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ASSESMENT OF THE SLICK PHENOTYPE FOR THERMOTOLERANCE IN CATTLE THROUGH HSP70 GENE EXPRESSION ANALYSIS

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THOMPSON RIVERS UNIVERSITY

ASSESMENT OF THE SLICK PHENOTYPE FOR THERMOTOLERANCE IN CATTLE THROUGH HSP70 GENE EXPRESSION ANALYSIS

by

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ABSTRACT

Climate change poses a significant challenge for agricultural production, especially livestock. As cattle are unable to adapt as fast as global temperatures are rising, heat stress has become of growing concern. Heat stress in cattle often causes a decrease in appetite and weight gain. In addition to underweight cattle, the adverse effects of heat stress also include a decreased reproduction rate, increased susceptibility to disease and poor-quality meat and dairy products. With the growing demand for high quality meat and dairy products, mitigating the effects of heat stress in cattle has become a priority. A promising solution to this problem is the introduction of the SLICK phenotype for cattle production, which has been associated with a shorter haircoat to enhance heat dissipation. This study aimed to evaluate the effectiveness of the SLICK phenotype in alleviating heat stress by comparing Heat Shock Protein 70 (HSP70) gene expression levels in Red Angus and Angus-Senepol hybrid (SLICK) cattle. In addition, haircoat properties, including length and diameter were compared to determine the difference in haircoat morphology between the two groups. RNA was extracted from hair follicles during different environmental conditions to generate cDNA for quantitative PCR (qPCR) analysis of HSP70 gene expression. Results confirmed that SLICK cattle exhibited significantly shorter haircoats than Red Angus cattle, suggesting an increased ability for thermoregulation. HSP70 gene expression analysis did not show significant differences between the two groups after experiencing a maximum temperature humidity index (THI) of 76.4. Although gene expression results were inconclusive, the findings from this study highlight the potential for selecting heat tolerant traits to improve heat stress resistance. This research supports the idea that the SLICK phenotype offers a promising strategy for enhancing the sustainability of cattle production in temperate climates.

Thesis Supervisor: Associate Professor Joanna Urban

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
GDP	Gross Domestic Product
CBT	Core Body Temperature
THI	Temperature Humidity Index
DMI	Dry Matter Intake
LH	Luteinizing Hormone
PRLR	Prolactin Receptor
EVHL	Evaporative Heat Loss
HSP	Heat Shock Proteins
HSP70	Heat Shock Protein 70
HSF1	Heat Shock Factor 1
ACC	Animal Care Committee
cDNA	Complementary Deoxyribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
Ct	Cycle Threshold
mL	Milliliter
μL	Microliter
ng	Nanogram
μg	Microgram

1 INTRODUCTION

1.1 The Economic Importance of Cattle

The Canadian economy relies heavily upon agriculture for revenue and stability (AAFC, 2023). Cattle are essential to the economy as the Canadian beef sector contributes approximately $24 billion annually (2020-2022) to Canada's gross domestic product (GDP) (Canfax Research Services, 2023). Canadian cattle farms and beef processing provides a reliable supply of meet for Canada, which produced 3.61 billion pounds of beef in 2021 (Canfax Research Services, 2023). In 2022 Canadians consumed 967,166 metric tons of Canadian beef, a 3% increase from 2021, with continuous increases projected for the coming years (Statistics Canada, 2023). Not only does the beef sector provide for Canadians, but approximately half of productions are exported to over half a dozen countries, with 49% of total beef products exported in 2022 (Statistics Canada, 2023; AAFC, 2023). Canada is projected to be the 8th largest beef exporter in the world as exported products were valued at $4.68 billion in 2022, a 5.1% increase from 2021 (Statistics Canada, 2023).

Canadian beef is also recognized as a sustainable food source that contributes to both domestic and international food security (CAA, n.d). However, the global demand for Canadian meat and dairy is expected to increase by 57% and 48% in 2050, respectively, due to the foreseeable increase in the global population and urbanization (Ominski et al. 2021). Overall, maintaining the health and productivity of cattle is essential to meet consumer demand, ensure a secure food supply, and sustain its role as a major contributor to the Canadian Economy.

1.2 Climate Change Impacts on Cattle Production

Climate change has become an increasing concern to multiple areas of Canadian agriculture. As the average temperature continues to increase, and situations of extreme heat such as the 2021 B.C. heat wave are expected more often (Beugin, 2023), the agri-food sector will be challenged. Global warming and climate variability not only affect feed and water resources, but animal health and production (Godde et al. 2021). These changes can significantly impact cattle farming which is critical to both the Canadian economy and food security. This creates another major pressure on the Canadian agri-food sector as the demand for more livestock products is met with less land, water, and feed for production (Ominski et al., 2021).

Specifically, rising temperatures can have a detrimental impact on the beef production sector due to the onset of heat stress and the associated health affects it causes for cattle (Godde et al., 2017; Cheng et al., 2022). Heat stress is a major concern in the livestock sector, particularly for beef cattle, as they are more susceptible than other livestock species. This is largely due to being raised primarily outdoors, providing them with little environmental protection, in addition to their reduced water retention capacity (Archana et al., 2017; Islam et al., 2023). Heat stress affects meat production for all commercial livestock (Gonzalez-Rivas et al., 2020) as animal body size is compromised and can lower meat quality (Summer et al., 2019; Cheng et al., 2022). In the early 2000's the estimated economic loss in the United States due to heat stress in cattle production was estimated at $1.7 billion annually (St-Pierre et al., 2003). In the absence of current data, it is assumed the costs of heat stress for the cattle industry are likely greater, especially considering climate change has become more prevalent. On a global scale, it is predicted that cattle production could lose up to $40 billion annually due to increases in temperature extremes (Thornton et al.,

2022). The physiological challenges brought on by heat stress can lead to significant economic losses in addition to threatening food security.

Multiple reviews have stated that finding ways for farmers and ranchers to combat climate change affects will become increasingly necessary to ensure animal welfare and to keep up with economic demands (Osei-Amponsah et al., 2019; Thornton al., 2022; Cheng et al., 2022; Khan et al., 2023; Kwon et al., 2024). Selective breeding has traditionally been used to improve production efficiency but could also become an attractive solution for mitigating heat stress experienced by cattle (Renaudeau et al., 2012; Osei-Amponsah et al., 2019; Cheng et al., 2022).

