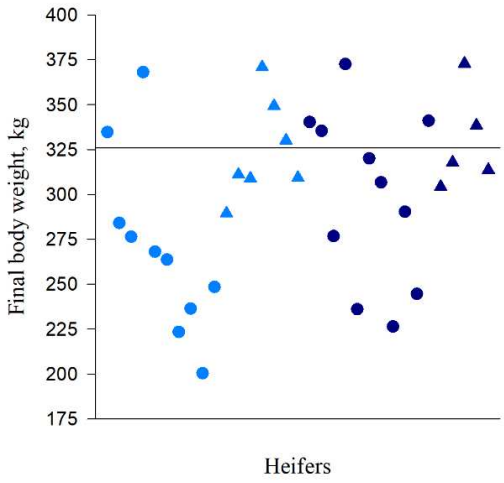


Fig 7. Final body weight of Holstein × Gyr heifers submitted to no rbST or rbST treatment. Light blue circles and triangles represent no rbST animals, both non-pubertal

(circles) and pubertal (triangles), and dark blue circles and triangles represent rbST animals, both non-pubertal (circles) and pubertal (triangles). The constant line indicates the average weight of heifers at puberty (326.5 kg).

In summary, our results suggest that heifers who entered puberty at some point in this trial did not have heavier initial or final body weight nor a higher average daily gain. Also, we cannot suggest that rbST injections acted to hasten the onset of puberty. The results observed indicate that for the assessed Holstein × Gyr heifers, puberty was a response effect of endocrine factors, management practices adopted during this period, and the genetic selection of reproductive characteristics, as suggested by Sartori et al. [26].

Plasma progesterone and estrogen concentration

Usually, puberty is defined by the first day when plasma progesterone levels exceed 1 ng/mL [2]. Nevertheless, puberty can also be identified by the presence of a *corpus luteum* in one of the ovaries [6,31]. According to Cooke and Arthington [32], progesterone criteria established as an indicator of luteal function and consequent onset of puberty is typically restricted to *Bos taurus* cows and heifers, with normal estrus cycles with a functional *corpus luteum*. However, the results showing that progesterone concentration was above 1 ng/mL only on experimental day 85 can be explained by the fact that not every heifer entering puberty had cycled before experimental day 85, and due to their distinct estrous cycle. We also must acknowledge that to assess puberty by progesterone concentration correctly, the most indicated management would be to collect blood samples at least two days a week, every week, until the identification of a *corpus luteum*.

Thus, because of the results obtained for plasma progesterone concentrations, we considered that the best option to identify the onset of puberty in our animals was rectal palpation and ultrasonography confirmation of a viable *corpus luteum*. Therefore, the absence of treatment difference in progesterone levels agrees with the results obtained by

ultrasonography, which indicates no effects of rbST on the onset of puberty. Moreover, the absence of treatment and condition effect on estrogen concentrations can be more related to the randomness of the estrous cycle of each heifer.

Reproductive measurements

According to Sartori et al. [33], *Bos taurus* and *Bos indicus* breeds have specific differences regarding their reproductive traits. Usually, *Bos indicus* females present smaller dominant follicles, increased levels of circulating progesterone and estrogen, and the follicle acquires ovulatory capacity at a smaller diameter, around six to seven millimeters. To our knowledge, no study compares the number and size of follicles between Holstein × Gyr and Holstein heifers, nor ovary size between these breeds. Therefore, we will not focus the discussion on these differences but on results obtained from the evaluation of the follicular population in Holstein × Gyr heifers and the assessment of rbST shots in follicular and ovarian characteristics according to the reproductive condition.

The higher number of follicles observed on experimental day 78 for non-pubertal heifers compared to the pubertal ones is possibly associated with the recruitment wave of the cycle. Moreover, the reduced average number of follicles presented by pubertal heifers was also a pattern observed by Gasser et al. [34], which, according to the authors, could be explained by an increased period of follicle dominance associated with an extended follicular wave. Nevertheless, the greater number of follicles was not assessed according to a specific size; it was accounted as an overall increase, including all sizes. Even though Adams and Singh [35] stated that the number of follicles is more related to the intrinsically individual factors, Gong et al. [10] demonstrated that heifers treated with rbST had an increased number of antral follicles (2 to 5 mm). Nevertheless, our results fail to demonstrate an effect of rbST on the number of antral follicles in Holstein × Gyr heifers.

