UNIVERSIDADE VILA VELHA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA ANIMAL

IDENTIFICATION OF NOVEL SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS) IN THERMOTOLERANT HOLSTEIN COWS AND EVALUATION OF THESE ANIMALS' REPRODUCTIVE CAPACITY

TRACY FERREIRA LACERDA

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Dissertação apresentada à Universidade Vila Velha, como pré-requisito do Programa de Pós-graduação em Ciência Animal, para a obtenção do grau de Mestre em Ciência Animal.

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Aprovada em 29 de julho de 2016,

Banca Examinadora:

Mauricio Gomes Favoreto – UVV

Rute Beatriz Garcia Clemente Carvalho – UVV

Bárbara Loureiro – UVV
Orientadora

DEDICO...

À minha família que me ensinou que a união faz a força.

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LACERDA, T.F., Universidade Vila Velha – ES, julho de 2016. **IDENTIFICATION OF NOVEL SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS) IN THERMOTOLERANT HOLSTEIN COWS AND EVALUATION OF THESE ANIMALS' REPRODUCTIVE CAPACITY.** Orientadora: Bárbara Loureiro

Atualmente, o estresse térmico é um grande problema que afeta a indústria bovina em todo o mundo. Fatores ambientais, tais como temperatura, umidade, radiação e velocidade do vento podem causar uma incapacidade dos animais em manter as condições homeotérmicas e levar a manifestações patológicas de estresse térmico. No Brasil, onde as temperaturas médias, na maior parte do território, permanecem elevadas durante todo o ano, este é um problema que afeta massivamente os animais. O objetivo do presente estudo foi identificar novos SNPs em vacas da raça Holandesa consideradas termotolerantes e sensíveis ao estresse térmico, e avaliar a reprodução destes animais através da inseminação artificial (IA). Temperaturas vaginais pertencentes a 70 animais da raça Holandesa de duas propriedades diferentes foram aferidas usando um termômetro automático durante o período de verão no Brasil. Os animais foram classificados como termotolerantes (TT), intermediários (INT) ou termossensíveis (HS), dependendo da temperatura corporal. Amostras de pelo da cauda foram coletadas de todas as vacas para a extração de DNA, PCR e sequenciamento de DNA. Para a detecção de polimorfismos, foram utilizados primers a partir de um fragmento do gene ATP1A1 e dois fragmentos, Ex2/3 e Ex6/8 do HSP90AB1 gene. Os dados de temperaturas, produção de leite e número de IA foram analisados por análise de mínimos quadrados da variância utilizando o General Linear Models do SAS (SAS para Windows, Versão 9.2, Cary, NC). As diferenças nos valores médios individuais foram analisadas por meio de

comparação aos pares (probabilidade de análise de diferença [pdiff]; SAS). Dos animais, 44 foram classificados no grupo HS, 10 foram classificados no grupo INT e 16 foram classificados no grupo TT. A temperatura máxima registada em cada grupo foi maior (P<0,0001) para os animais no grupo HS, quando comparado com os grupos INT e TT. O CV medido para cada animal foi maior (P = 0,01) nas vacas HS em comparação com o grupo TT. A média de produção de leite não foi diferente entre os grupos. Não houve diferença estatística no número de Ias dentro dos grupos (HS, INT e TT); no entanto, houve uma correlação moderada e positiva (0,33; P <0,004) entre o CV da temperatura e número de inseminações. O intervalo entre partos não foi diferente entre os grupos. Três SNPs novos foram identificados na região do exon 19 do gene ATP1A1, e 4 novos SNPs identificados no exon 1, intron 2 e intron 6 do gene HSP90AB1. Os polimorfismos identificados na região de exon de ambos os genes provocou mutações não sinônimas. Em conclusão, os animais com menor CV requerem menos energia para a termorregulação e menos IA para ficarem prenhas. A produção de leite não foi diferente entre os grupos classificados, desse modo interferência na termorregulação dos animais não pôde ser estabelecida; o grau de sangue não interferiu nas variáveis estudadas. Novos SNPS foram encontradas em ambos os genes ATP1A1 e HSP90AB1, porem mais estudos são necessários, a fim de associá-los a termorregulação.

Palavras-chave: vaca, estresse térmico, reprodução, polimorfismos de base única.

ABSTRACT

LACERDA, T.F., University Vila Velha – ES, July 2016. **IDENTIFICATION OF NOVEL SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS) IN THERMOTOLERANT HOLSTEIN COWS AND EVALUATION OF THESE ANIMALS' REPRODUCTIVE CAPACITY.** Advisor: Bárbara Loureiro

Currently, heat stress is a major issue affecting the cattle industry worldwide. Environmental factors such as temperature, humidity, radiation, and wind speed can cause an inability to maintain homoeothermic conditions and cause pathological heat stress manifestations. In Brazil, where average temperatures, in most of the territory, remain high throughout the year, this is a problem that massively affects animals. The aim of the present study was to identify novel SNPs in Holstein cows considered thermotolerant and sensitive to heat stress, and evaluate these animals reproduction through artificial insemination (AI). Vaginal temperatures belonging to 70 Holstein animals from two different properties were gauged using an automatic thermometer during the summer period in Brazil. Animals were classified as thermotolerant (TT), intermediate (INT) or thermosensitive (HS), depending on body temperature. Tail hair samples were collected from all cows for DNA extraction, PCR and subsequent DNA sequencing. For polymorphism detection, primers from a fragment of the ATP1A1 gene and two fragments, Ex2/3 and Ex6/8 of the gene HSP90AB1 were used. Temperature, milk production data, and number of AI needed, were analyzed by least-squares analysis of variance using the General Linear Models procedure of SAS (SAS for Windows, Version 9.2, Cary, NC). Differences in individual mean values were analyzed through pair-wise comparisons (probability of difference analysis [PDIFF]; SAS). Of the animals, 44 were classified in the HS group, 10 were classified in the INT group and 16 were classified in the TT group. Maximum

temperature registered in each group were higher (P<.0001) for animals in the HS group when compared with the INT and TT groups. The CV measured in each animal was higher (P=0.01) in the HS compared to the TT group. Milk production mean was not different within groups. There was no statistical difference within groups (HS, INT and TT) regarding AI; however, there was a moderate and positive correlation (0.33; P<0.004) between temperature coefficient of variance and number of inseminations. The presumable calving interval was not different within groups. Three novel SNPs were identified in the exon 19 region of the gene ATP1A1, and 4 novel SNPs identified in exon 1, intron 2 and intron 6 of the HSP90AB1 gene. Polymorphisms identified in the exon region of both genes led to amino acid substitution. In conclusion, animals with lower CV require less energy for thermoregulation and less AI to get pregnant. Milk production was not different between classified groups so interference in the animals' thermoregulation could not be established; blood degree did not interfere in the studied variables. Novel SNPS were found in both ATP1A1 and HSP90AB1 genes, but further studies are required in order to associate them to thermoregulation.