1.3 Identifying Heat Stress

1.3.1 Heat Stress in Cattle

Heat stress is the result of an imbalance between the metabolic heat produced inside an animal's body and the dissipation to its surroundings (Das et al., 2016). Homeothermy is a thermoregulation process performed by endotherms to maintain a stable internal body temperature; it requires minimal expenditure of energy if the animal's external environment is within the respective thermoneutral zone. Once the environmental temperature is outside of the thermoneutral zone, extra energy is required to thermoregulate, and the animal will experience stress to maintain homeothermy. (Nardone et al., 2006; Collier et al., 2015; Khan et al., 2023). During periods of excess heat in the animal's surrounding environment, homeostatic mechanisms like panting and sweating are activated to reestablish or regulate the animal's internal environment. When an animal is unable to dissipate excess heat, heat stress will occur (Collier et al., 2015; Herbut et al., 2019).

The average core body temperature (CBT) for cattle is found to range from 38°C to 39°C with a mean value of 38.6°C ± 0.5°C (Collier et al., 2015; Ammer et al., 2016; Herbut et al., 2019; Summer et al., 2019; Islam et al., 2023). Cattle are homeothermic and will maintain their body temperature over a range of conditions (Collier et al., 2015; Khan et al., 2023). In conditions outside of the thermoneutral zone, cattle may fail to dissipate self-generated and absorbed heat energy, causing an elevated CBT which leads to heat stress (Islam et al., 2023). In general., a temperature range of -0.5°C to 26°C is accepted as a thermoneutral zone for dairy cattle (Kadzere et al., 2002; Liu et al., 2019; Herbut et al., 2019), and as temperature passes 26°C, they begin to exhibit heat stress behaviors (Kadzere et al., 2002; Cheng et al., 2022). In addition to air temperature, relative humidity is an important factor that determines the exchange of heat between an animal's body and surroundings, therefore it must be accounted for when predicting heat stress (Herbut et al., 2019; Islam et al., 2023, VanderZaag et al., 2023).

1.3.2 The Temperature Humidity Index

To readily identify key heat stress points for cattle, a temperature-humidity index (THI) is commonly used (Dikmen et al., 2008; Kim et al., 2020; VanderZaag et al., 2023). This index represents the combined effects of air temperature and relative humidity to assess the risks of heat stress. (Wang et al., 2018; VanderZaag et al., 2023). A chart for how these two variables impacts the THI is displayed in Figure 1. Different thresholds for heat stress in cattle have been reported, with THI values as low as 68 found to impact the milk yield of high production dairy cattle (Zimbelman et al., 2009). Beef cattle tend to have a higher THI threshold than dairy cattle given that they have been found to tolerate temperatures up to 30°C while humidity is less than 80% (Summer et al., 2019). Overall, the heat stress threshold for cattle is THI 70 and the point where

beef cattle begin to experience mild heat stress, and cattle will elicit a response when THI is ≥72 (Dikmen et al., 2008; Liu et al., 2019; Herbut et al., 2019; Kim et al., 2020). As the THI increases, heat stress becomes more severe and will begin to cause adverse effects in cattle at indices of 80 and higher. However, even prolonged exposure to conditions of THI ≥ 72 can lead to chronic heat stress and physiological problems (Herbut et al., 2019; Kim et al., 2020; Cheng et al., 2022).

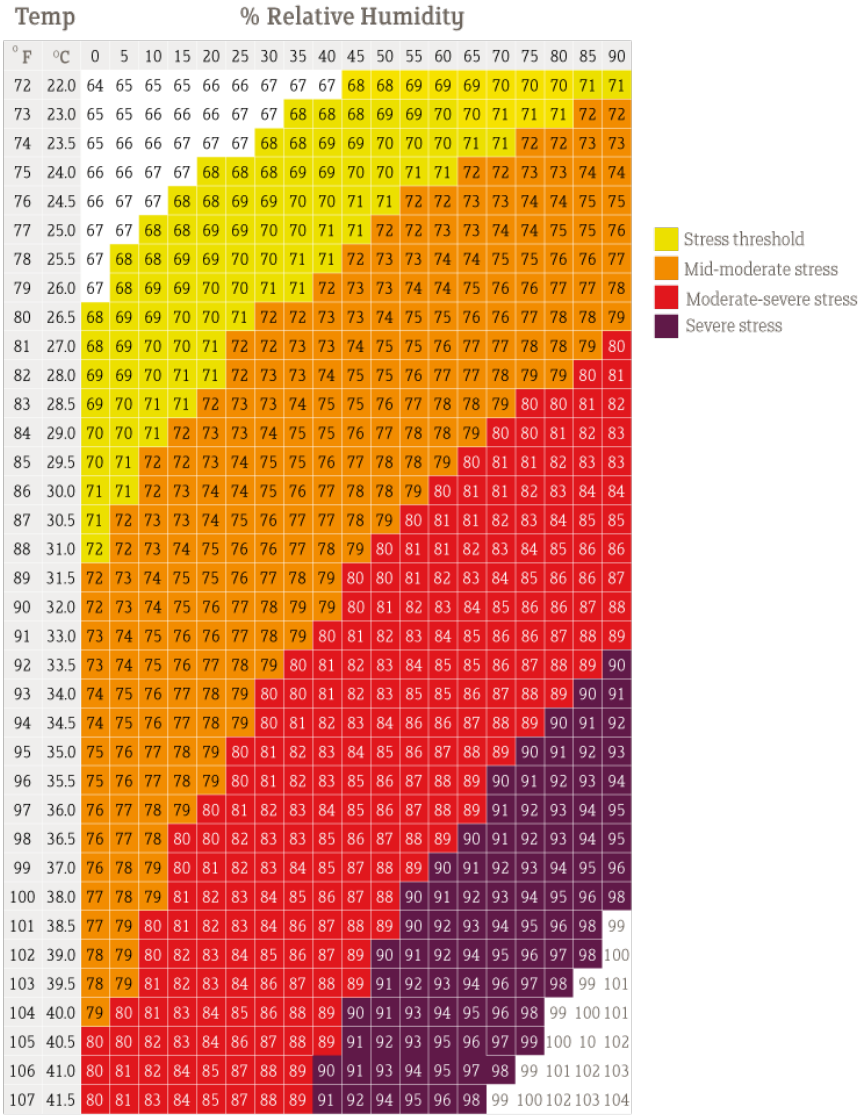

Figure 1. Temperature Humidity Index chart used by farmers to assess heat stress thresholds for cattle (University of Georgia, 2023).

