SEGREGATION OF HEAT SHOCK FACTOR-1, HEAT SHOCK PROTEIN-70 AND SIGNAL TRANSDUCER OF ACTIVATION OF TRANSCRIPTION 1 POLYMORPHISMS IN NORMAL AND SLICK COATED CATTLE AND ITS POTENTIAL ASSOCIATIONS WITH MOLECULAR BREEDING VALUES AND PHENOTYPICAL TRAITS

by

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ABSTRACT

Heat stress (HS) in dairy cattle is one of the major problems that contribute to farmer's financial losses in tropical and subtropical climates. Dairy cattle under HS have lower milk yields, deficiency in reproductive traits and immune depression. Genetic selection of animals that present better thermo-tolerance helps offset the adverse effect of HS. Previously, genes that can help mitigate heat stress have been identified. Among these is the prolactin receptor (*PRLR*), which determines the type of hair coat; the heat shock transcription factor-1 (*HSF1*), which increases the production of heat shock protein-70 (HSP70); *HSP70*, helping with cell protection against stress events; and the signal transducer of activation of transcription 1 (STAT1), involved in milk production and embryo survival. The objectives of this study were to assess the segregation of polymorphisms in the aforementioned genes and evaluate its associations with production traits and molecular breeding values (MBV) in slick (SL) and normal (WT) coat Holstein cows. Segregation of a previously reported *ApaL*I restriction fragment length polymorphism (RFLP) resulting of a thymine to cytosine transition (genotypes: TT, TC and CC) at intron 3 of *HSF1* (rs#133931291) was found. 2 STAT1 polymorphisms located at exon 11 (rs#209286339; aminoacid change phenylalanine to serine) and intron 11 (rs#211030653), respectively, were identified to be segregating in linkage disequilibrium (haplotypes: CC, CTCG and TG). *STAT1* haplotypes interacted with the *HSF1* genotypes to affect the MBV of productive life (PL) ($P = 0.0494$). Significant interaction was also found between *HSF1* genotypes and coat type for the same MBV ($P = 0.0456$). Out of 11 polymorphisms previously reported on the *HSP70* promoter, 6 were segregated in the estudied Holstein population and arranged in 12 haplotypes. Ultimately, 4 haplotypes were used for the correspondent statistical

analyses and a trend toward significance was found for the MBV of somatic cell score (SCS, *P = 0.0779*). For the same MBV, a trend toward significance interaction was observed for *STAT1* haplotypes with coat type (*P = 0.0809*). That interaction was significant for adjusted SCS determined by the Dairy Herd Improvement Program (*P = 0.0157*). Therefore, novel double interactions affecting PL molecularly were observed in Holstein cattle either associated or independently of coat type, and STAT1 seems to be involved with SCS phenotypically.

RESUMEN

El estrés por calor (HS) en el ganado lechero es uno de los principales problemas que contribuyen a las pérdidas económicas de los agricultores en climas tropicales y subtropicales. El ganado lechero bajo HS muestra rendimientos de leche más bajos, deficiencia en rasgos reproductivos y depresión inmune. La selección genética de los animales que presentan una mejor tolerancia térmica ayuda a compensar el efecto adverso de HS. Anteriormente, se han identificado genes que pueden ayudar a mitigar el HS. Entre estos se encuentra el receptor de prolactina (*PRLR*), que determina el tipo de pelaje; el factor de transcripción de choque térmico-1 (*HSF1*), que aumenta la producción de la proteína de choque térmico 70 (HSP70); *HSP70*, que ayuda a la protección celular contra eventos de estrés; y el transductor de señal de activación de la transcripción 1 (*STAT1*), que está involucrado en la producción de leche y la supervivencia del embrión. Los objetivos de este estudio fueron evaluar la segregación de polimorfismos en los genes antes mencionados y sus asociaciones con rasgos de producción y los valores de cría moleculares (MBV) en el ganado Holstein de pelaje corto (SL) y normal (WT). Se encontró segregación de un polimorfismo de longitud de fragmentos de restricción *Apa*LI (RFLP) previamente reportado y resultante de una transición de timina a citosina (genotipos: TT, TC y CC) en el intrón 3 de *HSF1* (rs # 133931291). 2 polimorfismos en *STAT1* localizados en el exón 11 (rs # 209286339; cambio de aminoácidos de fenilalanina a serina) e intrón 11 (rs # 211030653), respectivamente, se encontraron segregando en desequilibrio de ligamiento (haplotipos: CC, CTCG y TG). Los haplotipos de *STAT1* interactuaron con los genotipos de *HSF1* para afectar el MBV de vida productiva (PL; P = 0.*0494*). También se encontró una interacción significativa entre los genotipos de *HSF1* y el tipo de pelaje para el mismo MBV (P =

0.0456). Por otra parte, de 11 polimorfismos informados previamente en el promotor de *HSP70*, 6 segregaron en la población de Holstein estudiada y se organizaron en 12 haplotipos. Finalmente, se utilizaron 4 haplotipos para los análisis estadísticos correspondientes y se encontró una tendencia hacia la significancia para el MBV de la puntuación de células somáticas (SCS; P = *0.0779*). Para el mismo MBV, se observó una tendencia de significancia a la interacción para los haplotipos de *STAT1* con el tipo de pelaje (P = *0.0809*). Esa interacción fue significativa para el SCS ajustado determinado por el Programa de Mejoramiento del Hato Lechero (P = *0.0157*). Por lo tanto, nuevas interacciones dobles que afectan PL molecularmente se observaron en Holstein, asociadas o independientemente del tipo de pelaje, y *STAT1* aparenta estar envuelto con SCS fenotípicamente.

DEDICATORY

This is dedicated with all my love to my family. Thank you for all your support, especially my parents. They gave me the courage to finish this stage. Also, to my brothers who provided support to finish this goal. To my fiancé, thank you for your unconditional love and for always being there for me. All of you have a special place in my heart, thanks for the motivation and the love that you have given me, without you none of this would have been done.

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GLOSSARY OF TERMS

DHIP	Dairy Herd Improvement Program
DNA	Deoxyribonucleic Acid
HSF1	Heat Shock Transcription Factor 1
HSP70	Heat Shock Protein 70
InDel	Insertion/Deletion Polymorphism
MBV	Molecular Breeding Value
MP	Milk Production
PCR	Polymerase Chain Reaction
PL	Productive Life
PR	Pregnancy rate
PRLR	Prolactin Receptor
RFLP	Restriction Fragment Length Polymorphism
SCS	Somatic Cells Score
SNP	Single Nucleotide Polymorphism
SL	Slick Coat
STAT1	Signal Transducer of Activation of Transcription 1
THI	Temperature and Humidity Index
USDA – ARS-AGIL	United State Department of Agriculture – Agricultural Research Service - Animal Genomics and Improvement Laboratory
WT	Wild Type

CHAPTER 1–INTRODUCTION

The dairy industry is one of the most important agricultural industries worldwide According to the United Nations for Food and Agriculture Organization, in 2012, 759 million tons of milk were produced globally (FAO, 2018). Moreover, in recent years, the production and consumption of milk has risen worldwide. In Puerto Rico the dairy industry is the most important agricultural entrepreneur contributing approximately 214 million dollars annually (Agriculture Gross Income of Puerto Rico, 2014). Therefore improving the production efficiency of this industry would represent a significant impact for both, farmers and the local economy.

In order to accomplish this, it is necessary to overcome various challenges that dairy farmers continually experience with their herds, such as heat stress. Dairy cattle exposed to high environment temperatures and humidity may reach a state called heat stress. This effect is due to the incapacity of the cow to dissipated body heat. This hinders the productive and reproductive efficiency of the dairy cow (Hansen, 2013).

Another factor that influences the performance of dairy cows is their genetic. Animals with unsuitable genetics for milk production, reproduction or heat resistance are less efficient than those selected to be productive. Enhancing animal's productivity by genetic selection is not a quick process, due to the difficulty of improving specific characteristics, as they depend on the environment in which this animal interacts. Also, during the past decade, genetic improvements on dairy cattle have been more accurate and efficient to increase milk yields. However, selecting only for milk yield has made the animals more susceptible to environmental changes, diseases and impaired fertility (Ravagnolo et. al, 2000 and West, 2003. As part of this research, we will

be looking and studying mutations on candidate genes encoding proteins that may provide resistance to heat stress and greater reproductive and productive capacities.

CHAPTER 2 - LITERATURE REVIEW

2.1 Heat Stress

The geographical position of Puerto Rico is associated with elevated temperature and humidity index (THI) and solar radiation. These environmental conditions are very detrimental to dairy breeds developed under temperate weather, causing heat stress, decreasing their level of milk production, feed intake, growth rate and fertility (West, 2003 and Arias et al., 2008). The THI for dairy cattle are show in figure 2.1. To reduce such a negative effects, management strategies have been developed, such as cooling systems as a means to improve animal wellbeing and performance (West, 2003). In Australia, independently of all the strategies implemented in a dairy farm to reduce the effect of heat stress on farm profitability, during the summer from to 10 to 15% of milk yield is reduced (Dunshea et al. 2013).

It should be noted that heat stress in an organism occurs when the internal heat produce by metabolic functions and the heat absorbed from the environment exceed the animal's capacity for heat dissipation. High humidity and exposure to sunlight increases body heat, affecting essential metabolic functions (Kumar et al., 2015). Normally, when cattle are experiencing heat stress, it starts sweating to remove heat by evaporation, but if the relative humidity is high, sweat will not easily evaporate and the animal cannot cool by this mechanism. (West, 2003 and Hansen, 2013). This causes the body temperature to increase and heat stress worsens. When the animal's

ability to cool down cannot be accomplished by evapotranspiration, additional thermoregulatory strategies, such as reducing feed intake to reduce metabolic heat from fermentation, are implemented (Dunshea et al. 2013). However, when reducing feed intake milk yield is also reduced. Milk production is largely influenced by the amount of food the animal eats; if the voluntary intake decreases, milk production also decreases (Kadzere et al., 2002).

In addition, other strategy that the cow may use to alleviate heat stress is increasing respiration rate, to release heat by convection and evaporation. However, doing this for a long time may results in respiratory alkalosis or high blood pH (Kadzere et al., 2002). This occurs by excessive removal of CO_2 by hyperventilation. The CO_2 is produced by the chemical reaction of H+ and HCO_3-. Removing CO_2 excessively from the blood creates a disequilibrium between H+ and HCO_3 increasing the pH in blood due to the decrease of H+ in blood (Kadzere et al., 2002). This causes the animal to feel sick and stop eating.