Key words: cow, heat stress, reproduction, single nucleotide polymorphisms.

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1. INTRODUCTION

One of the issues that mainly influence the success of dairy farms is reproductive efficiency (SARTORI, 2007). High producing dairy cows are often subfertile. These animals' reproduction is decreasing due to an intense genetic selection for solid production, which led to a decrease in dairy herd fertility; in addition to a variety of physiological factors (LUCY, 2001), health issues (HERNANDEZ et al., 2012), as well as managing factors such as nutrition (LUCY, 2001; CHAPINAL et al., 2012), and heat stress (HANSEN, 2009), having an additive effect on reproductive efficiency.

Currently, heat stress is a major issue affecting the cattle industry worldwide (SILVA et al., 2013). Environmental factors such as temperature, humidity, radiation, and wind speed can cause an inability to maintain homoeothermic conditions and cause pathological heat stress manifestations (CARROLL et al., 2012). In Brazil, where average temperatures, in most of the territory, remain high throughout the year, this is a problem that massively affects animals (ROCHA et al., 2012). The economic losses due to infertility are high. Each pregnant cow yields the property a profit of $US 278, as an abortion generates a loss of $US 555 (DE VRIES, 2006). It should be noted that, the offspring of bulls selected for high milk production, are also more thermosensitive, so the continued selection for milk production can consequently lead to high susceptibility to heat stress (BOHMANOVA et al., 2005).

The aim of the present study was to identify novel SNPs in Holstein cows considered thermotolerant and sensitive to heat stress, and evaluate these animals' reproduction through artificial insemination.

2. LITERATURE REVIEW

2.1 Heat stress consequences in the cow

Reproductive disturbances due to heat stress may occur through two general mechanisms (HANSEN, 2009): 1) reproductive function may become impaired due to the homeokinetic changes that occur for body temperature regulation, such as blood flow redistribution to the body's periphery in order to increase sensible heat loss, as well as reduced consumption during heat stress. Feed intake reduction lowers metabolic heat production (WEST, 2002), but can also lead to alterations in energy balance (LUCY, 2001) and alter nutrients available, which may lead to cyclicity, pregnancy establishment, and fetal development issues (HANSEN, 2007; SENOSY et al., 2011) and; 2) homeokinetic system failure in reproduction regulation; increased body temperature can impair germ cell function, embryo in early development, as well as other reproduction cells.

Fertility reduction has been noted in a number of mammals following subsequent exposure to high temperatures. In dairy cows, conception rate prior to artificial insemination can range from 55% during months where ambient temperatures and humidity are low to less than 10% during months where temperatures and humidity are high. Reduction in estrus duration and intensity, and increased incidence of anestrus and silent ovulation, are caused due to adverse environment (BADINGA et al., 1993; KADOKAWA et al., 2012).

The luteinizing hormone (LH) plasma concentration, which is necessary for the dominant follicle's complete development, may reduce under high environmental temperatures. Dominant follicles developed in a reduced LH environment may have their final development and differentiation adversely affected (GUZELOGLU et al., 2001). Growth and development of ovarian follicles, which can be precisely monitored with the aid of an ultrasound, are also affected by elevated temperatures

(BADINGA et al., 1993). And there being an additive effect of thermal stress and increased milk production in reduced conception rates.

The pre-ovulatory follicle is imperative when it comes to the reproductive system and this functions deterioration may influence other reproductive events, such as gonadotropin secretion and subsequent development of the corpus luteum and embryo (GUZELOGLU et al., 2001). Heat stress affects follicular development by decreasing the quantity of follicle-stimulating hormone (FSH) receptors in granulosa cells (SHIMIZU et al., 2005) and by repressing the aromatase activity in these cells, following a low estradiol production capacity by these follicles (WOLFENSON et al., 1997; SHIMIZU et al., 2005). The first follicular wave dominant follicle is smaller in diameter and normally has a smaller quantity of follicular fluid in cows in lactation and suffering heat stress (BADINGA et al., 1993; GUZELOGLU et al., 2001).

Impaired follicles evolving in the ovaries of cows under thermal stress continue to grow. Subordinate follicles were seen in smaller sizes in cows suffering heat stress during the first follicular wave (WILSON et al., 1998; ROTH et al., 2000). Supposedly, these impaired follicles give rise to subfertile oocytes for numerous months after heat stress reduction (ROTH et al., 2001; AL-KATANANI et al., 2002).

In addition to altering the ovarian follicles, heat stress may also affect the corpus luteum expanding the luteal phase. This setback is due to reduced estradiol secretion by the dominant follicle. After stress discontinuation, cows present luteolysis and follicle development restarts normally, but oocyte improvement takes place a lot later (ROTH et al., 2002).

The effects of heat stress on reproductive functions are intensified by the upturn in metabolic heat production combined with lactation (SARTORI et al., 2002) and elevated humidity (WEST, 2003). Lactating cows are more vulnerable to elevated temperatures than heifers, because the metabolic heat originated in

lactating cows promotes hyperthermia during the hot season, while in heifers this heat production is smaller and, these animals most likely do not present hyperthermia under the same ambient conditions as cows (WILSON et al., 1998). This loss in the reproductive capacity of milk cows in the hotter seasons is correlated with reduction in body thermoregulation and genetic selection of the animals for higher milk yield (ROTH, 2008).

2.2 Heat stress consequences in embryo survival

Embryonic loss is one of the most important factors interfering in reproductive performance (CHEBEL et al., 2004; DISKIN et al., 2012). A decisive factor in embryonic development is the uterus's and oviduct's microenvironment. Disorders in such environments can cause alterations in cellular function and development failures. In dairy cows in lactation, in which the lactating metabolic requirements aggravate fertility loss (AL-KATANANI et al., 1999), stress caused by elevated temperatures disturbs embryonic development *in vivo* (PUTNEY et al., 1988; EALY et al., 1993). There are presumably innumerous motives for reduced embryo survival during stress, for example cutback in uterine blood flow and reduced hormonal secretion (WOLFENSON et al., 2000). Additionally, heat stress can cause a direct effect on the embryo, leading to alterations in its development (EDWARDS & HANSEN, 1997; SUGIYAMA et al., 2003; SAKATANI et al., 2004).

During the follicular growth period, heat stress may compromise the oocyte, either by direct action of high temperatures or due to changes in follicular function which might compromise their quality (HANSEN & ARECHIGA, 1999). Some studies show that the quality of oocytes collected from cows during the summer have a greater expression of genes related to apoptosis, which leads to an impairment in embryo development (CHEBEL et al., 2004; DISKIN et al., 2012).