1.4 Characteristics of Heat Stress in Cattle

Heat stress in cattle is demonstrated by various behavioral, metabolic and physiological changes that can significantly impact the animal's health and productivity (Collier et al., 2019). The primary autonomic responses include sweating and panting which are exhibited by cattle to increase the evaporative heat loss from the skin surface (Yadav et al. 2013; Collier et al., 2014). Cattle will also reduce feed intake to reduce heat gain, which is recognized as one of the first indicators of heat stress and is linked to many additional consequences (Godde et al., 2021). Reducing feed intake is also caused by decreased appetite and rumination time due to heat stress which directly results in weight loss, reduced fat thickness, and poor growth rates (Nardone et al., 2006; Cheng et al., 2022).

In addition to underweight cattle, reduced feed intake also results in lower amounts of nutrients and protein needed for proper physiological functions. Heat stressed cattle have decreased milk quantity and quality due to decreased fat and protein content. (Summer et al., 2019; Godde et al., 2021). Meat quality is also affected as it is reported to have less fat and therefore less water holding capacity, poor colour, tenderness, and marbling (Gregory 2010; Gonzalez-Rivas et al., 2019; Godde et al., 2021).

Chronic heat stress can also suppress immune function and impact the fertility of cattle as it begins to interfere with the endocrine system and hormonal pathways (Bagath et al., 2019; Lovarelli et al., 2024). Heat stress increases peripheral levels of glucocorticoids and blood cortisol which inhibit the synthesis and release of cytokines and ultimately suppress the immune system, resulting in cattle becoming more susceptible to infection and disease (Bagath et al., 2019; Godde et al., 2021).

Heat stress also disrupts the hormonal balance that is necessary for successful reproduction. In females, it reduces the luteinizing hormone (LH) and progesterone levels which leads to a decline in estrus behavior and problems with follicle development, compromised oocyte growth, poor embryo development, longer gestation periods and lower birthing rates. Furthermore, poor milk quality can compromise calf development and health. For males, semen concentration, spermatozoa motility and quality of fertile sperm are all reduced (Nardone et al., 2006; Khan et al., 2023).

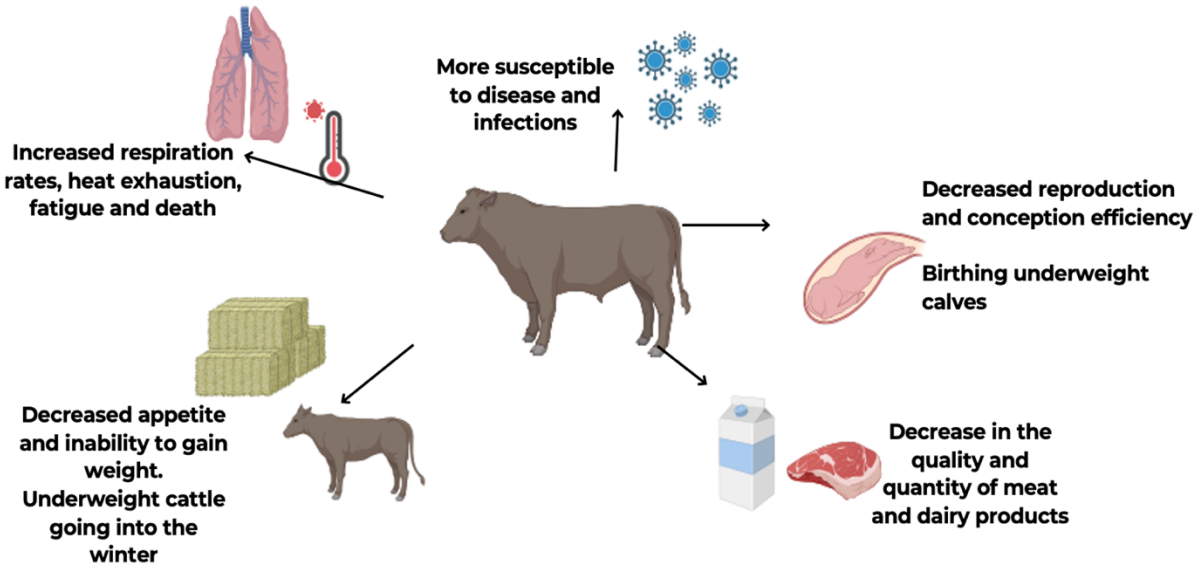

Figure 2. Representation of the adverse effects that can be caused by heat stress in cattle (BioRender, 2024).

1.5 The Significance of the SLICK Phenotype

1.5.1 Hair Coat Morphology and Evaporative Heat Loss

Cattle predominantly dissipate heat through latent heat exchange, known as evaporative heat loss (EVHL) in the form of sweating (Collier et al., 2008). The hair coat of an endotherm is one of the most significant factors that affect heat dispersion and moisture transfer from the animal to the environment. Physical properties of the hair coat such as hair length, diameter, coat density and thickness will all affect the efficacy of EVHL cooling from the skin (Collier et al., 2014). The variation in EVHL among different breeds of cattle suggest there is an opportunity to improve thermal tolerance (Dikmen et al., 2008; Collier et al., 2008).

1.5.2 The SLICK Phenotype

The SLICK phenotype is characterized by a short, sleek haircoat and larger sweat glands that provide cattle an increased resistance to heat stress (Olson et al., 2003; Dikmen et al., 2014). The SLICK1 mutation (c.1382del; rs517047387) follows a dominant inheritance pattern and has originated from the tropically adapted cattle breed Senepol, that arose in the Caribbean (Sosa et al., 2022). It is identified as a deletion mutation in the PRLR (prolactin receptor) gene on bovine chromosome 20 that causes a premature stop codon and results in a truncated C-terminal protein (Mariasegaram et al., 2007; Sosa et al., 2022). Mammalian hair growth is modulated by the prolactin hormone and its signalling pathways; a mutation to the PRLR gene affects the activity of prolactin and is linked to shorter hair morphology and the SLICK phenotype (Littlejohn et al., 2014). The SLICK phenotype has also been successfully inherited by other breeds of cattle as previous studies have shown that Senepol cattle and their crosses with Holstein and Angus animals are equally as heat tolerant (Mariasegaram et al., 2007). In fact, the gene-editing tool CRISPR-

Cas9 has been used to introduce an intentional genomic alteration (IGA) to the PRLR gene to create SLICK cattle, with the goal of enhancing heat tolerance for animal welfare purposes (Pozzebon et al., 2024; Cuellar., 2024). Cuellar et al. (2024) reported that SLICK animals achieved from gene editing not only had improved thermotolerance but were heavier on average compared to non-SLICK animals making them favourable for beef production. Furthermore, the IGA is also heritable and will be passed on through breeding with a PRLR-SLICK cattle lineage (Pozzebon et al., 2024). The gene editing approach is preferred over crossbreeding because it allows for the modification of only the target gene without potentially compromising other favoured traits (Cuellar et al., 2024).