Also, another effect of heat stress is the decrease of the reproductive functions in the animals. High milk yield has an adverse effect on the reproduction, making the cow more susceptible to heat stress (Kahtib, 2009c). During heat stress, cattle invest more energy for heat dissipation placing in secondary place their reproduction (Hansen et. al., 2009). The heat stress causes a decreased in expression of the normal estrus cycle (De Rensis, et. al., 2003). Another reproductive phenomenon that heat causes is embryonic deaths. It had been observed that an increment of cow's internal temperature causes a hostile uterine environment and inhibit embryo development (Roth and Hansen, 2004). The early stages of embryos are more susceptible to extreme heat condition (Hansen, et. al., 2001). To minimize these losses, new techniques had been developed, such as embryo transfer and the use of reproductive hormones to help maintain pregnancy and embryo survival (Hansen, et. al., 2001 and Hansen, 2007).

To mitigate these problems of great financial loss in livestock, candidates genes which can have a positive effect on dairy cattle are investigated. These genes encode proteins or transcription factors that can influence heat resistance or increase animal efficiency. In addition, these genes can influence the animal's reproduction, increasing fertility and tolerance against higher temperatures. These genes can be used as genetic markers to improve thermotolerance and fertility on dairy cattle.

Figure 2.1: Mild, moderate and severe heat stress of the dairy cow according to its temperature and humidity index

DAIRY COW TEMPERATURE HUMIDITY INDEX (THI)

	Humidity %																		
Temp °F	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
72	64	65	65	65	66	66	67	67	67	68	68	69	69	69	70	70	70	71	71
74	65	66	66	67	67	67	68	68	69	69	70	70	70	71	71	72	72	73	73
76	66	67	67	68	68	69	69	70	70	71	71	72	72	73	73	74	74	75	75
78	67	68	68	69	69	70	70	71	71	72	72	73	73	74	74	75	75	76	76
80	68	69	69	70	70	71	72	72	73	74	75	75	76	76	77	78	78	79	79
82	69	69	70	70	71	72	73	73	74	75	75	76	77	77	78	79	79	80	80
84	70	70	71	72	73	73	74	75	75	76	77	78	78	79	80	80	81	82	83
86	71	71	72	73	74	74	75	76	77	78	78	79	80	81	81	82	83	84	84
88	72	72	73	74	75	76	76	77	78	79	80	81	81	82	83	84	85	86	86
90	72	73	74	75	76	77	78	79	79	80	81	82	83	84	85	86	86	87	88
92	73	74	75	76	77	78	79	80	81	82	83	84	85	85	86	87	88	89	90
94	74	75	76	77	78	79	80	81	82	83	84	86	86	87	88	89	90	91	92
96	75	76	77	78	79	80	81	82	83	85	86	87	88	89	90	91	92	93	94
98	76	77	78	80	80	82	83	83	85	86	87	88	89	90	91	92	93	94	95
100	77	78	79	81	82	83	84	85	86	87	88	90	91	92	93	94	95	96	98
102	78	79	80	82	83	84	85	86	87	89	90	91	92	94	95	96	97	98	100
104	79	80	81	83	84	85	86	88	89	90	91	93	94	95	96	98	99	100	101
106	80	81	82	84	85	87	88	89	90	91	93	94	95	97	98	99	101	102	103
108	81	82	83	85	86	88	89	90	92	93	94	96	97	98	100	101	103	104	105
110	81	83	84	86	87	89	90	91	93	95	96	97	99	100	101	103	104	106	107

HUMAN HEAT INDEX

	Humidity %										
Temp °F	40	45	50	55	60	65	70	75	80	85	90
72											
74											
76											
78											
80	80	80	81	81	82	82	83	84	84	85	86
82	81	82	83	84	84	85	86	88	89	90	91
84	83	84	85	86	88	89	90	92	94	96	98
86	85	87	88	89	91	93	95	97	100	102	105
88	88	89	91	93	95	98	100	103	106	110	113
90	91	93	95	97	100	103	105	109	113	117	122
92	94	96	99	101	105	108	112	116	121	126	131
94	97	100	103	106	110	114	119	124	129	135	
96	101	104	108	112	116	121	126	132			
98	105	109	113	117	123	128	134				
100	109	114	118	124	129	136					
102	114	119	124	130	137						
104	119	124	131	137							
106	124	130	137								
108	130	137									
110	136										

Stress threshold for lactating cows. Respiration rate may exceed 60 BPM. Milk loses begin ~ 2.5 lbs/cow/day. Reproductive loses are detectable and rectal temperature exceeds 101.3°F.
Caution for people depending on age, exposure and activity. People may not feel heat stress until 80°F and 40% humidity.

Mild to moderate stress for lactating cows. Respiration rates may exceed 75 BPM. Milk loses ~ 6 lbs/cow/day. Rectal temperatures will exceed 102.2°F.
Extreme Caution for people depending on age, exposure and activity.

Moderate to severe stress for lactating cows. Respiration rate exceeds 85 BPM. Milk loses ~ 8.7 lbs/cow/day. Rectal temperature exceeds 104°F.
Danger for people depending on age, exposure and activity.

Severe stress! Life threatening conditions for lactating cows. Respiration rates are 120-140 BPM. Rectal temperatures may exceed 106°F.
Extreme Danger of heat exhaustion and/or heat stroke for people when working in these conditions.

Figure adapted from - http://www.thedairysite.com/articles/3712/an-introduction-into-heat-stress-in-dairy-cows/

2.2 Slick Hair

One of these improvements is the introduction of slick hair coat phenotype in to the dairy cattle temperate breeds. This phenotype is due to an autosomal mutation causing a deletion of one cytosine on the exon ten of the prolactin receptor (PRLR) gene on chromosome twenty (Littlejohn et al., 2014). This deletion is named p.Leu462* and causes a truncated protein limiting its function and resulting in the slick hair phenotype (Littlejohn et al., 2014). Studies indicated that this mutation derives from Senepol and Creole cattle (Dikmen et al. 2014). It also has been observed in Colombian and Venezuelan cattle, like the Romunsioano and Carora respectively. This dominant mutation makes dairy cows' hair much shorter and thicker than normal cows' hair, leading to better body heat dissipation (Dikmen, et. al, 2008, Dikmen, et. al, 2014 and Jimenez et. al, 2015).

Studies indicate that animals with shorter hair have a lower body temperature and are less affected by heat stress are less noticeable (Dikmen, et. al, 2008 and Curbelo et al. 2017). This reduction in temperature is due to the fact that the hot air is not enclosed between the hairs, thus causing better heat dissipation. In addition, SL Holstein has bigger sweat glands that contribute to a better thermoregulation (Contrera et al., 2017). In fact, is has been reported that slick hair Holsteins has rectal temperature 0.5 C lower than normal coat Holstein (Olson et al., 2003 and Curbelo et al. 2017). Also, SL cows on elevated THI shows lower vaginal temperature (Sanchez et al., 2015) This makes the slick cattle more resistant to hot temperatures and more productive in tropical conditions. Also during the summer, this type of cattle could have a better milk production than wild type cows (Patiño et. al, 2016).

In tropical climate, slick type Holstein is more efficient and productive, giving an advantage over the wild types. Farmers should select this type of animal for herds with higher efficiency. Similar to the slick phenotype, there are other aspects which can improve the heat resistance of these animals. There are other genes, which are being investigated to help improve the dairy herd to solve the problem of heat stress on the Island. Some of these candidate genes are Heat Shock Transcription Factor 1 (*HSF1*), heat shock protein 70 (*HSP70*), and Signal Transducer of Activation of Transcription Factor 1 (*STAT1*).

2.3 Heat Shock Factor 1

The *HSF1* is the major inducible transactivator factor of heat shock proteins (Xiao et al., 1999, Christians et al, 2000). There are three other HSF but none can substitute *HSF1* function (Pirkkala et al. 2001). The other HSF are: *HSF2*, avian *HSF3* and *HSF4*. The *HSF1* gene contains 13 exons and is located in bovine chromosome 13 (NCBI Gene ID: 506235, 2015). This factor is the first that responds to the heat stress; triggering a cascade of events for thermoregulation (Collier, et. al, 2008). The protein contains a region where it is anchored to the DNA of the cell called Heat Shock Elements (HSE), especially for the *HSP70* promoter (Pirkkala, et. al, 2001). *HSF1* gives the signal to express the *HSP70* gene and synthesizes the protein when a heat shock occurs (Li., et. al, 2011). The expression of *HSF1* is highly conserved in all the mammals' species giving similar effects in all of them (Xiao et al, 1999). The highly conserved region of the *HSF1* is the DNA biding region (Pirkkala, et al. 2001). Also, it can be regulate by *HSP90* and Heat Shock Biding Protein (HSBP). The HSP90 attaches to the protein

and controls the dimerization and activation of *HSF1* (Pirkkala, et al. 2001). Also, the HSBP attaches to the DNA binding domain of the *HSF1* inactivating the function (Pirkkala, et al. 2001).

In contrast, this factor had an important role on post natal growth and fertility. A study guide by Xiao (1999), demonstrated that mice lacking the *HSF1* gene exhibited growth retardation. In addition, studies demonstrated that female mice without the *HSF1* gene show defects on placenta development and reduced embryo survival rates (Xiao et al., 1999, and Christians et al, 2000). If there is a mutation in the protein coding region or in his promoter, it will probably change the expression, affecting the synthesis of the protein and decreasing the ability of protecting the animal against heat stress. Moreover, a mutation on gene can affect the reproduction and the normal growth of the animal.