Cows who suffer from heat stress prior to artificial insemination present low pregnancy rates (PUTNEY et al., 1988). Heat stress consequences in embryos are not visible until the late stages of development. Fertilized sheep and cow oocytes, when situated under elevated temperatures, *in vitro* and *in vivo*, suffer impairment; still, their development progresses, coming to die only in the critical stages of implementation (THATCHER et al., 2001). Most of the consequences due to heat stress on the oocyte and embryo function includes the direct effects of high temperatures on cellular function (HANSEN, 2015). Heat stress causes most harm to embryo survival when it takes place prior to the blastocyst stage (EALY et al., 1993; DEMETRIO et al., 2007). It has been shown, *in vitro,* that embryos are specifically affected by elevated temperatures when they are in the 2-cell stage, having a moderate effect on embryos with 4-8 cells, and minimum or absent effect on the development of morulae (EALY et al., 1995; EDWARDS & HANSEN, 1997). Nonetheless, when embryos are administered to elevated temperatures, *in vitro* as well as *in vivo,* up until its 7th day of development, these present reduced pregnancy ratios at day 30 (DEMETRIO et al., 2007; SILVA et al., 2012) and greater percentages of embryo loss at day 42 of gestation (DEMETRIO et al., 2007), indicating the temperature's vestigial effect regarding embryonic and fetal survival (LACERDA & LOUREIRO, 2015).

In comparison to most cells, embryos are especially susceptible to temperature changes. A temperature elevation to 41°C for 4.5 hours is enough to cause a reduction in the amount of embryos that progress in culture medium (KRININGER et al., 2002; HERNANDEZ-CERON, 2004). A further factor that does not directly affect the embryo, but still compromises its survival consists of reduced blood flow to the uterus (ROMAN-PONCE, 1978) compromising nutrient and hormone delivery to this organ (HOWELL et al., 1994). In addition, the embryo loses

the ability to inhibit uterine PGF2a (JAINUDEEN & HAFEZ, 2000), leading to premature embryonic loss (THATCHER et al., 2001).

Heat stress also reduces plasma estradiol concentrations, effect consistent with low concentrations of LH as well as reduced follicle dominance (RENSIS & SCARAMUZZI, 2003), and as a consequence to alterations in the follicular development, the subsequent corpus luteum and therefore progesterone (P4) concentrations may be impaired (WOLFENSON et al., 1995). The conceptus' development depends directly on P4 concentration, for it acts on pregnancy recognition and also in embryo implantation (LONERGAN, 2011). Studies show that there is a direct relationship between plasma progesterone concentrations and the possibility of embryo survival (DISKIN et al., 2006). One likely effect of low progesterone concentrations is premature oocyte maturation, compromising embryonic development after fertilization (DISKIN et al., 2012).

2.3 Thermotolerance in Holstein cows

Thermotolerance is defined as maintenance of rectal temperature below 39.1 °C, due to this temperature being considered the threshold for heat stress in dairy cows (BERMAN et al., 1985; WEST, 2003). Body temperature is managed through modulation of the metabolic heat produced as well as heat lost by the body (SAILO et al., 2015). The evaporation mechanism happens through water loss by respiration as well as evaporation through sweat glands. Heat stress resistance relies on the maximum and minimum temperatures that determine the animal's thermal comfort. The maximum ambient temperature for dairy cows varies from 25-28.4 °C; exceeding this range the animal's body temperature starts to elevate (BERMAN et al., 1985; DIKMEN & HANSEN, 2009).

The animal's ability to withhold its body heat may impact both pregnancy rate and maintenance (VASCONCELOS et al., 2006) and this capacity can be seen even in dairy animals of high production. In a recent study, VASCONCELOS et al. (2011) showed that high producing Holstein cows (>35 kg/day), which managed to maintain rectal temperatures inferior to 39 °C, demonstrated pregnancy rates after embryo transfer, as good as animals with low milk production (< 35 kg/day).

It is unquestionable that *Bos indicus* (zebu) possess a better thermoregulatory capacity than *Bos taurus* (European). According to BÓ et al. (2003), Indian breeds are more resistant to environmental stressors, making the creation of crossbreds an interesting option for milk production in Brazil. In a study, BEATTY et al. (2006) comparing the effects lead by heat stress in *Bos taurus* and *Bos indicus*, found that *Bos indicus* had a higher heat tolerance, better feed intake, lower rectal temperatures, and lower respiratory rates in periods of heat stress. Yet, studies with Holstein animals (*Bos taurus*) have demonstrated that some individuals belonging to this breed can experience heat stress and not show severe fertility damage (VASCONCELOS et al., 2011). The ability to maintain low body temperature during heat stress shows a moderate hereditability (SEATH 1947; DIKMEN et al., 2012); and therefore selection for thermoregulation should reduce heat stress consequences (DIKMEN et al., 2015).

According to SAILO et al. (2015) heat stress is genetically managed and heat shock proteins (Hsp) influences stress in animals. Heat shock proteins are categorized into families (HSP70, HSP90, HSP110, among others) based on their molecular weight and play an important role in normal physiological conditions as well as in systemic and cellular stress situations in all living organisms (KREGEL, 2002).

Although synthesis of these proteins occurs in response to stress, mechanism shown by most cells; animals differ individually in relation to their capacity to manage this stress, due to naturally occurring nucleotide alterations and therefore demonstrating different levels of thermotolerance (BASIRICÓ et al., 2011).

Heat stress resistance/tolerance has been associated to single nucleotide polymorphisms (SNPs) in genes involved in the response to heat stress or those responsible for homeostasis (RAVAGNOLO & MISZTAL, 2002). SNP markers are based on alterations in the DNA molecule, or mutations in a single base of the nitrogenous base chain (adenine, cytosine, guanine, and thymine), where the most common mutations are transitions, occurring exchanges of a purine by another purine (A-G) or a pyrimidine by another pyrimidine (C-T) (CAETANO, 2009).

Studies have demonstrated the connection between genetic polymorphisms and response to heat stress in dairy cows (CHRENCK et al. 2003; LIU et al., 2011), as well as their relation to reproduction (ORTEGA et al., 2016) and production (COCHRAN et al., 2013). Recently, DIKMEN et al., (2015), using Holstein cows identified genetic markers associated to genetic variations in thermoregulation during heat stress. Holstein heifers with a polymorphism in the intron 3 of the HSP90AB1 gene showed an upgrade considering thermotolerance (CHAROENSOOK et al., 2012). A new SNP was found in the Exon 3 of the HSP90AA1 gene in Sahiwal cows, where animals with AA genotype showed a lower heat tolerance coefficient (HTC) followed by animals with genotypes AG and GG, in which higher HTC values indicate animals more sensitive to heat stress (KUMAR et al., 2015). A polymorphism was seen in the gene HSF1 belonging to Holstein cows, and in an association study, presented an improved thermotolerance in these animals (LI et al., 2015). Holstein cows with polymorphisms in the gene ATP1A1 that encodes for the Na+,K+-ATPase, which maintains the Na/K electrolyte balance, also showed greater thermotolerance

(LIU et al., 2011). Acorrding to LIU et al. (2010), among various candidates in selection for dairy cows, Na+,K+-ATPase is a potential candidate for anti- heat stress in these animals. The obtainment of SNPs (C/- and G/T) in the 5' region of the gene HSP70.1 improves the response and heat stress tolerance in mononuclear cells belonging to Holstein cows (BASIRICO et al., 2011). A SNP located in the 5' region of the HSP70 gene was attributed to apoptosis in lymphocytes. The expression of BCL2 and HSP70 were notably higher in cows with polymorphism, as well as the apoptosis percentage (CAI et al., 2005). The data shows that it is possible to select animals for the phenotype thermotolerance using molecular markers.