1.6 Heat Shock Proteins

Heat shock proteins (HSPs) are a family of chaperone proteins that are produced by cells in response to stressful conditions such as heat stress (Archana et al., 2017). The expression of HSPs is a strong indicator of heat stress at the molecular level. Within the HSP family, heat shock protein 70 (HSP70) has the most sensitive response to environmental change and has been identified as the ideal biological marker for quantifying heat stress in animals (Archana et al., 2017; Herbut et al., 2019; Guzman et al., 2023).

When an animal experiences heat stress, a highly conserved process of protein activation and gene expression occurs to mitigate the impacts and sustain normal cellular function (Collier et al., 2008). The upregulation of chaperone genes like the HSP70 gene is mediated by heat shock transcription factors (HSFs) and an activation-attenuation cycle controlled by HSPs that is illustrated in Figure 3. (Pessa et al., 2023). In unstressed conditions the HSF1 monomer is bound to a HSP in the cytoplasm. The stress induced accumulation of misfolded proteins causes the HSF1

monomer to dissociate and bind with others for HSF1 trimerization, which is translocated into the nucleus. Within the nucleus, the homotrimetric HSF binds to the heat shock element – a sequence in the promoter region of heat shock genes – which results in the expression of HSP mRNA (Collier et al., 2008; Archana et al., 2017; Pessa et al., 2023). In mammalian cells, HSF1 will also recruit RNA polymerases and chromatin remodellers to ensure heat inducible genes are in an open state (Pessa et al., 2023).

The expression levels of HSP genes, particularly HSP70, have been used to establish the severity of heat stress experienced by cattle (Collier et al., 2008; Kim et al., 2020; Guzman et al., 2023). Tolerance to heat stress and THI thresholds can be identified through the quantification of HSPs (Guzman et al., 2023).

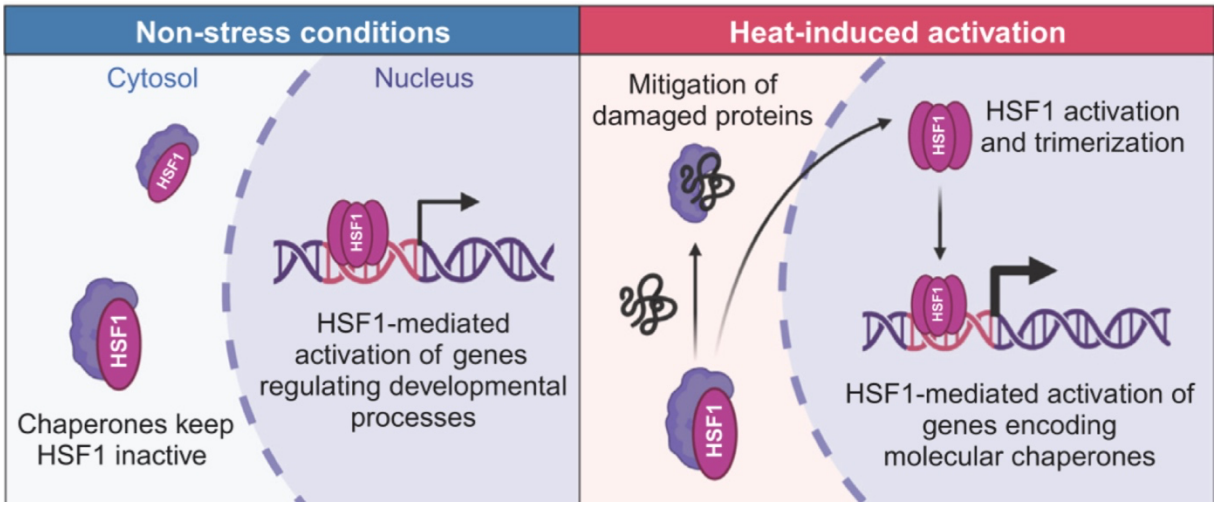

Figure 3. Schematic representation of the HSF1 and HSP activation-attenuation cycle that mediates the expression of HSP genes (Pessa et al., 2023).

1.7 Objective

The analysis of HSP70 expression provides a reliable alternative to monitoring behavioral traits in cattle for assessing heat stress. Given that HSP70 expression levels serve as a clear indicator of the onset and severity of heat stress, it can be utilized as an optimal biomarker to identify environmental conditions based on the Temperature-Humidity Index (THI) where heat stress poses a threat to cattle welfare and productivity. Recognizing the potential impact of heat-stressed cattle on Canadian beef production, this study aims to determine whether the presence of the SLICK phenotype incorporated into an Angus herd offers an advantage in mitigating heat stress. In order to confirm that the two groups used – traditional Angus type and Senepol-Angus hybrids – are phenotypically different, haircoat characteristics will be investigated by measuring hair length and diameter. To determine the difference in heat stress experienced, HSP70 gene expression levels will be quantified and compared between two cattle groups.

2. MATERIALS AND METHODS

2.1 Ethical Approval and Biosafety

An application for the use of animals in research was submitted to TRU's Animal Care Committee (ACC). Ethical approval for using the cattle was granted by ACC at Thompson Rivers University (File Number: 103700).

A Biohazardous Materials Application was submitted and approved by the Biosafety Committee at Thompson Rivers University (File Number: 103740). Biosafety modules were also

completed to work in a CL2 lab and approved by the Office of Safety & Emergency Management at Thompson Rivers University.

2.2 Herd Access and Animal Populations

All cattle used for this project were sampled at a farm in Heffley Creek, British Columbia (50.84632° N, 119.96735° W) with permission from Joanne Niklas, the farm owner. During two of the earlier sampling dates, 9 of the cattle used for testing spent time at Tod Mountain, before moving to the sampling location in August. Specifically, in June there were 5 SLICK cattle and 5 Angus type, while in August, there were 12 SLICK and 7 Angus type cattle. All cattle were approximately 4 months old at the beginning of sampling in June, and reported to be in good health during and between sampling times.