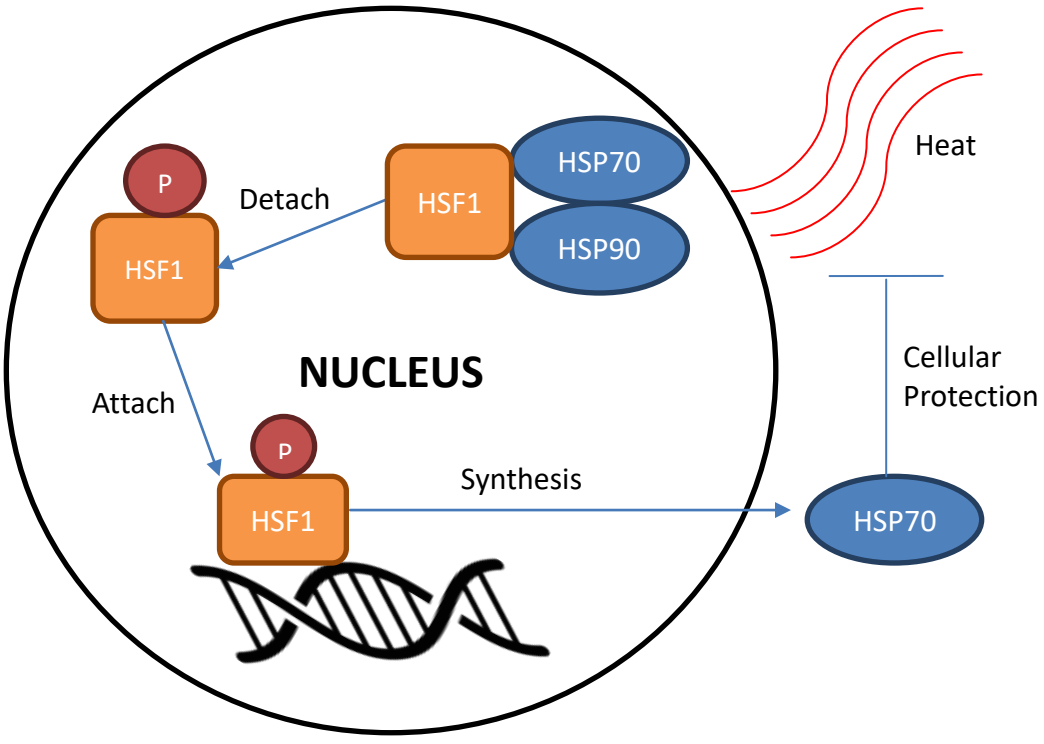


Figure 2.2: Mechanism of action of the HSF1 activating HSP70
Adapted from: Wang X, et. al 2014

2.4 Heat Shock Protein 70

The gene for Heat Shock Protein 70 contains only one exon on chromosome 23 (NCBI Gene ID: 281825, 2015). It is regulated by a cascade of events lead by HSF1 which bind to the promoter area of the HSP70 gene (Pirkkala, et al, 2001). This protein is part of a highly conserved heat shock protein family that has a weight of 70 kDa. (Berre et al., 2001 and Li et al, 2010). It is a chaperone protein that is synthesized when a heat shock occurs and is responsible for verifying that other proteins have been synthesized and folded correctly (Berre et al, 2001 and Basirico et al., 2011). After stressful events, body proteins are denatured; HSP70 is responsible for arranging the body proteins to keep them going under stress (Collier, et. al, 2008). This protein plays an important role in protecting cells from apoptosis by thermal stress or other trauma (Berre et al., 2001 and Deb, et. al, 2013). How HSP70 regulates apoptosis is unclear but it seems to be involved in the inactivation of caspase, an apoptotic protein. HSP70 blocks the recruitment of procaspase to Apaf-1 of apostome (Beere et al., 2001). Also, it has an important role on oocyte and embryo survival; studies show that HSP70 can block the caspase's apoptosis pathway on oocytes and early stage embryos (Beere et al., 2000 and Matwee et al, 2001). In another study with *Bos taurus* and *Bos indicus* oocytes, demonstrated that *Bos taurus* oocytes express more HSP70 than *Bos indicus,* showing that the *Bos indicus* breeds are more acclimated to the tropical environment (Camargo et al, 2007). It has been reported that the promoter region of HSP70 is polymorphic; this area regulates the expression and synthesis of the protein. A study found eleven SNP that had an effect on the calving and on the calving interval on dairy cattle (Roserkrans et al., 2010). These mutations affect the expression of the protein affecting the stress response decreasing the cow fertility. Higher expression of this gene may be a signal of

thermotolerance in hot climates and a better performance (Bhanuprakash et al., 2016). Expression of HSP70 brings a better reproductive and productive performance. If there is a mutation affecting the expression or conformation of it, the animal would be exposed to serious stress damage.

2.5 Signal Transducer of Activation of Transcription 1

Signal transducer activation of transcription factor 1 (STAT1) is a member of cytoplasmic proteins stimulated by cytoquinine, growth factors and hormones located in chromosome 2 (Darnell, 1997). There are seven different types of STATs in mammals: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6; each one with a specific role of gene expression (Darnell, 1997). The action mechanisms of these proteins are mediated by a cascade of events starting with the phosphorylation of Janus protein tyrosine kinase (JAK) (Darnell, 1997 and Akira, 1999). These proteins activate STAT1 to separate it from its receptor complex to form hetero or homodimer which goes to the nucleus to activate promoter and control genes expressions (Darnell, 1997 and Akira, 1999). Investigations in dairy animals show that *STAT1* and *STAT3* have higher expression in mammary tissue (Cobanoglu et al, 2006). This makes the *STAT1* a candidate gene for milk production improvement in dairy cows.

Variants of this protein and mutations on the promoter region contribute to milk quality increasing milk protein and milk fat (Cobanoglu, et al, 2001 and Gholam et al, 2013). Also, the elimination of *STAT1* on mice shows a decrease in the immune system and susceptibility to bacterial infection (Akira, 1999). Moreover, this protein had an effect on the response on interferon's proteins that contribute to cellular protection (Darnell, 1997). This effect can be

combined with cellular protection of HSP70. It also suggests that STAT1 can be used to improve bacterial infection resistance on the udder (Akira, 1999). Also, this protein had a positive effect on fertility helping on the survival rate of the oocyte (Khatib et al, 2009). In addition, according to Kumar et al. (2015) *STAT1* is highly expressed during pregnancy and lactation.

The technological advances helped us to have a better approach to genomic improvement. Investigation of these genes results in a major step towards the genetic improvement in cattle. Increasing the ability to tolerate heat stress directly increases the productive life of the animals in the livestock and benefits the country financially. The objective of this study is to determine segregation of SNPs in genes HSF1, HSP70 and STAT1 on slick and wild type Holstein and to know if those mutations had an effect on the molecular breeding value traits and dairy herd improvement that contribute to an increasing in milk production and fertility. In the future, these genetic enhancements will make healthy, fertile and productive dairy cattle.

CHAPTER 3 – METHODOLOGY

3.1 DNA Sample and variables to evaluate

DNA was extracted from white blood cells of a total of 215 Holstein cows from three DHIP participating dairy farms located in the northwestern and southwestern of Puerto Rico. Those cattle were segregated as SL and WT by genotyping for a *PRLR* variant allele containing a nonsense and dominant mutation causative of SL phenotype in Holstein (Littlejohn et al., 2014). *HSF1* and *HSP70* promoter, genotyped polymorphisms were previously reported by Li-Ling et al. (2011) and Rosenkrans et al. (2010), respectively. STAT1 candidate polymorphic regions were

selected based on a whole gene bioinformatics assessment done by Dr. Steven Schroeder at the USDA-ARS-AGIL. The totals of animals genotyped were 159 for HSF1, 198 for HSP70 and 215 for (STAT1). The aforementioned data was used for a genotype/haplotype association study with MBV obtained commercially (Igenity, Neogen Corp®) for PR, PL, MP and SCS. Also, potential associations with DHI traits (calving interval, milk production adjusted at 305d and adjusted SCS) were evaluated.

3.2 PCR amplification, enzyme digestion and Sanger sequencing

HSF1 and *HSP70,* PCR amplification was performed using oligonucleotide primers designed by Li et al. (2011) and Rosenkrans et al. (2010), respectively. The primer sequences and fragment lengths evaluated for the three candidate genes are shown in the Table 3.1. The optimal conditions for PCR amplification of *HSF1*, *STAT1* and *HSP70* are show on table 3.2. The *HSF1* PCR product was digested with the restriction endonucleases *Apa*LI (Figure 3.1). Enzyme digestion was performed with a reaction volume of 15.5 uL. The digested products were verified with a 3% agarose gel stained with ethidium bromide. *HSP70* and *STAT1* PCR products were sent for Sanger nucleotide sequencing to MCLAB (San Francisco, CA, USA). The electrophoretograms were analyzed with the biological sequence alignment editor BioEdit®. For both genes, segregating polymorphisms were arranged on haplotypes for the correspondent statistical analyses. For *HSP70,* four out of a total of twelve haplotypes were numerically representative and ultimately used to evaluate potential associations with MBV and DHIP selected traits. Similarly, for *STAT1* three haplotypes were used.

Table 3.1: PCR primers sequence, annealing temperature, and fragment size of *HSF1*, *HSP70* and *STAT1*

Gene	Primer sequence (5'-3')	Length (bp)	Annealing temperature (^{o}C)
HSF1[a]	F: TGCCAAGCCTGCTTTCTACC R: ACGAAGTTCTTTCTGGAACCCT	415	57.5
HSP70[b]	F: CCTACGCAGGAGTAGGTGGT R: GCCAGGAAACCAGAGACAGA	539	59
STAT1	F: CAGCACCTTAAGCAATGTATGG R: GACTTTCGTGTGTTGCAGTG	354	59

a. Primer sequence obtained from Li et al. (2011)
b. Primer sequence obtained from Rosenkrans et al. (2010)
c. Primer sequence designed by me.

Table 3.2: Optimal conditions for PCR amplification of *HSF1*, *HSP70* and *STAT1* studied regions

	Condition		
	HSF1	*HSP70*	*STAT1*
	Volumen : Concentration		
AccuStart™ II PCR Super Mix 2X	12.5ul : 1X	6.25ul : 1X	6.25ul : 1X
FW	1.5ul : 300nM	.75µl : 300nM	.75µl : 300nM
RV	1.5ul : 300nM	.75µl : 300nM	.75µl : 300nM
ddH_2O	5.5 ul	2.75µl	2.75µl
DNA	4 ul : 10ng/ul	2µl : 10 $^{ng}/_{\mu l}$	2µl : 10 $^{ng}/_{\mu l}$

3.3 Statistical analysis

The statistical analysis was performed using PROC MIXED (SAS/STAT®, version SAS University Edition software). A variance analysis was made using a randomized complete design to determine associations between *HSF1*, *HSP70*, *STAT1* and coat type (SL or WT) with MBV (PR, PL, MP, SCS) and DHIP traits (calving interval, milk yield adjusted to 305d and adjusted somatic cell score). The lineal model was:

$Y_{ikl} = \mu + T_i + G_k + TG_{ik} + e_{ikl}$ where:

Y_{jkl} = *l* observation represented on the phenotypic type *i (*SL or WT), on genotype/haplotype *k* (*HSF1*, *HSP70* and *STAT1*) and on the interaction of type and genotype/haplotype *ik.*