The differences observed for follicle size of pubertal heifers are attributed to the rbST, suggesting an influence of this exogenous hormone on follicle development. As reported by Lucy [25], the GH receptor can be mostly found in large *corpus luteum* cells, but it can also be identified in granulosa, oocytes, and cumulus cells [11] and [36]), thus improving follicle development [37]. Moreover, we observed greater size of ovaries for rbST heifers on experimental days 36, 43, 50, and 57, which can be associated with the size of follicles, mainly on experimental days 50 and 57.

Due to the presence of GH receptors on oocytes, we supposed that exogenous somatotropin injections would improve oocyte recovery rate and quality. However, we observed no difference in follicles harvested, oocytes recovered, and oocyte quality, which was also reported by Sá Filho et al. [38]. Moreover, Roth et al. [39] demonstrated that cows treated with rbST injections did not present greater oocyte quality at the reproductive cycle of treatment, nor morphological impairment of these cells, but on the subsequent cycle, these cows presented improved oocyte quality. This finding suggests that, for lactating cows, the rbST can influence oocyte quality differently than for nulliparous heifers.

Gene expression

Ovarian gene expression is well-documented in the literature, but information on the cumulus-oocyte complex is less reported in studies [40]. Lucy [12] reunited results from research indicating the presence of IGF1, IGF2, and its receptors in granulosa and theca cells of bovines. However, as mentioned by Nuttinck et al. [40], it would not be correct to extrapolate the observation in granulosa and theca cells to cumulus-oocyte complex because the COC approximates more to undifferentiated granulosa cells and respond lower to steroid hormones than granulosa cells [41].

As discussed by Bevers and Izadyar [42], GH acts through cumulus cells on the *in vitro* maturation of bovine oocytes and IGF1 may not mediate it. Moreover, the same authors stated

that GH and FSH have opposite functions during meiosis: while GH accelerates it, FSH retards, and their addition to the maturation medium does not improve blastocyst formation. Khurchabilig et al. [41] discussed the relative mRNA expression of FSHR and IGF1R. According to them, FSHR level is positively correlated to IGF1R expression, and their synergistic effect contributes to the differentiation of COC to granulosa cells. Despite their importance on specific periods, Khurchabilig et al. [41] concluded that higher expression of FSHR and IGF1R is likely to result in poor developmental competence of blastocysts, corroborating with the results reported by Bevers and Izadyar [42]. Therefore, we can suggest that exogenous injections of rbST helped to improve COC development and expansion, as reported by Izadyar et al. [11], and although we did not evaluate blastocyst development in our study, it might be possible that oocytes from rbST-treated heifers would better develop into blastocysts due to the lower expression of IGF1R in COC.

CONCLUSION

The use of rbST in pre-pubertal Holstein × Gyr heifers affected some reproductive characteristics, such as the size of ovaries and follicles and the gene expression of IGF1R in the COC. The exogenous somatotropin injections acted in ovaries promoting their greater size on specific days of the trial, which coincided with days with greater follicles number and sizes. Additionally, it did influence steroid hormone concentration and did not hasten the onset of puberty, nor the age, weight, wither, and rump heights at puberty. The evidence amassed in our study substantiates our hypothesis that the utilization of rbST could indeed affect the reproductive traits of Holstein × Gyr heifers. However, due to the observed inconsistency in these results, we advocate for additional research to delve deeper into GH's role in reproductive characteristics, particularly focusing on gene expression of COC within the Holstein × Gyr

heifer population since this breed holds significant productive and economic importance, particularly in tropical countries.

ACKNOWLEDGMENTS

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4. CHAPTER THREE

MODELLING MILK YIELD LOSSES ASSOCIATED WITH MASTITIS OCCURRENCE: WHEN DOES MILK PRODUCTION START TO DROP AND WHEN IS IT REESTABLISHED?[3]