The two groups of cattle that were used include a traditional Angus type (Wild Type), and a Senepol-Angus hybrid (SLICK) that is composed of ¼ Angus and ¾ Senepol. The Senepol-Angus hybrid carries the SLICK1 mutation and exhibits the SLICK phenotype being assessed. The initial hybrid embryos were synthesized approximately 20 years ago and supplied to TRU by Davis Rairdan Embryo Transplants in Calgary, Alberta (Davis Rairdan, 2024).

2.3 Genotyping

To determine if cattle carried the SLICK1 mutation, all animals were genotyped for the rs517047397 deletion. Blood samples were taken from each calf and stored at 5°C until processed. DNA was extracted from each blood sample following the protocol supplied with the Qiagen DNeasy Blood & Tissue Kit (Qiagen). PCR amplicons were produced using a reaction containing: 0.1uL Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific), 2uL of 5X buffer, 1

uL 0.4mM dNTPs, 1 uL of each primer at 5pmol, 2 uL Betaine, 2 uL of 1.5 ng/uL to 20 ng/uL of template DNA, and 0.9 uL dH2O. The forward primer and reverse primer were 5' – CCTGGATCTTGACAGTGACTCTG – 3' and 5' – TTTGGGAACAGAGCCAGCAC – 3', respectively. All reactions were carried out with an annealing temperature of 62°C in a SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific). The ExoSAP-IT™ Express Kit (Thermo Fisher Scientific) was used to cleanup all PCR amplicons in preparation for Sanger sequencing. The sequencing reactions were carried out using the BigDye™ Terminator V3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and purified with the BigDye Xterminator™ Purification Kit (Thermo Fisher Scientific). Sanger sequencing was preformed using a SeqStudio™ machine (Thermo Fisher Scientific) and genotypes were determined by using the electropherograms that were produced. Only electropherograms with Phred scores above 30 were confidently used to determine the rs517047387 genotype.

All genotyping was completed by Robert J. Wester, MSc, at the Applied Genomics Centre, Kwantlen Polytechnic University.

2.4 Hair Measurement with ImageJ

Hair clippings were taken from animals using electric clippers by shaving a small portion (~5 x 5 cm) at the shoulder, and saving in separate paper envelopes for transportation. To accurately measure the length and diameter of hair strands, the imaging software ImageJ-2 (National Institutes of Health, USA) was used. When taking pictures of the hair pieces, a small ruler was placed in the frame to use as a reference for measurements and to convert from pixels to mm. Photos were uploaded to the computer and imported into ImageJ in JPEG format. To measure the diameter, images of hair samples were taken while using a dissecting microscope for better

resolution (Figure 4). To set a reference scale, the straight-line tool was selected and drawn across a known distance in mm, the number of pixels that related to that measurement was recorded. To measure the subject of interest, the straight-line tool was used, and the number of pixels related to that measurement was recorded. To measure the length of hair strands, each piece was taped with clear tape to a blank surface to ensure steady measurements. If hair pieces were wavy, the segmented-line tool was used to trace over each piece of hair, and the total pixel measurement was recorded. 5 hairs were selected from each individual for measurement of diameter and length.

Figure 4. Image taken of a hair follicle underneath a dissecting microscope and used for measurement with ImageJ-2.

In order convert the pixel measurement to metric measurements, the following formula was used:

$$Measurement\ in\ mm = \frac{Measurement\ in\ pixels}{Pixels\ per\ mm}$$

2.5 HSP70 Gene Expression Analysis

2.5.1 Collection of Hair Samples

For gene expression analysis, hair from the tail of each calf was used. Calves were contained in cattle-squeeze prior to pulling hair, and hairs were grasped as close to the base as possible to ensure the follicles were attached. After pulling hair samples from the tail, they were immediately placed in sterile 10mL Falcon tubes and the follicles were submerged in RNA*later*™ Stabilization Solution (Themo Fisher Scientific) to protect the RNA. The Falcon tubes were placed in a cooler at the collection site and then moved to Thompson Rivers University for long term storage at -20°C.

2.5.2 THI Measurements

Environmental conditions for the sample site at Heffley Creek, was obtained by entering the location into Visual Crossing Weather Data (Visual Crossing Corporation, 2024), and requesting the historical hourly weather data for the dates of interest. Once the query was approved, hourly weather data was downloaded and exported to Excel. The downloaded data was also compared with historical data supplied by other weather databases such as Weather Underground, AccuWeather, and The Weather Network. The herd was sampled at three separate points throughout the summer months and the environmental parameters from each collection are displayed on Table 1. Environmental conditions that the herd would have experienced in the 72 hours prior to sampling have also been collected. The formula developed by Ravagnolo et al. (2000) that is widely used to calculate THI measure heat stress in cattle (Dikmen et al., 2008;

Davila et al., 2019; Kim et al., 2020; Ekine-Dzvinu et al., 2020) is as follows:

THI = (1.8x T + 32) – [(0.55-0.0055 x RH) x (1.8 x T – 26)],

where T = dry bulb temperature (°C) and RH = relative humidity (%).

Table 1. Environmental conditions during hair sample collections.

Collection Date	Time	Temperature (C°)	Humidity (%)	THI
June 13th, 2023	9:00 AM	19.4	51.1	64.5
August 2nd, 2023	9:30 AM	20.5	36.0	56.9
August21st, 2023	10:00 AM	15.5	45.2	57.46

2.5.3 RNA Extraction

Hair samples frozen in RNA*later*™ (Thermo Fisher Scientific) were thawed on ice prior to homogenization. Approximately 15-20 hairs were placed follicle first into a 2.0 mL BeadBug™ (Millipore Solutions) tube and were cut approximately 1cm in length to fit. 1mL of TRIzol™ was added to each tube and samples were placed on ice. To homogenize the samples, they were placed on a desktop vortex machine at the maximum speed for a runtime of 30 seconds and rest time of 30 seconds on ice, which was repeated 3 times.

For phase separation, 200 μL of chloroform was added to each sample and hand shaken vigorously for 15 seconds, incubated at room temperature for 3 minutes and then centrifuged at 12,000 x g for 15 minutes at 4°C using a High Performance Refrigerated Microcentrifuge (Labnet International, Z233 Series). After centrifugation, the solution separated into a clear layer containing the RNA and a pink layer which contains DNA. 400 μL of the clear phase was

transferred into a RNase-free microcentrifuge tube with an equal volume of 70% ethanol and vortexed to disperse any visible precipitate that had formed.