μ = Total mean

T_j = Phenotypic type

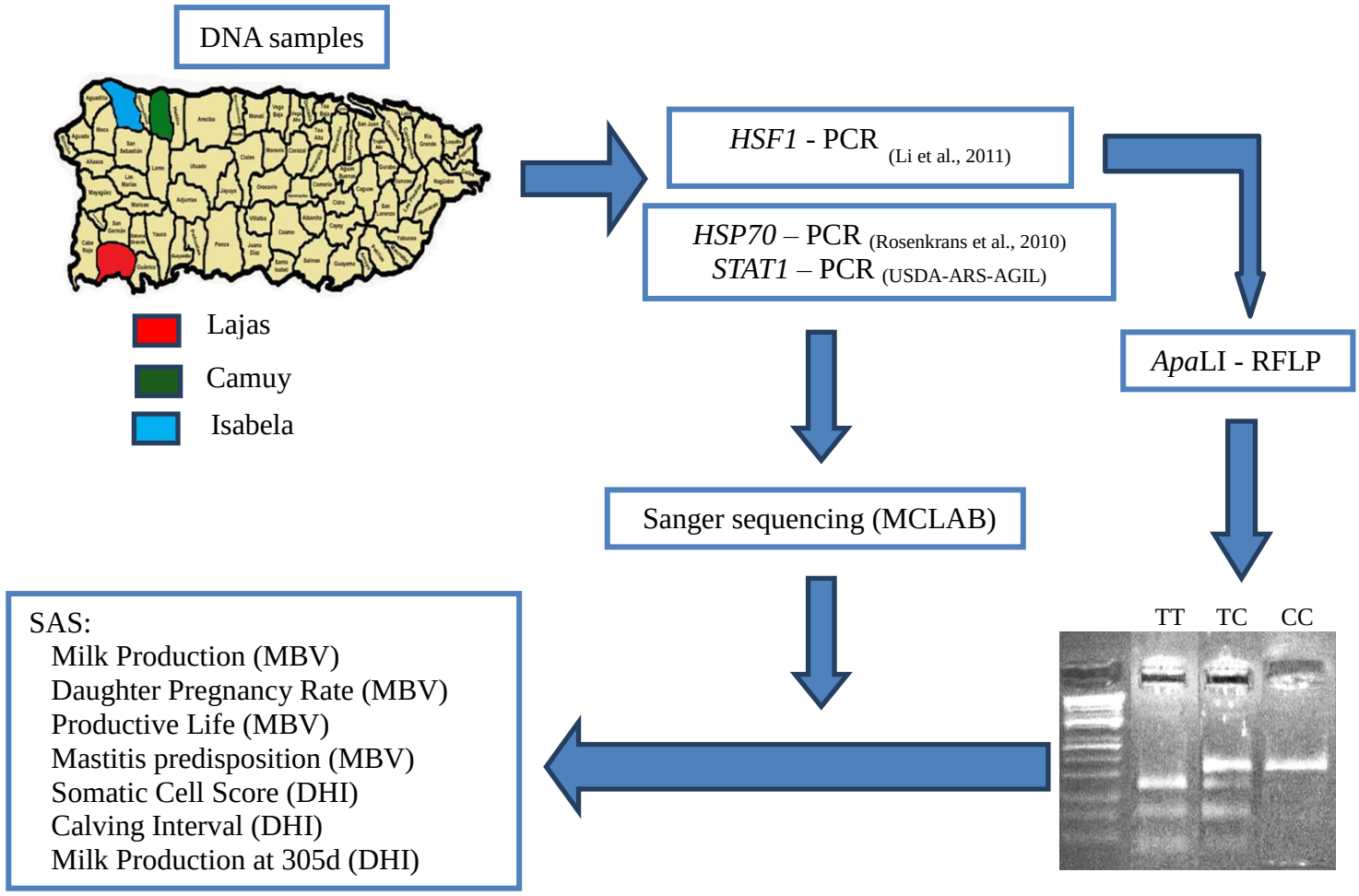
G_k = Genotype/Haplotype (HSF1, HSP70 or STAT1)

TG_{jk} = Interaction between phenotypic type *i* and genotype/haplotype *k*

E_{jkl} = Experimental error

Genotypic, haplotypic, and allelic frequencies were calculated using PROC FREQ and statistical differences were determined using the Chi Square test.

Figure 3.1: Concept map of experimental procedures used in the investigation



CHAPTER 4 – RESULTS AND DISCUSSION

4.1 Genomic and allelic frequencies of *HSF1, HSP70* and S*TAT1* overall and by coat type.

159 animals were genotyped for a SNP located at *HSF1* intron 5 (Figure 4.1). Three genotypes: TT, TC and CC with a frequency of .5912 (94), .3585 (57) and .0503 (8), respectively, were observed with a different overall distribution (P<.0001). Those genotypic frequencies did not differed between coat type (P = .1451). The allelic frequencies were T/.77045 and C/.22955 (table 4.1). For *HSP70,* five SNP and one InDel were found to be segregated in the genotyped population (n=198). Those polymorphisms corresponded to a deletion of cytosine, three transversions (A/C, G/T and C/G) and two transitions (A/G and C/T). For the InDel, three genotypes were identified (CC, CD and DD; Figure 4.2), where D represented the allele with the cytosine deletion. The genotypic frequencies observed were CC/.5274, CD/.3035 and DD/.1692, respectively (P<.0001) with allele frequencies of C/.67885 and D/.32095 (Table 4.2a). In both, SL and WT, the C allele was in higher proportion. The first *HSP70* transversion (Figure 4.3) had frequencies of AA/.4194, AC/.3687 and CC/.2121 (P<.0010) with A/.60355 and C/.39645 (Table 4.2a). The second transversion also presented three segregating genotypes in differing proportions (GG/.4343, GT/.3535, and TT/.2121; P = .0005) with allele frequencies of G/.6115 and T/.3885 (Table 4.2b). Additionally, it was found that the second and third SNP were in linkage disequilibrium (Figure 4.3). Also, the fourth (Figure 4.5) and fifth SNP (Figure 4.6) presented the same phenomena. The sixth SNP of HSP70 had 3 genotypes: CC, TC and TT (Figure 4.6) with frequencies of .1667, .3788 and .4545,

respectively (P<.0001). The allelic frequency was .3561 for allele C and .6439 for allele T. Meanwhile, in WT, SNP 2, 3 and 6 resulted in allele frequencies more numerically close than in SL and in dissimilar proportion when comparing both types of coat (P=.10 for SNP2; P =.0364 for SNP3; P=.0315 for SNP6). The *HSP70* polymorphisms were grouped in haplotypes resulting from twelve different combinations (Table 4.3). For *STAT1*, 215 cows were genotyped for two SNP corresponding to a cytosine to guanine (C/G) transversion and a cytosine to thymine (C/T) transition (Figures 4.7 and 4.8). Both SNP were on linkage disequilibrium, therefore similar genotypic (CC-CC/.2372, CG-CT.5674, GG-TT/.1953) and allelic frequencies (C/.5210 and .4790 for G and T) were obtained (Table 4.4). In addition, three haplotypes were established CC, CGCT and GT (Table 4.4).

4.2 Productive life

The *HSF1* significantly interacts with coat type (Figure 4.9) and with *STAT1* haplotypes (Figure 4.10). SL cows of *HSF1*-TC genotype had higher PL-MBV than WT of *HSF1* TT genotype (1.0155 months difference). Similarly, animals of CC homozygote haplotype (*STAT1*)/TT genotype (*HSF1*) combination presented lower PL-MBV (1.7907 months difference) than animals with the TG homozygote haplotype (*STAT1*)/TC genotype (*HSF1*) combination (P=.0090). Moreover, animal with the *STAT1* double heterozygote haplotype CTCG/TT genotype *(HSF1)* had lower (2.3707 months less) PL-MBV than the TG (*STAT1*)/TC *(HSF1)* combination (P=.0004). Likewise, a tendency toward significance interaction was observed between *STAT1* haplotypes and coat type (P= .0809). Animals of TG haplotype and SL had more desirable PL-MBV than WT cattle with the CC or CTCG haplotype (Figure 4.11).

4.3 Somatic Cell Score - MBV and DHIP traits

A tendency toward significance of *HSP70* haplotypes with SCS-MBV was found (P= .0774). *HSP70* haplotype CAGCAT showed more desirable (lower) MBV for the trait when compared with other three haplotypes (CAGCAT<CDACGTCATC<DCTCAC< CACGTCGACTC; Figure 4.12). Meanwhile, the interaction between *STAT1* haplotypes and coat type was significant for SCS as determine by the DHIP (P = .0157). Animals with *STAT1* haplotype CC and SL had less SCS than animals CC and WT (P= .009). On the other hand, animals with haplotypes CTCG or TG and SL had higher SCS than animals WT of the same two haplotypes.

4.4 Discussion

It is well known that extreme heat conditions for long periods of time produces heat stress, which ultimately affects the productivity of dairy cattle (Dikmen et al 2013). According to Das et al. (2016), heat stress decreases feed intake, milk yield, reproductive performance and can result in immune depression. In Puerto Rico, the cattle are always under moderated to severe heat stress condition according to the THI. In consequence, it could shorten the productive life of the cattle and compromised the immune response in the cattle used locally for milk production. Genetic improvement for increased productive life and thermo-tolerance is possible due to recent advances in genetic marker assisted selection and the growth of the field of animal genomics. Camargo et al. (2007) documented that low expression of *HSP70* in oocytes of *Bos indicus* cattle was an indicator of thermo-tolerance. In that sense, oocytes resistant to heat had a lower

production of HSP70. On the other hand, higher expression of *HSP70* on peripheral blood in dairy cow contributes to a better respond on a heat stress event (Basirico et al., 2010). Therefore, this *HSP70* evidence suggests that differences in expression of that gene can provide a scenario for better productivity and a longer productive life in cattle because they can adapt favorably to hot climate. Also, in a recent study conducted by Bhanuprakash et al. (2016), the proliferation of HSP70 in native cattle was higher than in crossbreds when climate stress was provided, contributing to a better thermostability. In the present study, six polymorphisms located in the *HSP70* promoter were found to be segregated in SL and WT Holsteins, which resulted in four representative haplotypes. *HSP70* haplotype CAGCAT presented the lower genetic predisposition to have mastitis resulting from a more desirable (lower) MBV for SCS (P = 0.0774). That haplotype was the one in higher representation in SL and WT Holsteins (Table 4.3). In addition those haplotypes had a tendency toward significance on 365 adjusted milk productions (P=0.0626). Where, the haplotype CAGCAT (12040 lbs ± 323.20 lbs) had lower milk production than haplotypes CDACGTCA (12959 lbs ± 345.78 lbs) and DCTCAC (13334 lbs ± 410.70 lbs). Another change that results in a better adaptation to hot climates is the SL coat itself. Littlejohn et al. (2014) described the mutation that causes this phenotype in Senepol cattle, confirming that these animals had a better thermoregulation. Also, Dikmen et al. (2008) showed that SL dissipates heat more efficiently which gives them an advantage in tropical climates. No interaction between *HSP70* haplotypes and type of coat was observed in the studied population for any of the MBV and DHIP traits evaluated. The second candidate gene evaluated, *STAT1*, contributes to a better milk production (Cobanoglu et al., 2006). This may provide to a longer productive life. According to Akira (1999) this gene plays an important role on the immune system. This may help cattle to have better health, ultimately improving productive life. The