[3]This manuscript was written to be submitted to *JDS Communications.*

ABSTRACT

Despite the suggested daily and overall losses in lactation due to clinical mastitis cases reported in the literature, no recent study attempted to model daily milk losses before and after mastitis onset and the exact moment when it begins. Thus, we aimed to describe the impact of mastitis on milk production based on mastitis level and moment of occurrence. We used data from thirteen dairy farms, generating a databank of 908,816 daily individual milk test records from 3,508 cows in different lactation orders, from January 2017 to December 2022. For modeling milk drop and recovery relative to mastitis, we followed three steps: firstly, we removed milk recorded at the diagnosis day of mastitis from the databank and fitted a Wood's Curve for each cow and lactation order. Secondly, we returned the mastitis data to the database and estimated the residual milk loss due to mastitis from six days before and after the mastitis event. Thirdly, we used a meta-analytical approach, including farm as a random control effect, to estimate residual milk loss. The model developed is strongly influenced by mastitis level, and milk yield. In short, considering the occurrence of two mastitis cases level 1 at the first and last third of lactation, we will have a total loss of 170.8 kg of milk; if these mastitis cases were level 2, the total loss would be of 273 kg of milk. The estimations suggest that milk drop occur three to four days prior to mastitis onset and can last until ten to twelve days from the diagnosis. Thus, our model shows that milk loss estimated at mastitis day is greater than values referenced in the literature and differ due to mastitis level. To conclude, the novelty of our study does not

only indicate when and to what proportion mastitis drop and recovery occur, but it also brings new perspectives to mastitis modelling.

Mastitis is one of the most common causes of economic losses on dairy farms (Silva et al., 2021), known to depress milk yield, reduce fertility, and increase culling rates (Daros et al., 2019). This multifactorial disease initiates when pathogenic microorganisms enter the teat canal and surpass mammary gland immunity defenses, which can increase the vascular permeability of the mammary tissue and result in altered milk composition, depending on the severity of the infection (Nielsen, 2009). Clinical cases of mastitis present visible signs of the infection, such as clots in the milk, discomfort while milking, swelling, fever, and loss of appetite (Nielsen, 2009). This disease can also cause systemic reactions in the cow's body and a constant production loss for each one-unit increase in SCS (Ruegg, 2017). Additionally, mastitis has a more prominent impact on highly productive females from the second lactation onwards (Wilson et al., 2004).

According to Puerto et al. (2021), the highest reductions in positive clinical mastitic cows were observed during late- and mid-lactation (1,137 and 506 kg of milk, respectively), resulting in losses of $710 to $324 of cumulative milk value. van Soest et al. (2016) acknowledged that the average milk production loss of cows suffering from clinical mastitis was 336 kg per case per year, approximately €240 per lactating cow per year. For Heikkilä et al. (2018), clinical mastitis can cause a daily reduction in milk yield of 1.4 to 3.5 kg, depending on the pathogen.

Lescourret and Coulon (1994), Rajala-Schultz et al. (1999), and Nielsen (2009) observed that milk losses due to mastitis initiate two to four weeks before the diagnosis and can be influenced by previous production, lactation week, and parity. Additionally, Nielsen (2009) estimated that, on mastitis day, primiparous cows could lose 5 kg of milk, while multiparous cows can have their production reduced from 1 to 8 kg. Moreover, Lescourret and Coulon (1994) and Rajala-Schultz et al. (1999) observed that milk recovery could occur over two or

four weeks passed the disease identification, although milk yield become compromised for the entire lactation. However, despite the references suggesting daily and overall losses in lactation due to clinical mastitis cases, no recent study attempted to model daily milk losses before mastitis onset and the moment when it begins; similarly, more information is necessary regarding daily milk production reestablishment and recovery time after the mastitis identification.

Therefore, considering the welfare impairment and the production losses that this disease can trigger in animals, it is of utmost importance to develop a new model with more robust data regarding mastitis incidence on dairy farms, which will estimate milk loss and the recovery time after the diagnosis of this disease. Thus, we aimed to describe the impact of mastitis on milk production based on mastitis degree and moment of occurrence.

In this retrospective study, we used data from thirteen dairy farms (one from Canada, three from Spain, four from the United Kingdom, and five from Brazil) and the databank consisted of 908,816 daily individual milk test records from 3,508 cows in different lactations, with an average milk yield of 35.34 ± 0.053 kg, from January 2017 to December 2022. Lactations with less than five records, milk yield (**MY**) and days in milk (**DIM**) equal to zero or any missing data were removed from the database. Additionally, cows should have at least one milk record before 60 DIM and one after 150 DIM to be used in the model. Thus, a total of 3,367 cases of mastitis from 1,832 cows were assessed.