A PureLink™ RNA Mini Kit (Thermo Fischer Scientific) was used for column purification of the sample. 400 µL of the sample was transferred to a Spin Cartridge and centrifuged at 12,000 x g for 15 seconds at room temperature; the flow-through was discarded. This step was repeated until the entire sample was processed through the Spin Cartridge. Next, 350 µl of Wash Buffer I was added to the Spin Cartridge, which was then centrifuged at 12,000 x g for 15 seconds at room temperature, and the flow-through was discarded. 80 µl of a DNase Treatment containing 80 µL of 10X DNase 1 Buffer, 100 µL of Resuspended DNase, and 620 µL of RNase Free Water was added directly onto the membrane and incubated at room temperature for 20 minutes. After incubation, 350 µl of Wash Buffer I was added to the Spin Cartridge, which was then centrifuged at 12,000 x g for 15 seconds at room temperature; the flow-through and collection tube were discarded. 500 µl of Wash Buffer II was then added to the Spin Cartridge, which was centrifuged at 12,000 x g for 15 seconds at room temperature, and the flow-through was discarded. This wash step was repeated once.

To dry the membrane with bound RNA, the Spin Cartridge was centrifuged at 12,000 x g for 1 minute. The collection tube was discarded, and the Cartridge was placed in a Recovery Tube. Then, 39 µl of RNase-free water was added to the center of the Spin Cartridge, incubated at room temperature for 1 minute, and centrifuged at 12,000 x g for 1 minute at room temperature. 1 µl of RiboLock RNase Inhibitor (40 U/µl) was added to the eluted sample, and the samples were placed in the -80°C freezer.

The purity and concentration of RNA samples was determined by using a Nanodrop One (Thermo Fischer Scientific), and gel electrophoresis was preformed to visualize the integrity of RNA samples.

2.5.4 cDNA Generation

A High-Capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific) was used to generate cDNA samples from RNA extractions. Prior to reactions, all regents from the kit and RNA samples from the -80°C freezer were thawed on ice. The High-Capacity cDNA Reverse Transcription Kit user manual was followed to prepare all reactions (Thermo Fischer Scientific, 2018). A 2X Master Mix was prepared for each reaction using the following components: 2.0 µl of 10X RT Buffer, 0.8 µl of 25X dNTP Mix (100 mM), 2.0 µl of 10X RT Random Primers, 1.0 µl of Multiscribe Reverse Transcriptase, and 4.2 µl of nuclease-free water, resulting in a total volume of 10 µl. The 2X Master Mix was gently mixed and kept on ice until use.

The volumes for this Master Mix are for one reaction and were upscaled based on how many samples were being processed.

For the reverse transcription reactions, 10 µL of the 2X Master Mix was pipetted into each 0.2 mL RNase-free PCR tube along with 10 µL of RNA sample. The capacity of the Reverse Transcriptase Kit was 2 µg of RNA, therefore some samples required dilution prior to carrying out the reactions. The reactions were briefly vortexed to ensure thorough mixing and then centrifuged to remove any bubbles. The tubes were kept on ice until they were loaded into the thermocycler.

The reverse transcription reaction was performed using the following thermal cycling conditions: Step 1 at 25°C for 10 minutes, Step 2 at 37°C for 120 minutes, Step 3 at 85°C for 5 minutes, and Step 4 at 4°C (or 10°C) for holding. The purity and concentration of cDNA samples was determined using a NanoDrop One. cDNA samples were diluted in autoclaved water and used directly for qPCR.

2.5.5 Quantitative PCR

Relative gene expression of HSP70 was measured through Quantitative PCR (qPCR). Custom primers for HSPA1A (HSP70 gene) and the endogenous controls, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-tubulin isoform 5, were designed with the Primer Express 3.0.1 Software (Life Technologies) and ordered through Custom Oligo Design Tools (ThermoFisher Scientific) in a dry format. Primer sequences for each gene are displayed in Table 3. Primer design was completed by Robert J. Wester, MSc, at the Applied Genomics Centre, Kwantlen Polytechnic University.

All qPCR reactions were performed in triplicate in a total reaction volume of 20µL per well, in a 96-well plate and consisted of 10µL of 2X PowerTrack™ SYBR Green Master Mix (Thermo Fisher Scientific), 1µL of each primer at 10µM, 2µL of autoclaved water, and 6µL of cDNA at approximately 20ng/µL. Thermal cycling conditions for all reactions are as follows: initial incubation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds. After, samples were heated to 95°C for 10 seconds and cooled to 65°C for 5 seconds, then reheated to 95°C at a rate of 0.5°C/second to produce a melt curve. All reactions were carried out and analyzed using a QuantStudio™ 3 (Thermo Fisher Scientific).

Table 2. Primer sequences used for gene expression analysis.

Gene	Primer Component	Sequence (5'-3')	Fluorescence
HSP70	Forward Reverse	CCGGTGCCCTGCCTTT GGCCGTTTTCAGGTTTGAAG	SYBR
GAPDH	Forward Reverse	CCCTCCACGATGCCAAAGT GGCGTGAACCACGAGAAGTATAA	SYBR
β-Tubulin	Forward Reverse	GCTGTTCTTATTCTGCACGTTGA CGTGGGCGGATGTCCAT	SYBR

The QuantStudio 3™ instrument and corresponding Design and Analysis Software 2.8.0 (Thermo Fisher Scientific), were used to collect and analyze cycle threshold (Ct) values. Analysis of gene expression data was completed through normalization of the target gene to control genes by utilizing part of the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). To examine the relative expression of HSP 70 between wild-type and SLICK cattle relative to the control genes the following equation is used:

$$\Delta Ct = (Ct_{target}) - (Ct_{control})$$

where Ct_{target} is the mean Ct value for HSP70 gene expression and $Ct_{control}$ is the mean Ct value for endogenous control genes, GAPDH and β-tubulin. The ΔCt values for HSP 70 Angus type and SLICK cattle were compared using a t-test to determine the presence of a significant difference in expression levels. To display the relative expression between the two groups, values were displayed using: $2^{-\Delta Ct}$.

2.6 Statistical Analysis

To assess the statistical significance between groups for both hair coat characteristics and gene expression levels, independent t-tests were performed. These analyses were conducted in Microsoft Excel, employing a two-tailed distribution with a two-sample unequal variance (heteroscedastic) assumption. A p-value threshold of 0.05 was used to determine statistical significance between selected data sets.

3. RESULTS

3.1 ImageJ Hair Measurements

Hair samples were taken for measurement from animals exhibiting both traditional Angus, and SLICK cattle (Senepol x Angus cross) phenotypes. All hair pieces selected for measurement included the follicle and natural end of strand. Table 3 reports values for hair length and diameter from each group as mean ± standard deviation. Overall, there was a large variation in hair length observed in both groups and an expected difference in the average hair length between groups (P = 0.0287). Less variation in diameter was measured and there is no significant difference between the two groups (p = 0.841).