The ability to maintain body temperature even under thermal stress is a valuable resource for dairy cattle in tropical and subtropical countries (GANAIE et al., 2013). Although variations in thermal tolerance between breeds and between crossbreeds have been studied (BEATTY et al., 2006), few studies have attempted to understand the mechanism involved in thermotolerance and heritability of this trait. The data indicates that there is a genetic variation in Holstein cows regarding thermotolerance and this feature can be included in these animals' selection criteria if a genetic marker is identified. However, studies relating thermotolerance, SNPs, and the reproductive capacity of these animals have not been shown.

3. MATERIAL AND METHODS

3.1 Animal classification as for thermoregulation

The experiment was conducted during the months of January to March (summer in Brazil) of the years 2014 and 2015, using 70 lactating Holstein cows with blood degree varying between 75 to 100 % Holstein crossbreed with Gir, cows had no apparent reproductive diseases (uterine infection, retained placenta, mastitis), and were animals with 30-336 (mean 96.9) days postpartum and body condition score

between 3 and 3.5. The animals were from two different properties, one in Santa Teresa- ES and another in Venda Nova do Imigrante- ES, Brazil, however all animals were kept in a free-stall under the same conditions, in sheds with ventilators and sprinklers. To measure the body temperature of each cow an automatic thermometer (iButton Maxim, California, USA) coupled to a placebo intravaginal implant was used, which was kept in each cow for 3 days gauging the temperature every 5 minutes. Between each reuse the implants were washed with a chlorhexidine solution and autoclaved. On the day of insertion the implants were sprayed with oxytetracycline along with hydrocortisone (Terra-Cotril, Pfizer, São Paulo, Brazil).

Classification was as follows: thermotolerant (TT) animals, those who showed no temperature event (at least 30 minutes) above 39 °C; intermediate (INT), animals showing one or more events from 39.1 to 39.5 °C; and thermosensitive/heat stressed (HS) those in which the temperature exceeded 39.5 °C for at least one event of a half hour. Furthermore, the coefficient of variation of the temperatures within all the measurements from each animal was calculated as the ratio of the standard deviation to the mean. The highest temperature registered in each animal within the three days was considered in the analysis.

Each animal's mean milk production on the day of implant insertion was registered.

A thermometer was maintained in the cow shed under the shade throughout the experiment for ambient temperature verification.

The animals used in the experiment were inseminated (AI) without hormonal synchronization, 12 hours after presenting estrus signs. Semen from 29 bulls belonging to Holstein and Gir breeds were used. After 30 days pregnancy diagnosis was performed through ultrasound examination. All animals diagnosed as non-pregnant or animals showing estrus before the ultrasound examination were

inseminated again, until diagnosed pregnant. The number of inseminations necessary, since the last calving, for each animal to be diagnosed pregnant was calculated.

3.2 DNA extraction, PCR and sequencing

Tail hair samples were collected from all cows used in the experiment. The genomic DNA extraction was performed using the DNeasy® Blood & Tissue kit (Qiagen, Venlo, Netherlands); first, hair samples were cut so that only the bulbs were used (0.1 mm), they were placed in 1.5 ml microtubes and 180 µl of ATL buffer and 20 µl of proteinase K were added followed by a 15 second vortex and centrifugation at 8000 rpm for 1 minute. These samples were placed in an incubator at 56 °C. After 2 hours a manual maceration was performed using a polypropylene pistil with a cone shaped extremity, followed by a vortex and centrifugation, and again left in the incubator overnight. The following day, the hair samples were macerated again and 5 µl of proteinase K were added to each of the samples, they were then vortexed for 15 seconds followed by a centrifugation at 8000 rpm for 1 min and placed back in the incubator for 2 hours at 56 °C. The following steps for extraction performance were according to the manufacturer's instructions; 200 µl of Buffer AL were added followed by a 15 second vortex, and again samples were placed in the incubator. After 10 minutes, 200 µl of ethanol (96-100%) were added to the microtube, followed by a further vortex. Subsequently, the mixture, without hair follicles, was placed in the DNeasy Mini spin column and centrifuged at 8000 rpm for 1 minute. The liquid was then discarded and the column placed back into the collection tube. The buffer AW1 (500 µl) was added to the column followed by 500 µl of buffer AW2, with a 1 minute centrifugation and liquid discard between the two additions. After addition of the buffer AW2 a 3 minute centrifugation at 14000 rpm was performed followed by

disposal of the liquid as well as the collection tube. The column for each sample was placed into a new clean tube, where 100 µl of buffer AE were added followed by a 1 minute wait and centrifugation at 8000 rpm for one minute, this step (addition of the buffer, 1 minute wait, and centrifugation) was repeated; finally, the liquid from each sample was then transferred into new 1.5 ml microtubes. The quality and quantity of the extracted DNA was verified on 1% agarose gel, stained with SYBR® Safe (Invitrogen, São Paulo, Brazil) and visualized under an ultraviolet light.

The primers used for polymorphism detection are shown in Table 1. Polymerase chain reactions (PCR) were performed using a total volume of 25 µl (genomic DNA, dNTP mix, Taq-polymerase enzyme (LongRange PCR enzyme mix-Qiagen), $MgCl_{2,}$ and specific primers for each gene). Amplification was performed in a Apollo ATC 201 (Nyx Technik) thermocycler, consisting of initial denaturation at 93 °C for 3 minutes followed by 35 cycles of denaturation at 93 °C for 15 seconds, annealing at 55 °C for 30 seconds (HSP90AB1) or 50 °C for 30 seconds (ATP1A1) and extension at 68 °C for 3 minutes. The PCR products were electrophoresed on 1% agarose gel with SYBR® safe (Invitrogen) for visualization of the fragment sizes. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). The purified products were visualized and quantified on 1% agarose gel, and finally, sent to the Multiuser Genotyping and sequencing Laboratory - LMGs of the University of Campinas for sequencing.