For modeling milk drop and recovery relative to mastitis, days were computed relative to the day when the first clinical sign was observed (from – 6 days to + 6 days, not including zero, and the mastitis day was coded as day 1). Mastitis severity was then coded as 1(one) – mild or 2 (two) – severe mastitis. The mastitis was considered mild if only gargots (flakes or clots in milk) were observed during the teat stripping test. If any additional symptoms of mastitis (redness, inflammation, fever, pus, blood, etc.) were observed by the milker, the mastitis was

considered a severe case. Although several studies have used three grades for mastitis intensity (Wenz et al., 2006; Tomazi et al., 2018; Nagasawa et al., 2019), our preliminary analysis did not result in good estimates when coding three levels for mastitis; therefore, moderate, and severe cases were coded as severe mastitis. Moreover, a mastitis case was considered new if it appeared within fourteen days of difference between the current and the previous mastitis case (Tomazi et al., 2018).

Then, we modeled the effect of mastitis based on the drop and recovery of milk yield following three steps. Firstly, we removed milk recorded at the diagnosis day of mastitis (day 1) from the databank and fitted a Wood's Curve (**WC;** Wood, 1967) for each cow and lactation order. This approach would eliminate the effect of cow and lactation order since we modeled the milk yield loss and not milk itself at the diagnosis of mastitis.

$$MY = a \times wk^{b} \times exp^{(-c \times wk)} \quad [1]$$

Where: MY = milk yield; a, b, and c = constants; and wk = week of lactation.

Secondly, we returned the mastitis data to the database and estimated the residual milk loss (**RML**) due to mastitis from 6 days before to 6 days after the mastitis event:

$$RML = pMY - MY \quad [2]$$

Where: RML = residual milk loss; pMY = predicted milk yield by Wood's Curve; and MY = actual milk yield.

Thirdly, we used a meta-analytical approach, including farm as a random control effect, to estimate RML. We used a multiple regression using the mastitis level (**ML**; 1 or 2), days relative to the mastitis event (Day, from - 6 to 7), and predicted milk yield (**pMY**) to determine RML, which is the theoretical milk yield of health cows, without any mastitis case during the lactation. The pMY was included as a covariate, and the day was tested both as linear and quadratic effects to address possible curvilinear responses. Then, we tested the effect of ML on all parameters of

the model (Equation 3). To reach the final equation, we performed a backward selection of the significant parameters at $P < 0.05$. At first, we tried to set one model for both drop and recovery of milk relative to the mastitis day; however, because of non-biological estimates, we changed the approach and fitted one model for milk yield drop (Day ≤ 1), and another model for milk yield recovery (Day ≥ 1), which consistently corrected our estimation problem. Lastly, we checked our model to ensure that on day 1 (mastitis day), the estimates of RML were similar between our two models (drop and recovery models). The model was run using PROC MIXED (SAS University Edition) and parameters were considered significant when $P < 0.05$.

$$RML = \beta_0\, ML + \beta_1\, Day + \beta_2\, pMY + \beta_3\, ML \times pMY + \beta_4\, ML \times Day + \beta_5\, Day \times Day + \beta_6\, ML \times Day \times Day \quad [3]$$

Where: RML = residual milk loss; ML = mastitis level; Day = days relative to mastitis event; pMY = predicted milk yield; and β_0, β_1, β_2, β_3, β_4, β_5, and β_6 are parameters.

A total of 1,832 cows had a mastitis case recorded at some point of their current lactation. There were 2,483 cases of mild mastitis and 884 cases of severe mastitis, and the number of lactations when mastitis occurred varied from 1st to 11th lactation. Also, there was a considerable variability of mastitis occurrence among weeks of lactation (1st to 49th weeks; Figure 1).