Table 3. Measurements for hair characteristics pulled from Angus and SLICK cattle.

	n	Hair Length	Hair Diameter
SLICK	65	37.059 ± 8.836	0.084 ± 0.0083
Angus Type	30	51.549 ± 10.735	0.082 ± 0.0243
p value		0.0287	0.841

3.2 Genotypes

Genotypes for all animals were successfully obtained from blood samples to determine the presence of the SLICK1 mutation.

Table 4. Animals used in the assessment of the SLICK Phenotype with corresponding genotypes.

Cattle ID	PRLR_387 Genotype	Phenotype
H1	CC	Wild type
H2	CC	Wild type
H3	CC	Wild type
L1	NN	SLICK
L2*	NC	SLICK
L3*	NN	SLICK
L4	NC	SLICK
L5	NN	SLICK
L6*	NN	SLICK
L7*	NN	SLICK
L8*	NC	SLICK
L9	CC	Wild type
L10	NN	SLICK
L11	NC	SLICK
L12*	NC	SLICK
L13*	CC	Wild type
L14*	NC	SLICK
L15*	NC	SLICK
L16	CC	Wild type

Note: N represents the deletion associated with the SLICK phenotype. NN and CC indicate homozygous, NC indicates heterozygous, Cattle ID* indicate cattle that were not available for all sampling date.

3.3 RNA Extractions

All RNA extractions that were preformed using samples that had been properly stored were successful and yielded intact RNA for further application. RNA concentrations ranged from 254.2 ng/µL to 658.1 ng/µL with an average of 435.9 ng/µL. 260/280 ratios ranged from 1.85 to 2.10, with an average of 2.03. Better readings for the 260/280 ratios were obtained when the PureLink RNA columns were treated with DNase for 20 minutes, rather than 15 minutes. All RNA extractions were run on a 1% agarose gel in 1X TBE buffer for visualization; 10 extractions are displayed in Figure 5. The most noticeable and brightest bands are consistent with the highly conserved 28S and 18S rRNA bands that should be predominant in eukaryotic organisms. Other bands present are as expected from other mRNAs, small RNAs and long-noncoding RNAs.

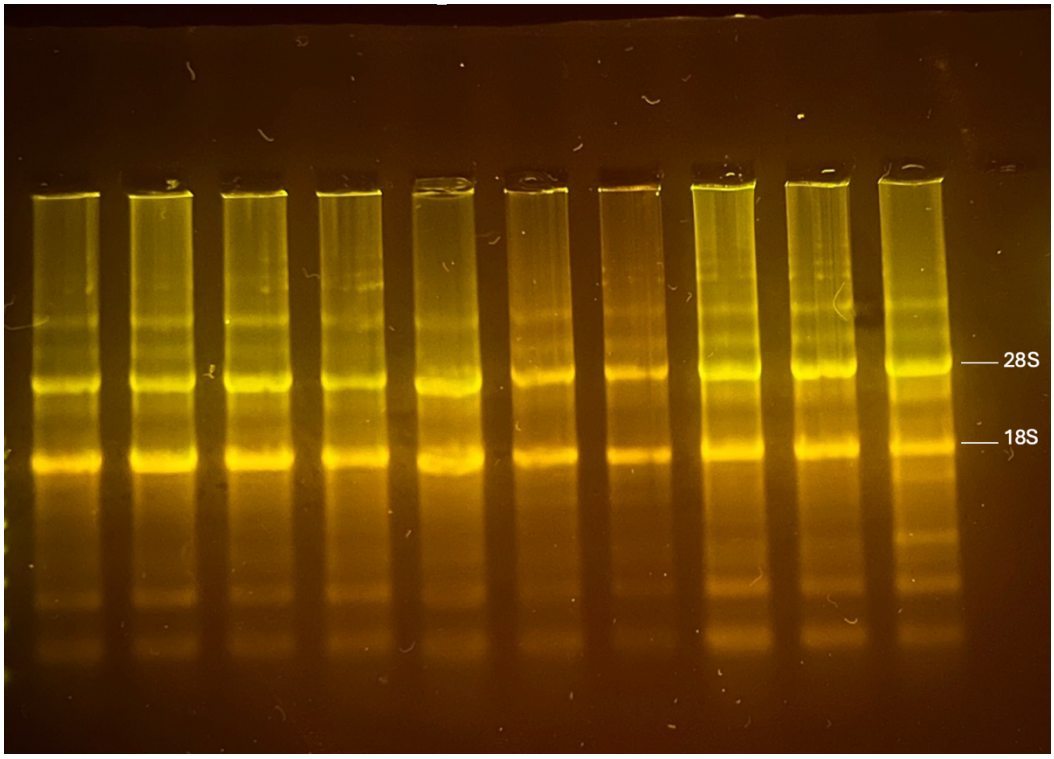

Figure 5. Gel electrophoresis of 10 RNA extractions used to visualize the integrity and quantity of samples.

3.4 cDNA Generation

All reverse-transcription reactions were performed by following the manufacturers instruction manual to generate single stranded, first strand cDNA as the final product. The reverse-transcription PCR conditions did not include amplification cycles and the MultiScribe™ Reverse Transcriptase has RNase H activity, therefore a 1:1 conversion of RNA to cDNA was assumed. 260/280 ratios of cDNA samples ranged from 1.84 to 1.99, with an average of 1.89. Figure 6 includes gel electrophoresis that confirmed cDNA generation and functioning MultiScribe™ Reverse Transcriptase.