MBV for productive life is used to predict the longevity of the cattle in the herd. VanRaden (2002) mentioned that a cow could remain in the herd only if her profit surpasses the feed cost, veterinary expenses, labor and other cost related to her production. For that reason, it is convenient to raise cows with high longevity in order to produce substantial profit on the long run. The C allele of the *HSF1* had a positive effect in the MBV for PL when is combined with SL or *STAT1* haplotypes CTCG or TG. The change of base pair in the intron 3 of *HSF1* may result in an enhanced activation of *HSP70* and a better response to heat stress and a higher apoptotic protection (Pirkkala et al., 2001). That SNP was reported by Li et al. (2011). In that study, significant differences in potassium content in erythrocytes (PCE) were observed between genotypes. For that matter, the CC and TC genotype had lower PCE than TT suggesting a better thermal tolerance for animals with one or two copies of the C allele. Therefore, the *HSF1* C allele could give a better response to heat and improvement on longevity when interacting with PRLR genotypes and STAT1 haplotypes segregated in Holstein cattle from Puerto Rico. Patiño et al. (2016) demonstrated that SL cattle grown in the tropics had higher milk yields and lower calving intervals than WT. On the other hand, Cobanoglu et al. (2006) mentioned that STAT1 had a higher expression, possess an important role on mammary tissue formation and are located in a quantitative trait loci region affecting milk yield and composition. Moreover, an investigation conducted by Watson (2001) provided additional evidence that *STAT1* is regulated during mammary gland development and can be described as an oncogene. Furthermore, *STAT1* homozygote haplotype TG had a positive effect on the MBV of PL when combined with SL. Also, the *STAT1* haplotypes CTCG and TG presented lower adjusted SCS in WT animals ($P = 0.0157$). This effect may due to a better developed mammary gland and the role that STAT1 had on the immune system. STAT1 knockout mice provided evidence of a major role of that gene on

the innate defense against bacteria and viruses (Akira, 1999). Likewise, animals with *STAT1* CC homozygote haplotype and SL had lower adjusted SCS than WT of the same haplotype. This effect might result from the documented thermotolerance of the SL cattle (Curbelo et. al, 2017). Animals with lower stress are associated with less compromised and better immune system (West, 2003).

Similarly, HSP70 wild type haplotype CAGCAT had a lower probability to suffer mastitis's problems. On the other hand, the *HSP70* CAGGTCCACTC haplotype presented higher risk. It had been noticed that animals with a normal function in HSP70 had a better acclimatation on tropical environment and a better immune system response (Wallin et al., 2002 and Deb et al., 2013). Also, HSPs are involved in the activation of innate immune system. According to Wallin et al. (2002) HSPs attach to pathogens to mark them for T cells. Differences observed between HSP70 haplotypes in terms of the MBV for SCS might be indicative of differences in promoter activation during transcription and potential interaction in the other candidate gene polymorphisms used for the correspondent MBV estimation.

Figure 4.1: Representative agarose gel electrophoresis of a *Apa*LI enzyme digestion a *HSF1* intron #3 PCR amplified fragment (genotypes TT, TC and CC).

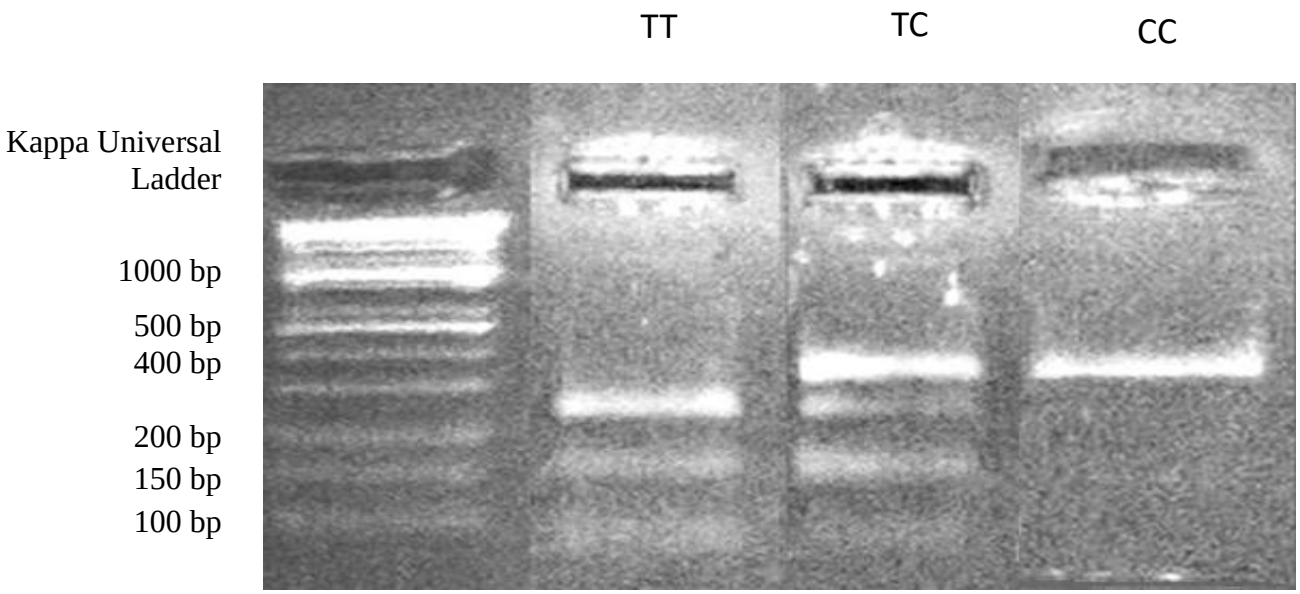


Figure 4.2: Nucleotide sequencing electrophoretogram of *HSP70* promoter polymorphism 1 (rs#447203792: a insertion/deletion of cytosine, genotypes CC, CD and DD)

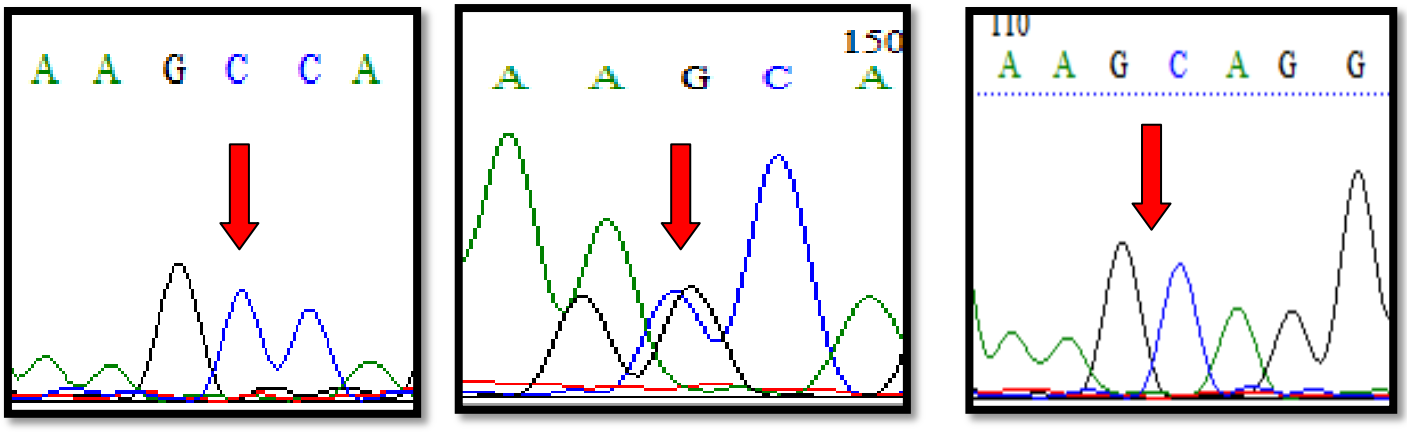


Figure 4.3: Nucleotide sequencing electrophoretogram of *HSP70* promoter single nucleotide polymorphism 2 (rs#478612967) and 3 (genotypes combinations due to linkage disequilibrium: AA/GG, AC/GT and CC/TT)

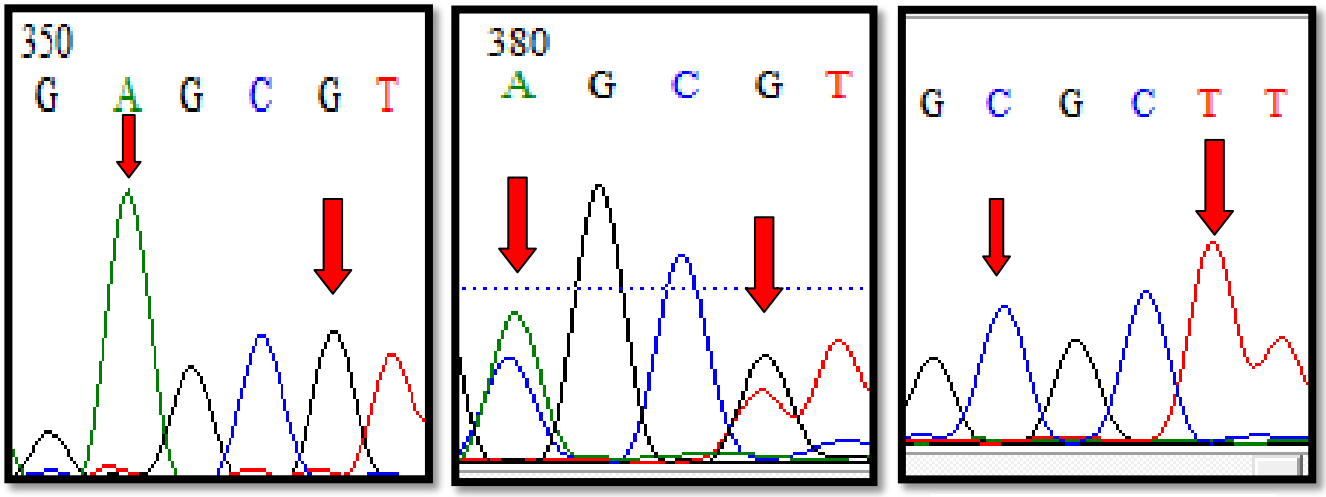


Figure 4.4: Nucleotide sequencing electrophoretogram of *HSP70* promoter single nucleotide polymorphism 4 (rs#471604061: genotypes GG and GA)