Table 1. Primers used for polymorphism detection of genes ATP1A1 and HSP90AB1.

Gene	Sequence (5' – 3')	Exon	Reference
ATP1A1	F1: AGT GCT GCG TGA AAC CTG	16	LIU et al., 2011
	R1: GTG ATG TGT GGA ATG TGT GC	16	LIU et al., 2011
HSP90AB1	F1: CCTGGATTGGAATGCCTAAC	2	CHAROENSOOK et al., 2012
	R1: TCAGGCTCTCATAGCGAATC	3	CHAROENSOOK et al., 2012
	F2: TCACCCAGGAGGAATATGGAG	6	CHAROENSOOK et al., 2012
	R2: AGAAGGACCGATTTTCTCACC	8	CHAROENSOOK et al., 2012

The obtained sequences were analyzed and edited using the program BioEdit Software (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). Sequence alignment was performed using the virtual tool *Clustal Omega*, available online at http://www.ebi.ac.uk/Tools/msa/clustalo/. The sequences obtained from experimental animals were aligned against the sequences available in the NCBI data bank for species *Bos taurus* (GenBank accession No. NM_001079637.1 and NM_001076798.1) and *Bos indicus* (GenBank accession No. FR847225.1 and KF286658.1), considering the genes HSP90AB1 and ATP1A1 respectively.

3.3 Statistical analysis

The effect of group (HS, IN or TT), blood degree and its interactions on the mean and maximum animal temperature, the coefficient of variation within each temperature measured, milk production, number of IA and calving interval were

analyzed by least-squares analysis of variance using the General Linear Models procedure of SAS (SAS for Windows, Version 9.2, Cary, NC). Differences in individual mean values were analyzed through pair-wise comparisons (probability of difference analysis [PDIFF]; SAS). To exclude the effect of sire on pregnancy rate a logistic regression analysis using the logistic procedure of SAS was performed.

The use of temperature coefficient variation as a parameter to analyze animal thermoregulation has been reported in the literature in only two articles (KOGA et al., 2004; GOURDINE et al., 2016), both use correlation analysis to evaluate its effect on specific traits. Here we analyzed the correlation between temperature coefficient of variation and number of inseminations using the correlation procedure of SAS.

All values are reported as the least-squares mean ± SEM. Differences were considered significant when $P < 0.05$, and values of $P \geq 0.05$ and ≤ 0.1 were taken to indicate a tendency.

4. RESULTS

4.1 Ambient temperatures

The minimum and maximum temperatures in the sheds where the animals were maintained throughout the study period were 19.7 °C and 36.1 °C respectively, with a mean temperature of 28.2 °C.

4.2 Reproductive and production traits

From the 70 animals used in this study, 44 were classified in the HS group, 10 were classified in the INT group and 16 were classified in the TT group.

The mean temperature measured during the three days that the thermometers were kept in the animals and the maximum temperature registered in each group were higher ($P<.0001$) for animals in the HS group when compared with the INT and TT groups. The INT group was not different from the TT group (Figure 1 and 2).

The coefficient of variance measured in each animal was higher (P=0.01) in the HS group when compared with the TT group. There was a tendency (P=0.064) for the INT group to be higher than the TT group (Figure 3).

The milk production mean was not different within groups (Figure 4).

The number of inseminations needed for the cow to get pregnant is showed on Figure 5. There was no statistical difference within groups (HS, INT and TT); however, there was a moderate and positive correlation (0.33; P<0.004) between temperature coefficient of variance and number of inseminations (Figure 6).

The presumable calving interval was not different within groups (Figure 7). Difference in blood degree did not influence any of the variables studied nor did sire influence pregnancy rates.

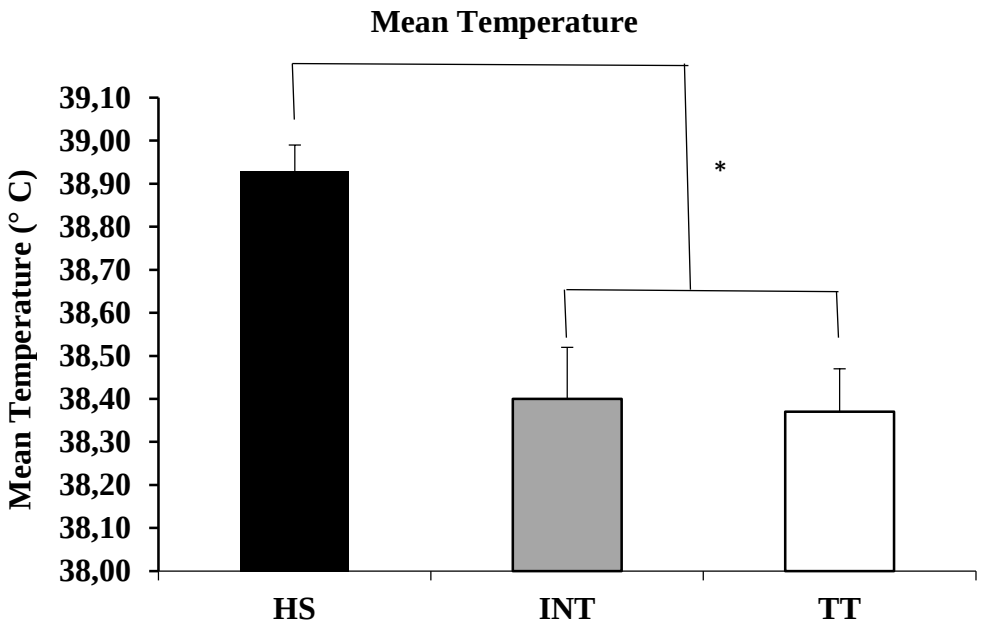

Figure 1. Mean temperature (°C) observed during the three days that the thermometers were maintained in the animals of each classified group (HS, INT and TT). Mean temperatures were higher (P<.0001) for animals in the HS group when compared with the INT and TT groups. The INT group was not different from the TT group. Differences were considered statistically significant when P<0.05.