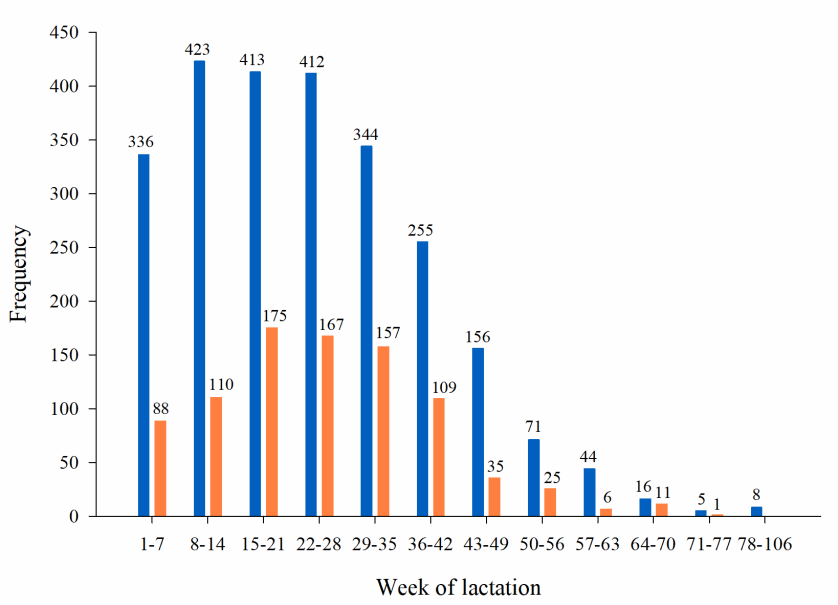

Figure 1. Frequency of mastitis cases according to lactation week (n = 3,367). Blue columns represent mastitis level 1 and orange columns represent mastitis level 2.

Although we expected to include all thirteen farms in both models (drop and recovery from mastitis), some had to be removed to ensure that RML was similar between drop and recovery estimations. Then, to improve our model, we considered the leave-one-out procedure to identify these questionable farms. Thus, one farm from Spain was removed from the milk drop model, and three farms from Brazil were removed for the milk recovery model. As a result, the mastitis level did not impact β_3 ($P = 0.681$), but it did affect all the other parameters ($P < 0.002$) while evaluating the drop in MY.

$$RML_{DML1} = (5.616 \pm 0.692) + (3.501 \pm 0.153 \times Day) + (0.078 \pm 0.014 \times pMY) + (0.375 \pm 0.028 \times Day^2) \quad [4]$$

$$RML_{DML2} = (7.560 \pm 0.753) + (4.676 \pm 0.139 \times Day) + (0.078 \pm 0.014 \times pMY) + (0.523 \pm 0.025 \times Day^2) \quad [5]$$

$R^2 = 0.996$; RMSE = 103.437.

Where: RML_{DML1} = residual milk loss during the drop of mastitis level 1; RML_{DML2} = residual milk loss during the drop of mastitis level 2; Day = days relative to mastitis event; and pMY = predicted milk yield.

While assessing the recovery after mastitis incidence, ML did not affect β_3, β_4, and β_6 ($P > 0.256$). Therefore, the equations to estimate RML during the recovery were:

$$RML_{RML1} = (4.694 \pm 7.332) - (0.246 \pm 0.090 \times Day) + (0.201 \pm 0.017 \times pMY) - (0.084 \pm 0.014 \times Day^2) \quad [6]$$

$$RML_{RML2} = (9.203 \pm 13.888) - (0.246 \pm 0.090 \times Day) + (0.201 \pm 0.017 \times pMY) - (0.084 \pm 0.014 \times Day^2) \quad [7]$$

$R^2 = 0.921$; RMSE = 130.180.

Where: RML_{RML1} = residual milk loss during the recovery of mastitis level 1; RML_{RML2} = residual milk loss during the recovery of mastitis level 2; Day = days relative to mastitis event; and pMY = predicted milk yield.

To illustrate the RML during the drop and recovery of mastitis, as an example, we estimated the lactation curve and losses related to mastitis occurrence on days 25 and 250 of lactation (Figure 2). ML did not influence pMY, but it affected the intercept and Day (quadratically) parameters while estimating MY drop. On the other hand, for recovery estimations, ML did not affect pMY or Day, only the intercept. This result could be attributed to the more severe drop in MY for ML 2 on mastitis day, which accounted for 17 kg of milk, approximately 4.5 kg more milk than in ML 1. Thus, during the recovery, cows from ML 2 already begin their first day of mastitis with higher milk losses, taking more time to reestablish their MY.