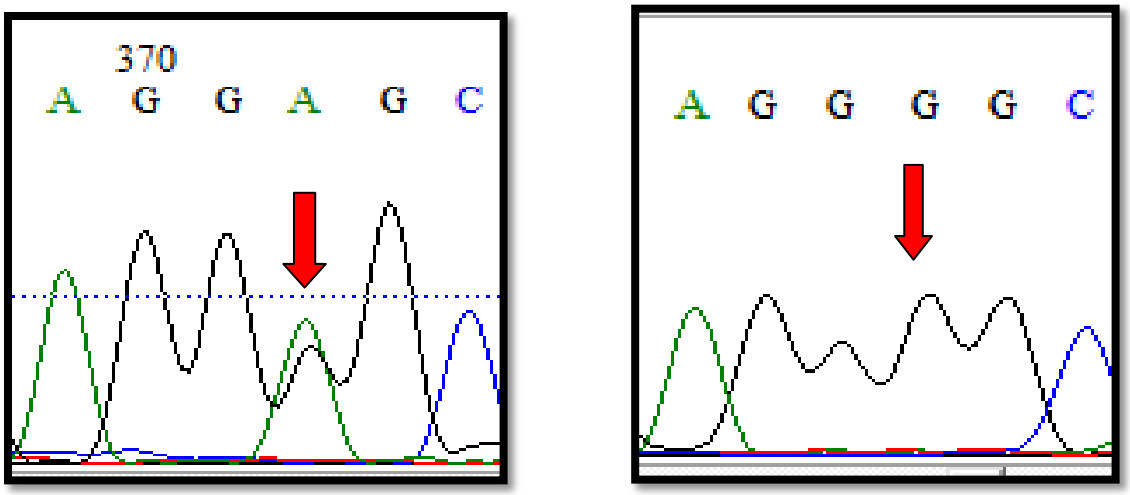


Figure 4.5: Nucleotide sequencing electrophoretogram of *HSP70* promoter single nucleotide polymorphism 5 (genotypes CC and CG)

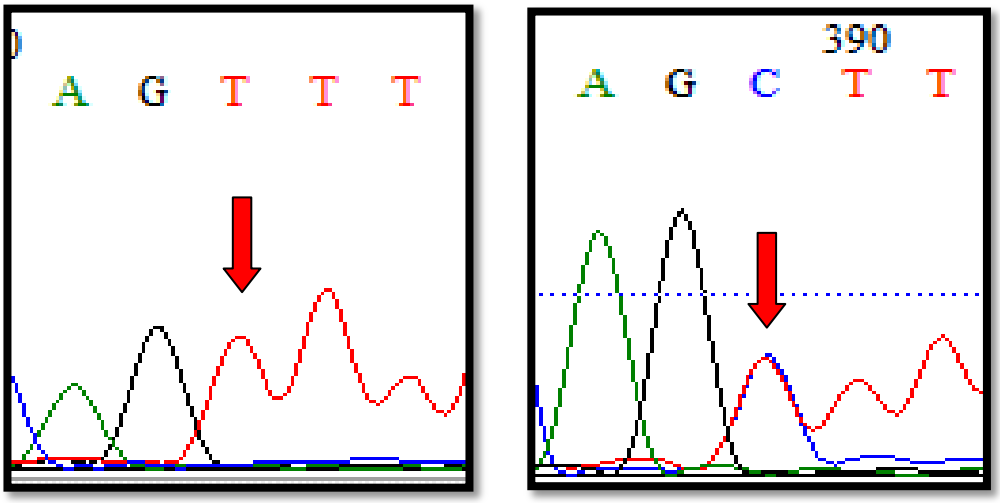


Figure 4.6: Nucleotide sequencing electrophoretogram of *HSP70* promoter single nucleotide polymorphism 6 (rs#473916108: genotypes TT, TC and CC).

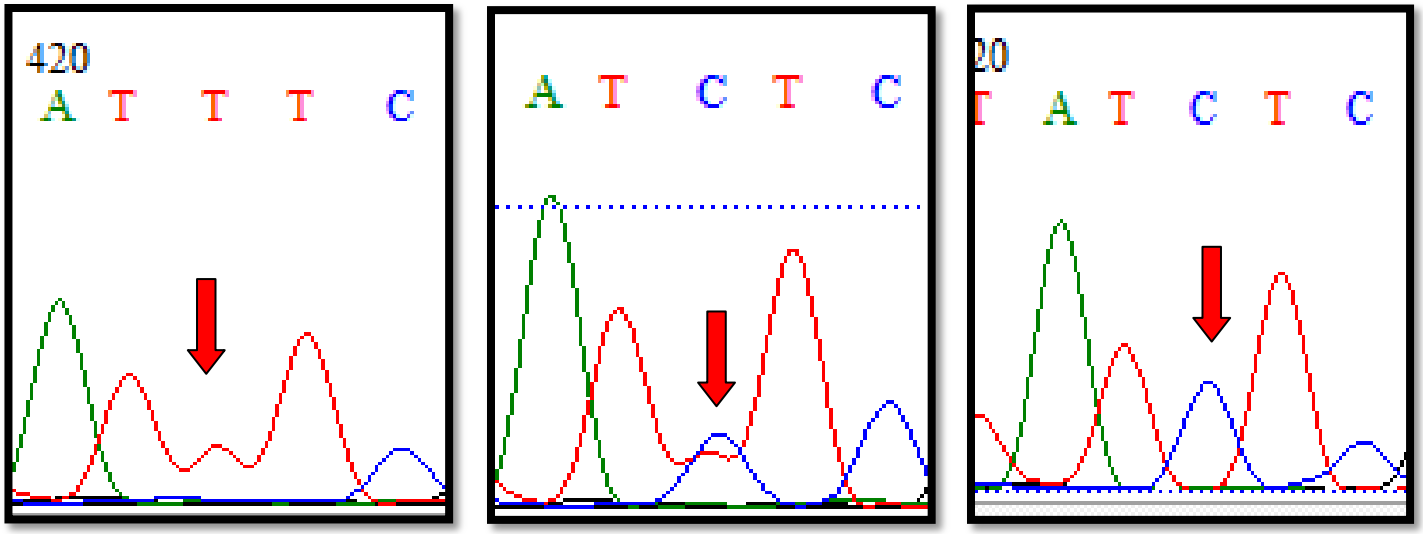


Figure 4.7: Nucleotide sequencing electrophoretogram of a *STAT1* transversion single nucleotide polymorphism at exon 11 (rs#209286339: genotypes TT, TC and CC)

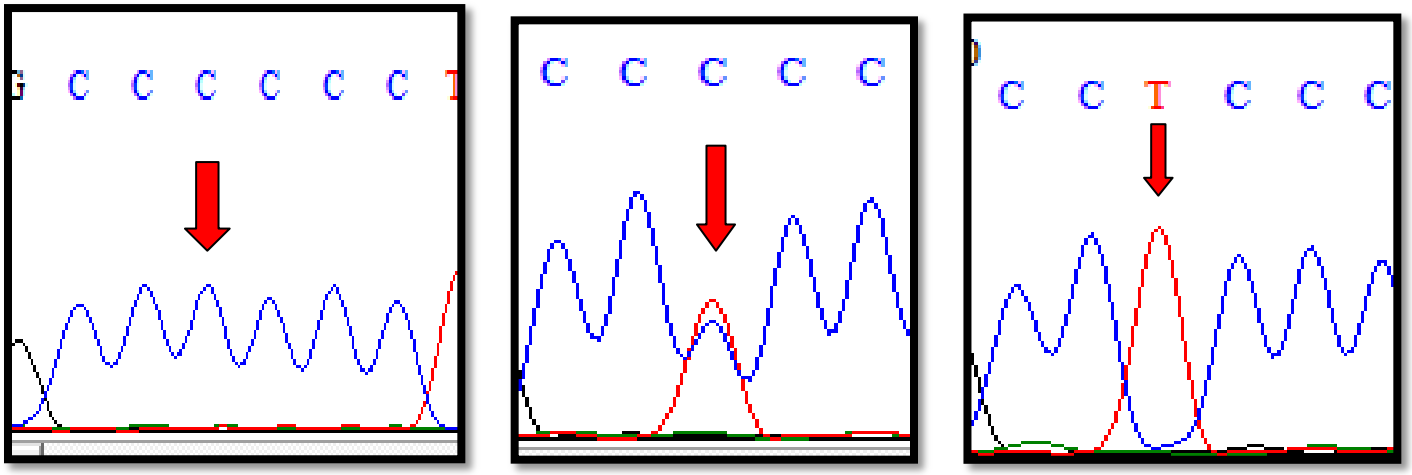


Figure 4.8: Nucleotide sequencing electrophoretogram of a *STAT1* transition single nucleotide polymorphism at intron 11 (rs#211030653: genotypes GG, GC and CC)

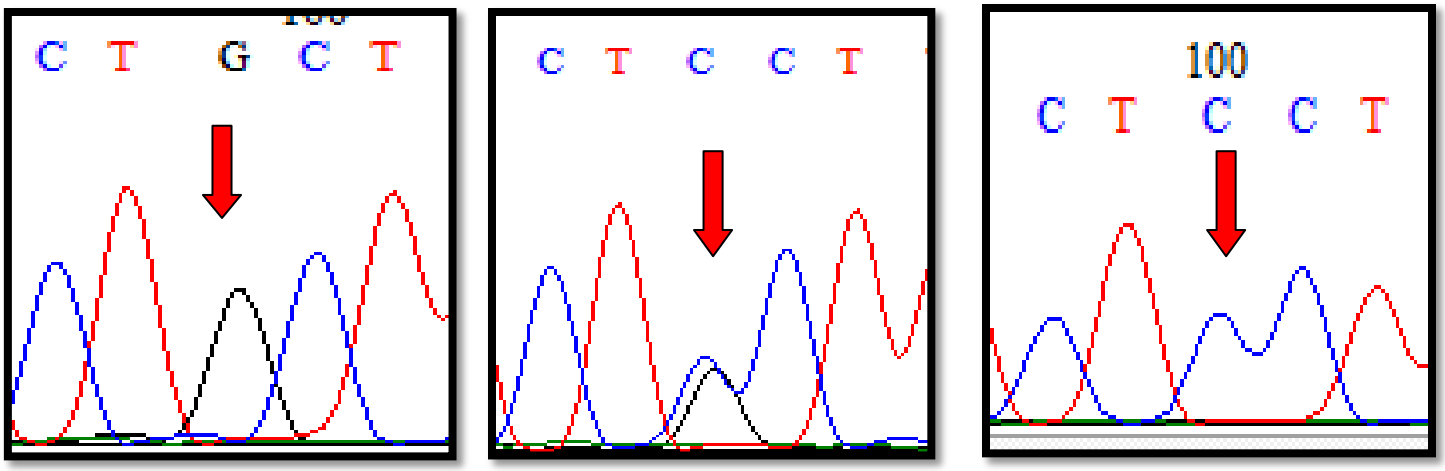


Table 4.1: Genotypic and allelic frequencies (overall and by coat type) of *HSF1* single nucleotide polymorphism