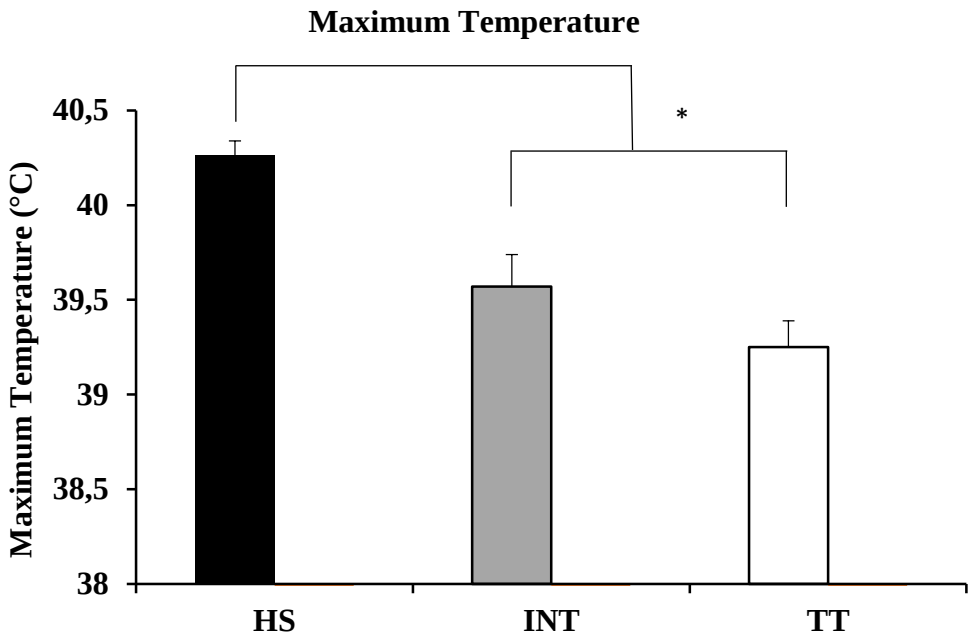

Figure 2. Mean maximum temperatures (°C) observed during the three days that the thermometers were maintained in the animals of each classified group (HS, INT and TT). Maximum temperatures were higher (P<.0001) for animals in the HS group when compared with the INT and TT groups. The INT group was not different from the TT group. Differences were considered statistically significant when P<0.05.

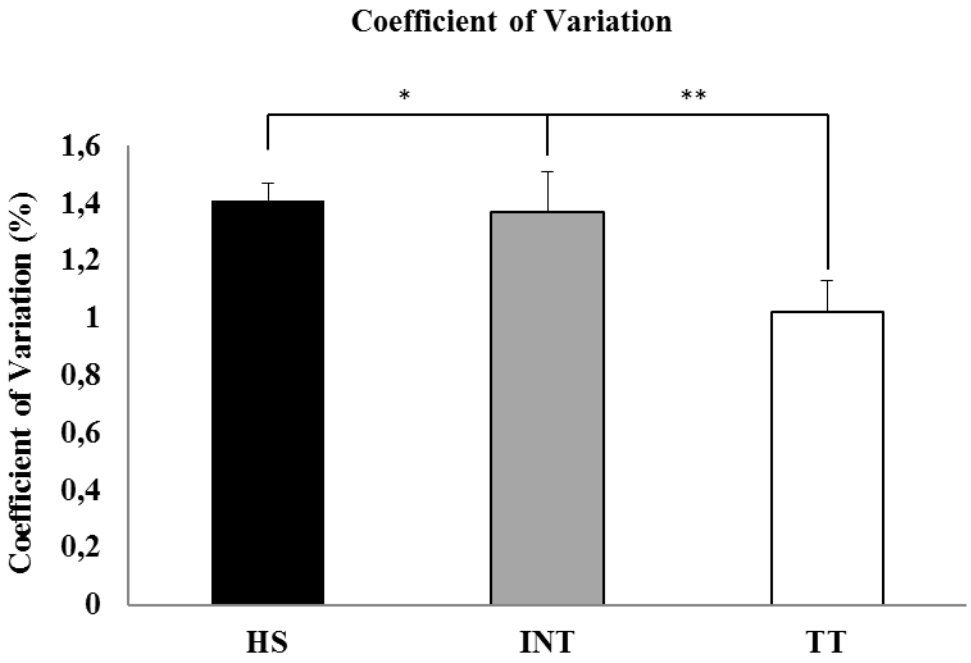

Figure 3. Coefficient of variance (%) in relation to each classified group (HS, INT and TT). Coefficient of variance measured in each animal was higher (P=0.01) in the HS group when compared to the TT group. There was a tendency (P=0.064) for the INT group to be higher than the TT group. Differences were considered statistically significant when P<0.05.

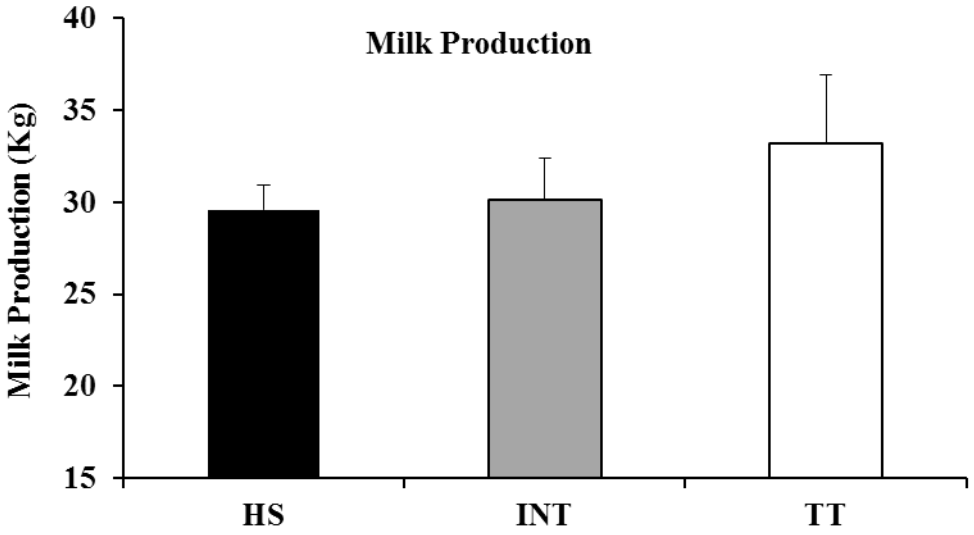

Figure 4. Mean milk production (Kg) observed in relation to each classified group (HS, INT and TT). No statistical difference was observed within groups. Differences were considered statistically significant when $P<0.05$.