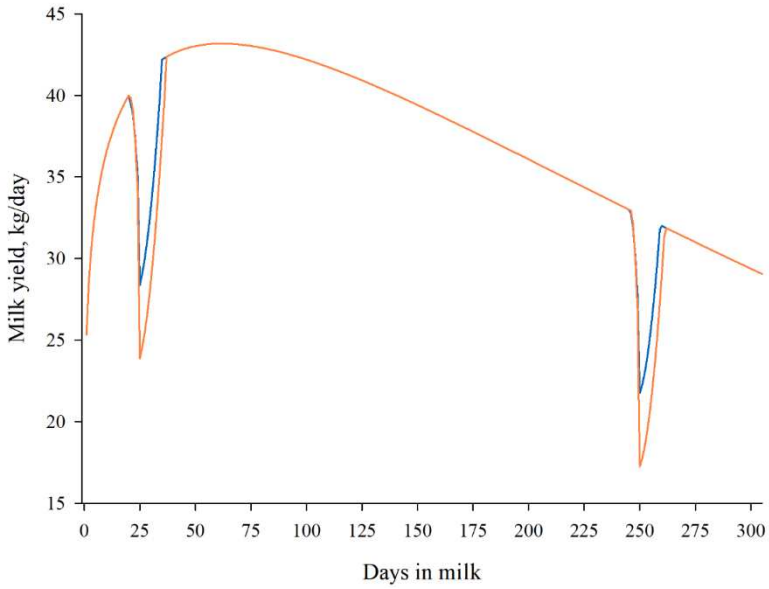

Figure 2. Lactation curve of Holstein cows diagnosed with clinical mastitis, level 1 (blue line) or 2 (orange line), in two different days of lactation (25 and 250 days in milk).

The model developed and presented here is strongly influenced by ML and MY, indicating that multiparous cows, closer to maximum MY, with a more severe level of mastitis, and producing more milk than their contemporaries, tend to present greater MY loss due to mastitis. Moreover, at the first third of the lactation curve, both ML 1 and/ or 2 can promote losses from four days before the mastitis diagnosis. However, moving on to the last third of the lactation curve, ML 2 can affect MY closer to the mastitis day. A light mastitis identified on day 25 promoted a MY loss of 95.8 kg from four days before until ten days after the day of mastitis; additionally, the same ML identified on day 250 promoted a reduction of 75 kg on MY within the same days (Figure 3). Nevertheless, a more severe mastitis occurring on lactation day 25 contributed to a sharper decrease in MY: a sum of 148.9 kg from four days before until 12 days after the days of mastitis. Another abrupt reduction in milk yield caused by ML 2 was also observed at 250 DIM; however, it was marked with 124.1 kg of milk loss relative to three days before to 12 days after the mastitis identification (Figure 3).

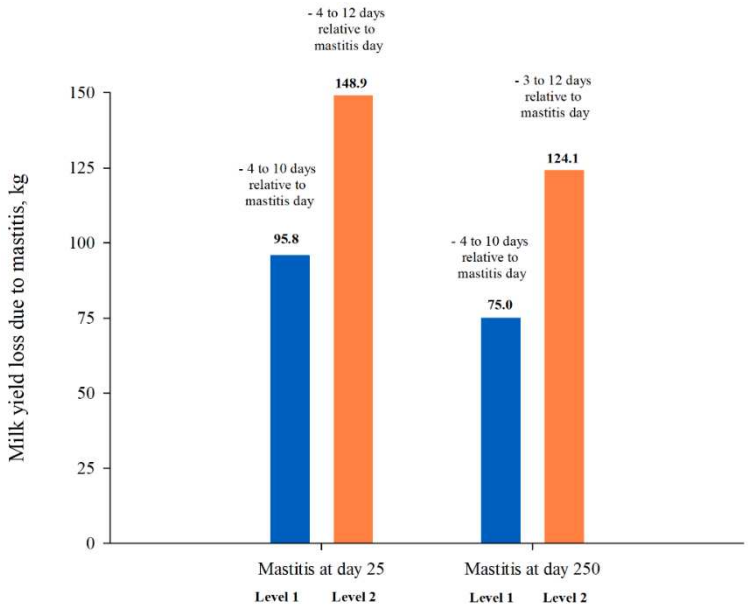

Figure 3. Milk yield drops, recovers, and losses caused by mastitis on days 25 and 250 of lactation. Blue columns represent mastitis level 1 and orange columns represent mastitis level 2.