Overall				WT		SL		Chi-Squre
Genotype	Genomic frequency	Allelic frequency	Chi-Square	Genomic frequency	Allelic frequency	Genomic frequency	Allelic frequency	
TT	.5912 (94)	T= .7705	P=<.0001	.5385 (49)	T= .7308	.6618 (45)	T= .8235	P= < *0.1451*
TC	.3585 (57)			.3846 (35)		.3235 (22)		
CC	.0503 (8)	C= .2295		.0769 (7)	C= .2692	.0147 (1)	C= .1765	
Total	1.00 (159)	1.00		1.00 (91)		1.00 (68)		

Table 4.2a: Genotypic and allelic frequencies (overall and by coat type) of *HSP70* promoter In/Del polymorphisms and SNP A/C

Polymorphism 1 (cytosine deletion)							
Overall				WT		SL	
Genotype	Genomic frequency	Allelic frequency	Chi-Square	Genomic frequency	Allelic frequency	Genomic frequency	Allelic frequency
CC	.5274 (106)	C= .6789	P=<.0001	.5804 (65)	C= .7054	.4607 (41)	C=..6461
CD	.3035 (61)			.2500 (28)		.3708(33)	
DD	.1692 (34)	D= .3211		.1696 (19)	D= .2946	.1685 (15)	D= .3539
Total	1.00 (198)	1.00		1.00 (112)		1.00 (89)	
SNP (2) A/C							
Overall				WT		SL	
Genotype	Genomic frequency	Allelic frequency	Chi-Square	Genomic frequency	Allelic frequency	Genomic frequency	Allelic frequency
AA	.4192 (83)	A= .6035	P=<.0010	.4679 (51)	A= .6193	.3596 (32)	A= .5843
AC	.3687 (73)			.3028 (33)		.4494 (40)	
CC	.2121 (42)	C= .3965		.2294 (25)	C= .3808	.1910 (17)	C= .4157
Total	1.00 (198)			1.00 (109)		1.00 (89)	

Table 4.2b: Genotypic and allelic frequencies (overall and by coat type) of *HSP70* promoter SNPs G/T, C/G and A/G

SNP (3) G/T							
Overall				WT		SL	
Genotype	Genomic frequency	Allelic frequency	Chi-Square	Genomic frequency	Allelic frequency	Genomic frequency	Allelic frequency
GG	.4343 (86)	G= .6115	P=<.0005	.4954 (54)	G= .6330	.3596 (32)	G= .5843
GT	.3535 (70)			.2752 (30)		.4494 (40)	
TT	.2121 (42)	T= .3885		.2294 (25)	T= .3670	.1910 (17)	T= .4157
Total	1.00 (198)	1.00		1.00 (109)		1.00 (89)	
SNP (4) and (5) C/G and A/G							
Overall				WT		SL	
Genotype	Genomic frequency	Allelic frequency	Chi-Square	Genomic frequency	Allelic frequency	Genomic frequency	Allelic frequency
CC/AA	.8990 (179)	C/A= .9545	P= .0001	.9175 (100)	C/A= .9586	.8764 (78)	C/A= .9382
CG/AG	.1110 (20)			.0825 (9)		.1236 (11)	
GG/GG	0	G/G=.0555		0	G/G= .0414	0	G/G= 0618
Total	1.00 (198)	1.00					

Table 4.2c: Genotypic and allelic frequencies (overall and by coat type) of *HSP70* promoter SNP T/C

SNP (6) T/C							
Overall				WT		SL	
Genotype	Genomic frequency	Allelic frequency	Chi-Square	Genomic frequency	Allelic frequency	Genomic frequency	Allelic frequency
TT	.4545 (90)	T= .6439		.5321 (58)	T= .6835	.3596 (32)	T= .5956
TC	.3788 (75)		P=.0001	.3028 (33)		.4719 (42)	
CC	.1667 (33)	C= .3561		.1651 (18)	C= .3165	.1685 (15)	C= .4044
Total	1.00					1.00	

Table 4.3: Haplotype frequencies (overall and by coat type) of *HSP70* promoter polymorphisms

HSP70 Haplotypes	Frequency overall	WT frequency	SL frequency

CACGCAT	.0101 (2)	.0183 (2)	0
CACGCATC	.0051 (1)	.0092 (1)	0
CACGTCAT	.0101 (2)	.0183 (2)	0
CACGTCGAT	.0758 (15)	.0550 (6)	.1011 (9)
CAGCAT	.4192 (83)	.4679 (51)	.3596 (32)
CCTCAT	.0152 (3)	.0275 (3)	0
CDACGTCATC	.2626 (52)	.1927 (21)	.3483 (31)
CDACGTCGTC	.0051 (1)	.0092 (1)	0
CDCTCAGT	.0101 (2)	.0183 (2)	0
CDCTCGAGTC	.0152 (3)	.0092 (1)	.0225 (2)
DCTCAC	.1667 (33)	.1651 (18)	.1685 (15)
DCTCATC	.0051 (1)	.0092 (1)	0

Table 4.4: Genotypic and allelic frequencies (overall and by coat type) of *STAT1* single nucleotide polymorphisms

SNP (1) and (2)							
Overall				WT		SL	
Genotype	Genomic frequency	Allelic frequency	Chi-Square	Genotype frequency	Allelic frequency	Genomic frequency	Allelic frequency
CC/CC	.2372 (51)	C/C= .5210		.2049 (25)	C/C= .4836	.2796 (26)	C/C= .5699
CG/CT	.5674 (122)		P= .0001	.5574 (68)		.5806 (54)	
GG/TT	.1953 (42)	G/T= .4790		.2377 (29)	G/T= .5164	.1398 (13)	G/T= .4301
Total	1.00 (215)			1.00 (122)		1.00 (93)	

Figure 4.9: Effect of an interaction between *HSF1* genotypes and coat type on the MBV for productive life

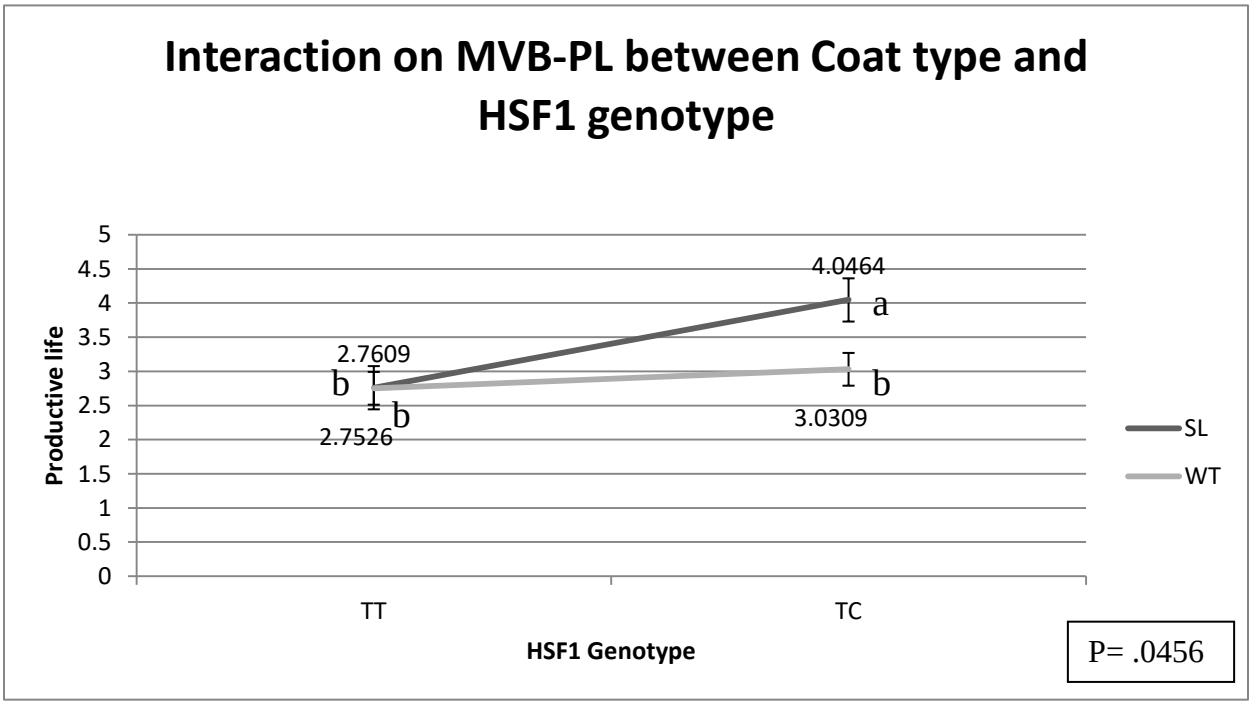


Figure 4.10: Effect of an interaction between *HSF1* genotypes and *STAT1* haplotypes on the MBV for productive life

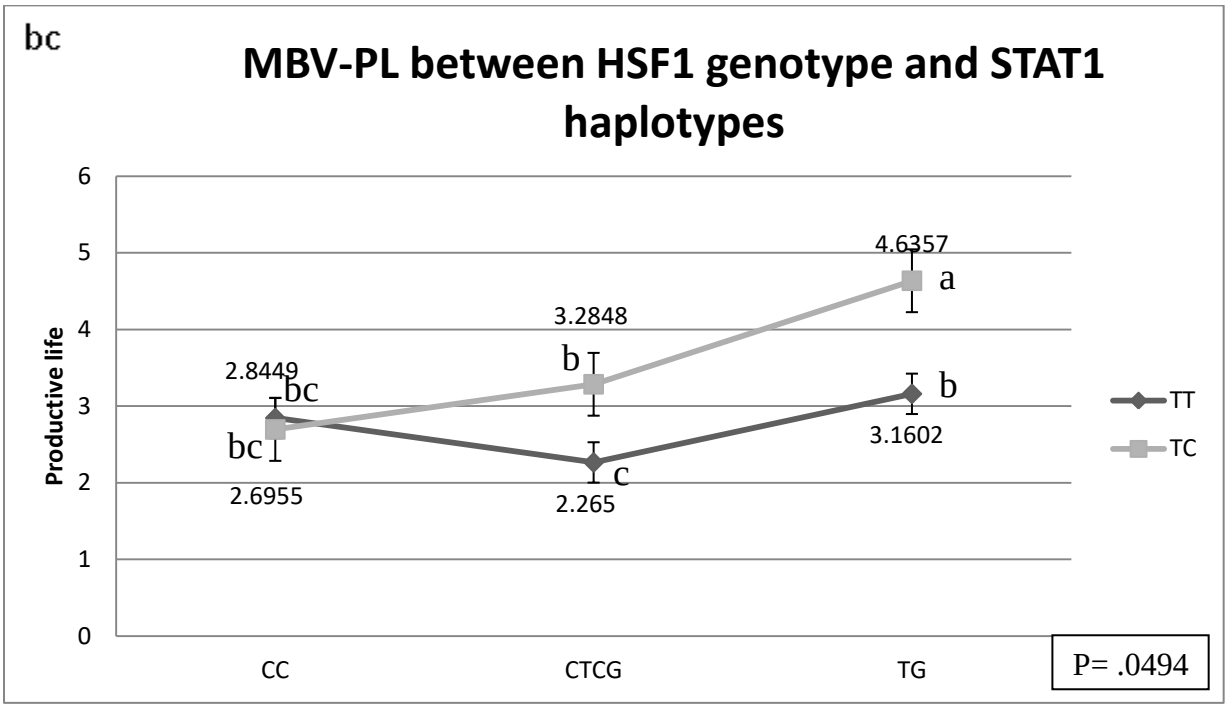


Figure 4.11: Effect of an interaction between *STAT1* haplotypes and coat type on the MBV for productive life