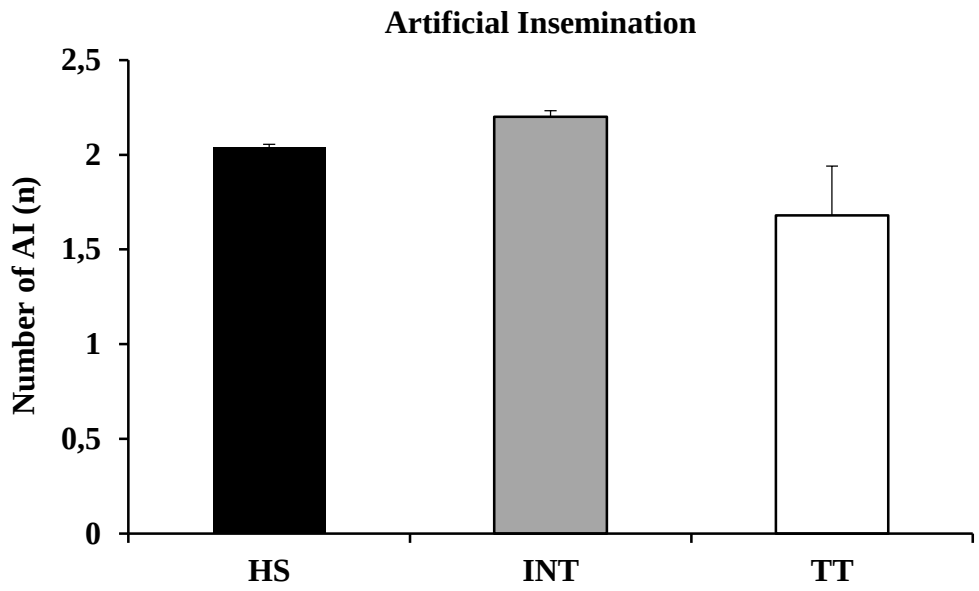

Figure 5. Number of inseminations needed for pregnancy diagnosis in each classified group (HS, INT and TT). No statistical difference was observed within groups. Differences were considered statistically significant when $P<0.05$.

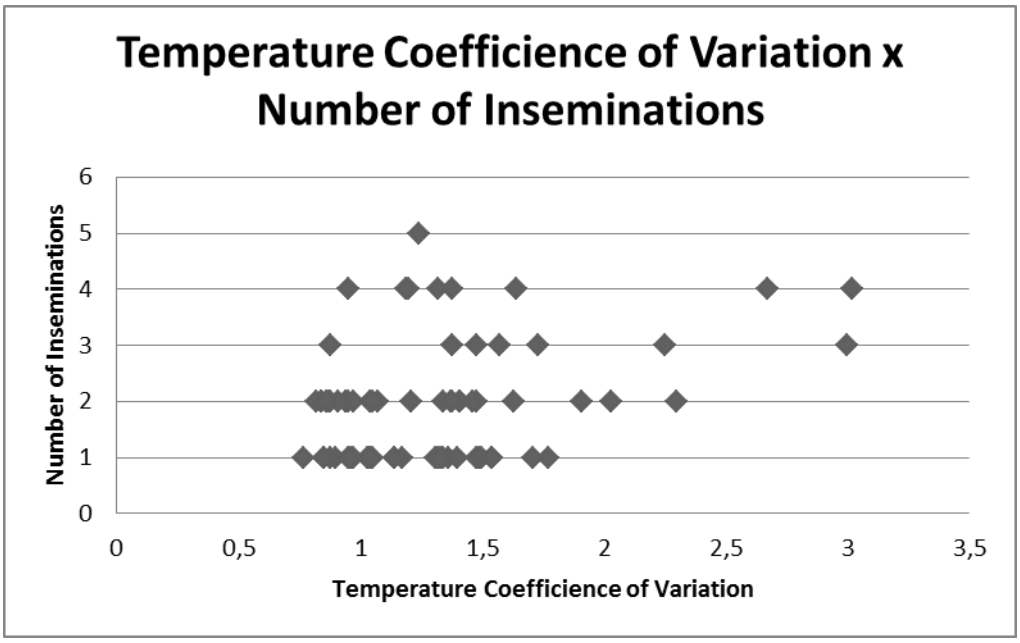

Figure 6. Correlation observed between temperature coefficient of variance and number of inseminations. Data shows a moderate and positive correlation (0.33; P<0.004). Differences were considered statistically significant when P<0.05.

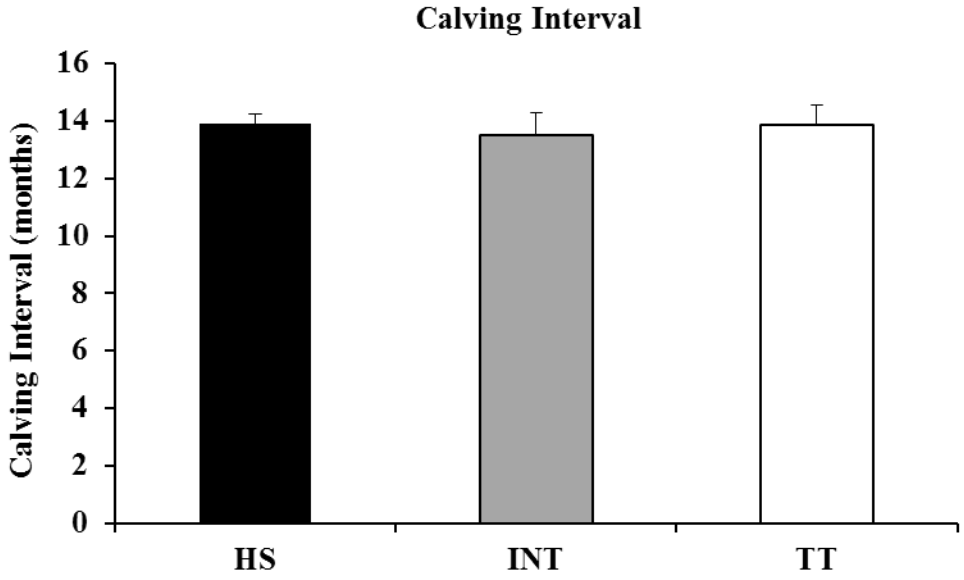

Figure 7. Calving interval (months) observed in relation to each classified group (HS, INT and TT). Presumable calving interval was not different within groups. Differences were considered statistically significant when $P<0.05$.

4.3 Polymorphisms

4.3.1 ATP1A1

Sequences from sixty animals were used to compare exon 18-19 of the gene ATP1A1 with the *Bos taurus* and *Bos indicus* sequences.

Three novel polymorphisms were identified in exon 19 of the ATP1A1 gene when the animals of the experiment were compared with the *Bos indicus* and the *Bos taurus* sequences. The first polymorphism was a G-A mutation at the nucleotide position 116,400,962 of the gene mRNA. This polymorphism was identified in five pure breed Holstein animals; four classified as HS and one as INT. This mutation represents an alteration from the amino acid aspartate to asparagine.

Two other polymorphisms in exon 19 of the ATP1A1 gene were identified in the 60 cows sequenced, ranging from 75 to 100 % Holstein blood, in which 36 were classified as HS, 10 as INT, and 14 animals classified as TT. These polymorphisms

were identified in nucleotide positions 116,400,985 and 116,400,990 being both a C-G mutation; however only being observed when compared to the *Bos indicus* sequence. Both polymorphisms led to an amino acid alteration, the first being an alteration from aspartate to glutamate and the second from threonine to serine.

4.3.2 HSP90AB1

Sequences from 63 animals were used to compare exons 1- 3 and 6-8 of the gene HSP90AB1 with the *Bos taurus* and *Bos indicus* sequences available in the NCBI data bank.