Studies estimating milk losses due to mastitis did not assess when milk losses begin prior to mastitis onset and how much milk is lost. However, they agree that milk losses can occur two to four weeks before mastitis day and that their recovery can be prolonged until four weeks after the disease diagnose (Lescourret and Coulon,1994; Rajala-Schultz et al., 1999; Nielsen, 2009). Moreover, Nielsen (2009) observed that MY loss caused by mastitis could be affected by the severity of the infection, DIM, parity, and production level. Overall, severe cases of mastitis can cause long-term damage to the mammary tissue and, when occurring before lactation peak, clinical mastitis cases are likely to interfere with the differentiation of secretory cells, which would result in yield impairment through the lactation.

It is noteworthy that our study considers daily losses along the lactation period, which can be estimated as a total MY loss in the entire lactation. However, while considering the recovery phase, at a certain point, after 10 or 12 days, cows can return to the same pMY if they did not have that mastitis case. This mathematical estimation may conflict with the biological

assumptions of mammary impairment, mainly on the first third of lactation. Therefore, estimating the recovery rates after a period of severe mastitic infection can be more complex than milk drop itself, considering that there may exist long-term biological effects.

In short, if we consider the exemplified occurrence of two mastitis cases level 1, at the first and last third of lactation (25 and 250 DIM), we will have a total loss of 170.8 kg of milk; however, if these mastitis cases were level 2, according to our estimations the total loss would be of 273 kg of milk. Østergaard and Gröhn (1999) observed MY losses of 65 kg for primiparous and 117 kg for multiparous cows; however, Østergaard and Gröhn (1999) considered these values underestimated, since estimations did not include losses after five weeks from the mastitis day. Compared to our study, MY losses and days to recover are very distinct, as well as the ones observed by Puerto et al. (2021) and van Soest et al. (2016). These differences could be explained by the models used to estimate milk yield reduction, by the more intense decrease in MY, and by the faster recovery rate, allowing animals to reestablish their production in approximately two weeks, as suggested by our data.

To conclude, the two models developed here present daily estimations of MY losses before, at, and after mastitis diagnosis. The estimations suggest that milk drop occur three to four days prior to mastitis onset and can last until ten to twelve days from the diagnosis, which would be the necessary time for a cow to reestablish their pMY. In addition, MY loss estimated at mastitis day is greater than values referenced in the literature and are distinguished due to mastitis level. Therefore, the novelty of our study does not only indicate when and to what proportion mastitis drop and recovery occur, but it also brings new perspectives to mastitis modelling.

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NOTES

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5. GENERAL CONCLUSION

The main objectives of this thesis were to assess the effects of exogenous rbST on digestibility, growth, mammary gland development, carcass composition, reproductive characteristics, hormones and metabolites concentration, and gene expression of the cumulus-oocyte complex of pre-pubertal Holstein × Gyr heifers fed high gain diets; and to describe the impact of mastitis on milk production based on mastitis degree and moment of occurrence.

We conclude that the administration of bovine somatotropin in pre-pubertal Holstein × Gyr heifers fed for high daily gain rates does not improve growth parameters, feed efficiency, or final body weight. However, bST injections can increase the IGF1 serum concentration and, as a result, we can expect improved metabolism, mammary parenchyma growth, and lean carcass gain of heifers. Moreover, the use of rbST affected some reproductive characteristics, such as the size of ovaries and follicles and the gene expression of IGF1R in the COC. It did influence steroid hormone concentration and did not hasten the onset of puberty, nor the age, weight, wither, and rump heights at puberty. The evidence amassed in our study substantiates our hypothesis that the utilization of rbST could indeed overcome the detrimental effects of high gain diets on mammary gland growth, improve lean carcass gain and affect the reproductive traits of Holstein × Gyr heifers. However, due to the observed inconsistency in the results, we advocate for additional research to delve deeper into GH's role in reproductive characteristics, particularly focusing on gene expression of COC within the Holstein × Gyr heifer population since this breed holds significant productive and economic importance, particularly in tropical countries.

Regarding the description of mastitis incidence and impact on dairy herds, the two models developed presented daily estimations of MY losses before, at, and after mastitis diagnosis. The estimations suggest that milk drop occur three to four days prior to mastitis onset and can last until ten to twelve days from the diagnosis, which would be the necessary time for a cow to

reestablish their pMY. In addition, MY loss estimated at mastitis day is greater than values referenced in the literature and are distinguished due to mastitis level. Therefore, the novelty of our study does not only indicate when and to what proportion mastitis drop and recovery occur, but it also brings new perspectives to mastitis modelling.