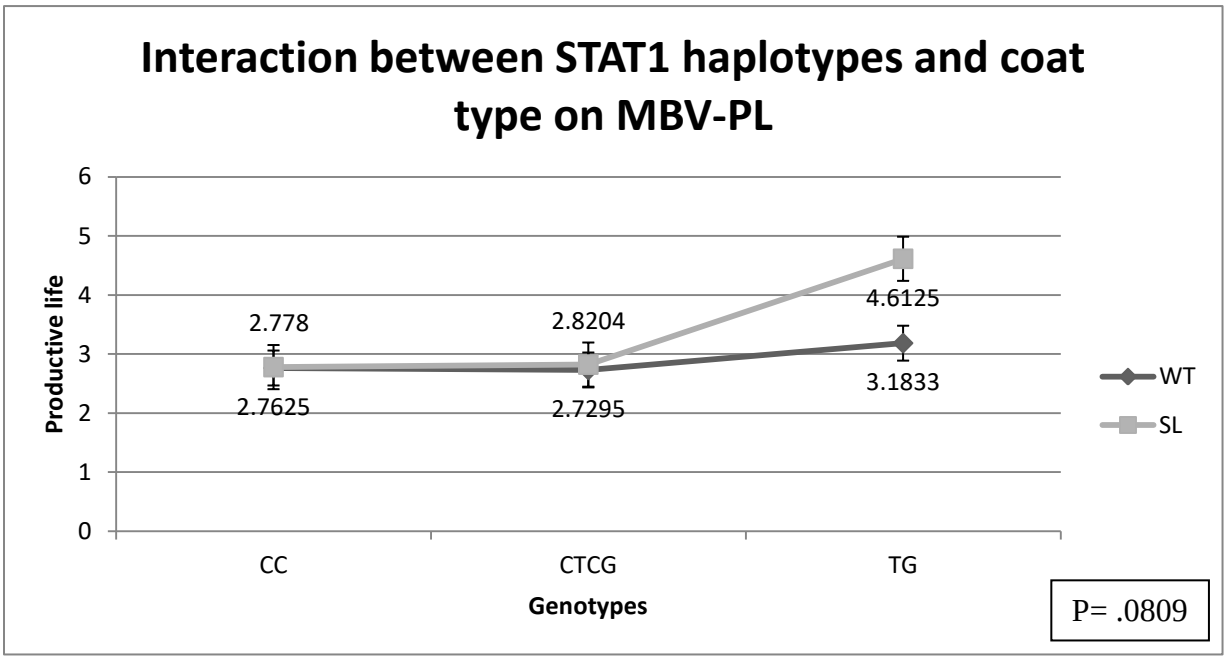


Figure 4.12: Single effect of four *HSP70* haplotypes on the MBV for somatic cell score

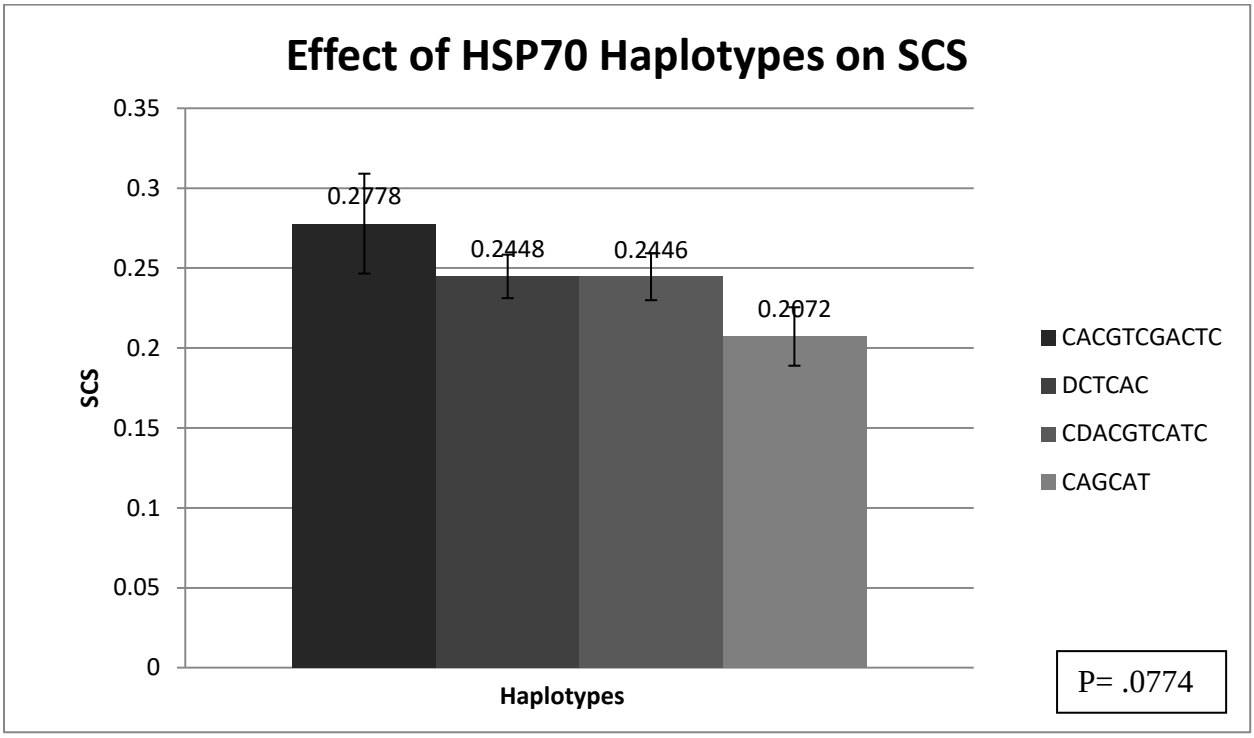
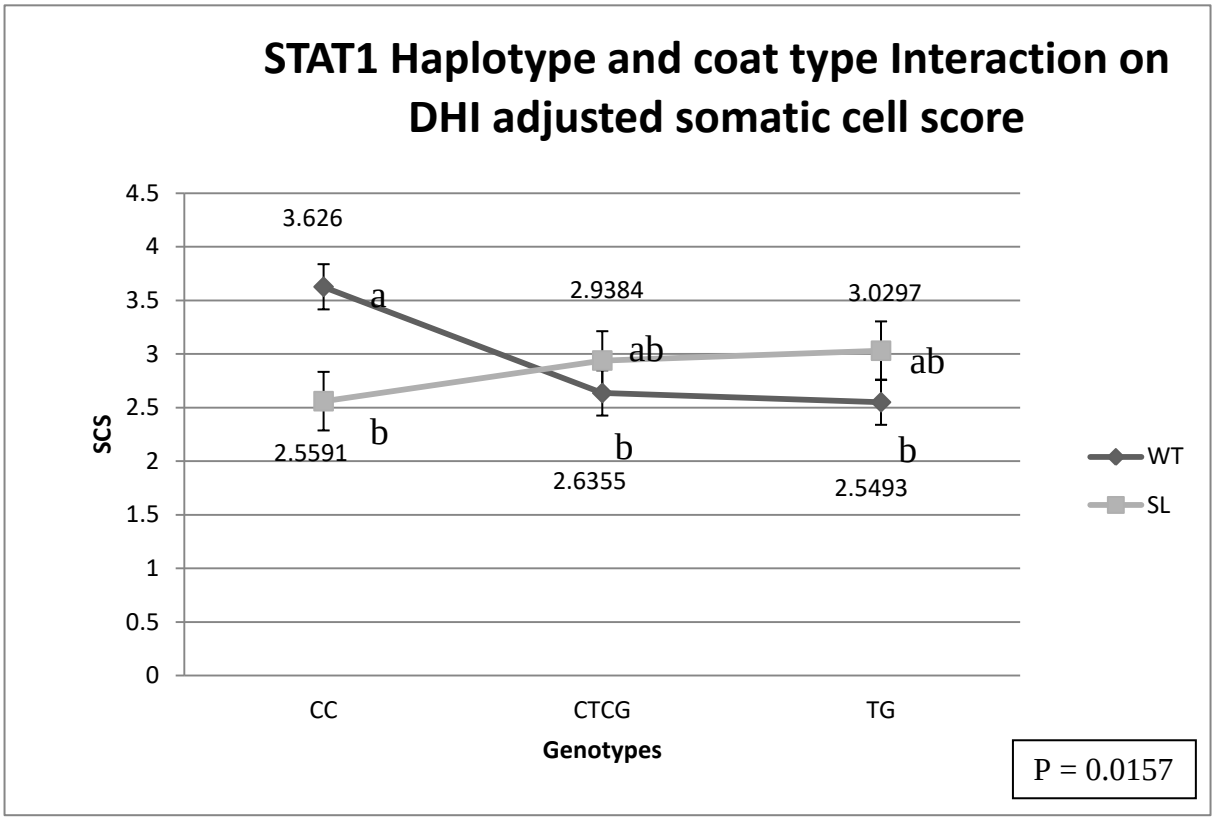


Figure 4.13: Effect of an interaction between *STAT1* haplotypes and coat type on adjusted somatic cell score.



CHAPTER 5 – CONCLUSION

The results of the present study validated the segregation in Holstein cattle from Puerto Rico of polymorphisms reported in *HSF1*, *HSP70* and *STAT1* and its usefulness as markers for selection in economically important dairy traits at the genome level and phenotypically. Novel two way interactions between those genes and with coat type affecting the molecular breeding values for productive life and somatic cell scores and actual milk somatic cell counts were documented. Further investigations are needed to have a better understanding of the pathway that these genes use to provide a better adaptation in hot climates in dairy cattle as it relates to productive life, somatic cells scores and milk production.

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