In nucleotide position 233 of exon 1 belonging to the HSP90AB1 gene, an A-T mutation was observed when compared to the *Bos indicus*; this polymorphism was seen in one pure breed Holstein animal classified as HS and led to an alteration from the amino acid tyrosine to phenylalanine.

In nucleotides 409 to 682, intron 2 of the same gene, the 63 animals (41 HS, 9 INT and 13 TT) with sequencing results showed 100% homology with *B. indicus* and 41% with *B. taurus.* Still referring the intron 2 region of this gene, in nucleotide positon 646, a A-G mutation was identified when compared to both *Bos indicus* and *Bos taurus*, in two animals (1 HS with 75% Holstein blood and 1 INT pure breed Holstein), in which these animals presented a 99% homology with *Bos indicus* animals and 40.6% with *Bos taurus*.

Furthermore, referring the gene's intron 6 region, 26 animals presented a G-A mutation at nucleotide position 1872 in relation to the *Bos indicus* sequence, where 14 animals where classified as HS, 4 animals as INT and 8 as TT.

From nucleotides 1833 to 1899 and 2200 to 2352, these same animals showed a homology of 100% with *Bos indicus* as well as with *Bos taurus* animals. Still in intron 6 of the HSP90AB1 gene, in nucleotide position 1918, nine individuals

(5 HS and 4 TT) showed a G-A mutation when compared to the *B. indicus* sequence. Two further mutations, both T-C, where observed in nucleotides 2017 and 2968, being the first seen in 2 HS animals, and the second (nucleotide 2968) seen in one TT animal.

5. DISCUSSION

5.1 Reproduction and production traits

A dairy cow that maintains its body temperature below 39.1 °C is considered thermotolerant (WEST, 2003), when the ambient temperature exceeds 25-28.4 °C the animal's body temperature begins to elevate (BERMAN et al., 1985; DIKMEN & HANSEN, 2009). The cows in this study were kept in a shed with ventilators and sprinklers to help keep the animals' body temperatures at a comfortable range; however during the summer the temperatures within the shed varied from 19.7 °C to 36.1 °C, explaining the increase in body temperature in the animals used here.

In this study, vaginal temperature was used to classify the cows as TT, INT or HS. Cows that had at least one event (minimum of 30 minutes) with temperatures above 39.5 °C were classified as HS. These cows also had a higher mean temperature and higher maximum temperature during the three days that the thermometer was kept in the animal.

Body temperature is managed through modulation of the metabolic heat produced as well as heat lost by the body (SAILO et al., 2015). Little variation in body temperature, expressed in this study as coefficient of variance (CV), even during high dairy producing periods, leads to low energy requirements for body temperature regulation (GOURDINE et al., 2016). The coefficient of variance results presented here, suggests that the animals classified as TT require less energy for

thermoregulation than the animals classified as HS. KOGA et al. (2004) showed that Brahman breed cattle presented a lower coefficient of variance and lower skin and rectal temperatures than buffaloes. In sows, those who presented low variation regarding thermoregulation responses presented high lactating performances (GOURDINE et al., 2016). In the present study, it was shown that cows with a higher coefficient of variation regarding temperature also needed more inseminations to get pregnant. However, the classification of the cows as HS, INT or TT based on the temperature measurements did not interfere on the number of inseminations needed for pregnancy. Maximum body temperature as well as body temperature variation (CV) are used in studies to indicate heat stress, and were both used in the present study, however, CV seems to be a better representative for HS since it indicates energy requirements for thermoregulation; which is managed through modulation of the metabolic heat produced as well as heat lost by the body.

High producing dairy cows are more vulnerable to high ambient temperatures due to the hyperthermia caused by the metabolic heat derived from lactation (SARTORI et al., 2002; ROTH, 2008). However, VASCONCELOS et al. (2011) showed that cows with lower rectal temperatures showed better pregnancy rates after receiving an embryo during the summer season, despite being high or low milk producing cows. The cows used in the present study are high producing animals with a milk production mean of 31 kg/day; however milk production did not differ between TT, INT and HS groups nor had any correlation with the variables studied, therefore we can consider that thermoregulation is not due to low milk yield in the TT group.

5.2 Polymorphisms

Single nucleotide polymorphisms are single nucleotide mutations in the genome, where the most common mutations are transitions, occurring alterations of a purine by another purine or a pyrimidine by another pyrimidine (CAETANO, 2009). In farm animals, these polymorphisms have been known to alter animal health, production and reproduction, constituting biodiversity and individual variability in response to environmental factors (IBEAGHA-AWEMU et al., 2008).

Single nucleotide polymorphisms can occur in two regions, the coding (exon) and the noncoding (intron) regions of the DNA; being most commonly seen in noncoding regions. In the present study, novel SNPs where found in both coding and noncoding regions. LIU et al. (2011), studying 160 Holstein cows observed a novel C-A polymorphism at the nucleotide position 2789 of the ATP1A1 gene, however this SNP did not alter amino acid. In another study, Holstein heifers with a polymorphism in the intron 3 of the HSP90AB1 gene showed an upgrade considering thermotolerance (CHAROENSOOK et al., 2012). When in the noncoding regions, these SNPs do not affect encoded proteins; however, they can alter gene *splicing* consequently altering gene expression. In a study SAILO et al. (2015), using Jersey crossbred, found 4 SNPs in the gene HSP90AB1 (targeting intron 7 to exon 11). The authors stated that one of the encountered polymorphisms can be used for increasing thermotolerance in Jersey animals.

On the other hand, when found in the coding regions, these SNPs can alter DNA transcription which may lead to protein alteration (KIM & MISRA, 2007), consequently altering gene function (COLLINS et al., 1998). The SNPs identified in the coding regions of the present study, all led to amino acid substitutions.

Regarding the fragments in the gene HSP90AB1 where animals showed

higher homology with the *B. indicus* than the *B. taurus* sequences found in the NCBI data bank, this fact may be one of the reasons for the similar results between the animals (HS, INT and TT) regarding thermoregulation, since *Bos indicus* animals are better adapted to heat stress. However, further investigations have to be performed to identify why these animals maintain parts of the *B. indicus* genome.

In the present study, novel SNPs were identified in both ATP1A1 and HSP90AB1 genes, however considering that cows from all three groups (HS, INT and TT) showed these polymorphisms, association with thermotolerance was not possible. Further studies are needed in order to evaluate the effects of these SNPs in thermoregulation.

In conclusion, animals with lower CV require less AIs to get pregnant. Milk production was not different between classified groups so interference in the animals' thermoregulation could not be established; blood degree did not interfere in the studied variables. Novel SNPS were found in both ATP1A1 and HSP90AB1 genes, but further studies are required in order to associate them to thermoregulation.

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