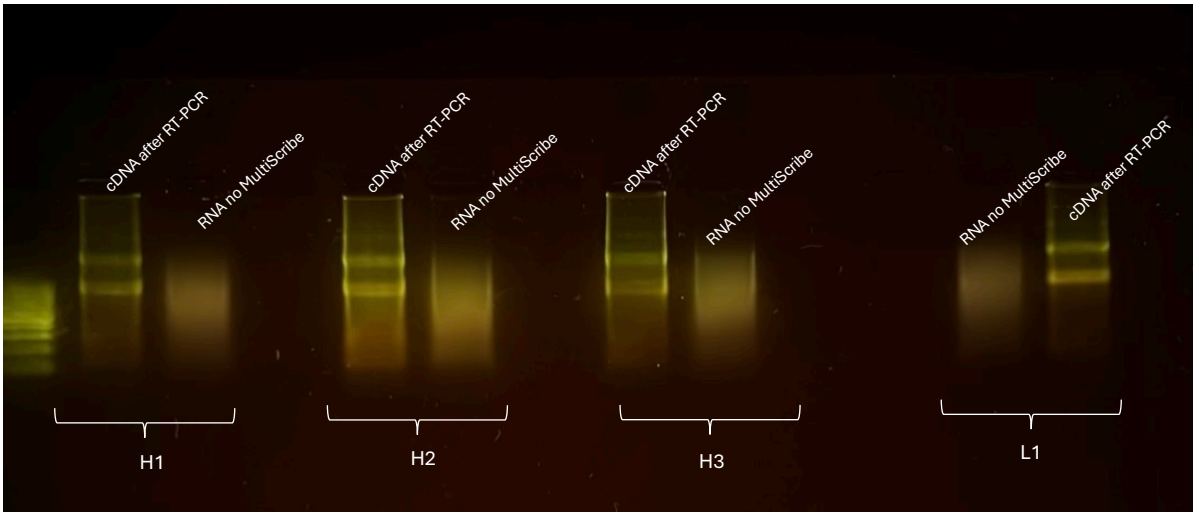

Figure 6. Reverse transcription products run alongside respective RNA sample run without MultiScribe Reverse Transcriptase.

3.5 HSP70 Gene Expression Analysis

HSP70 gene expression in cattle was determined by using qPCR and calculating the difference in expression to reference genes. The relative expression values for HSP70 between SLICK and Angus type cattle, were determined using $2^{(-\Delta Ct)}$ and are displayed in Figure 7. The difference in cycle threshold values (ΔCt) from the housekeeping genes is presented in Table 5 as mean ± standard deviation. All ΔCt values report that the HSP70 gene was downregulated in

respect to the housekeeping genes. Data collected on June 13th does not have a statistically significant difference of HSP70 gene expression between SLICK and Wild type cattle (p=0.210). Data from August 21st does report a statistically significant difference in HSP70 gene expression between the two cattle groups (p=0.048). The hourly THI for the 72 hours prior to sample collection is displayed in Figures 8a. and 8b. Samples collected August 2nd, 2023, were ineligible due to incorrect storage of RNA extractions.

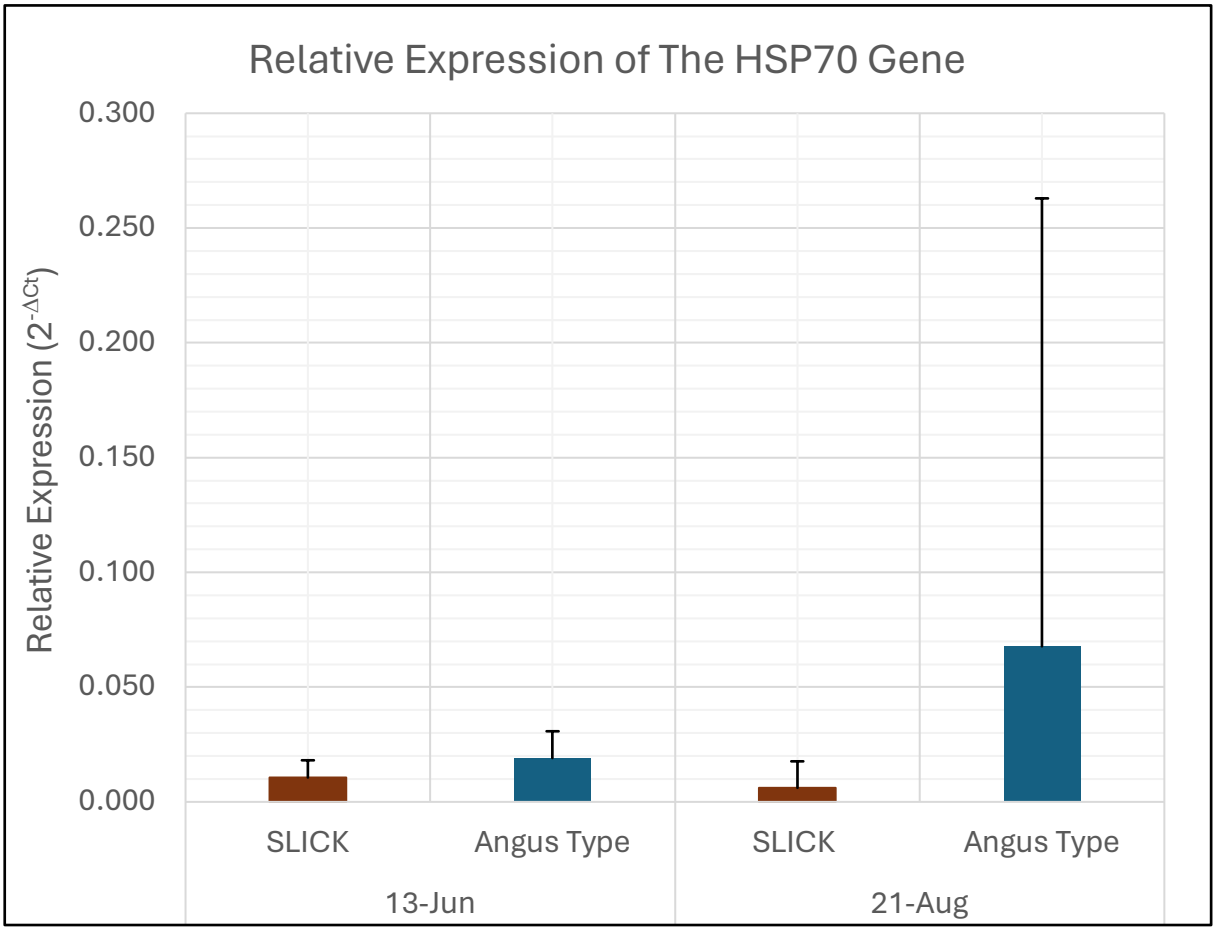

Figure 7. Bar graph of the median HSP70 gene expression ($2^{-\Delta Ct}$) for each cattle group during different sample collection dates.

Table 5. Difference in cycle threshold values between HSP70 and endogenous controls (ΔCt).

Sampling Date	Cattle Group	Mean Relative Expression (ΔCt)	Standard Deviation	p value
June 13th	SLICK	6.557	0.880	0.210
	Wild type	5.7096	1.072	
August 21st	SLICK	7.351	2.721	0.048
	Wild type	3.883	4.153	

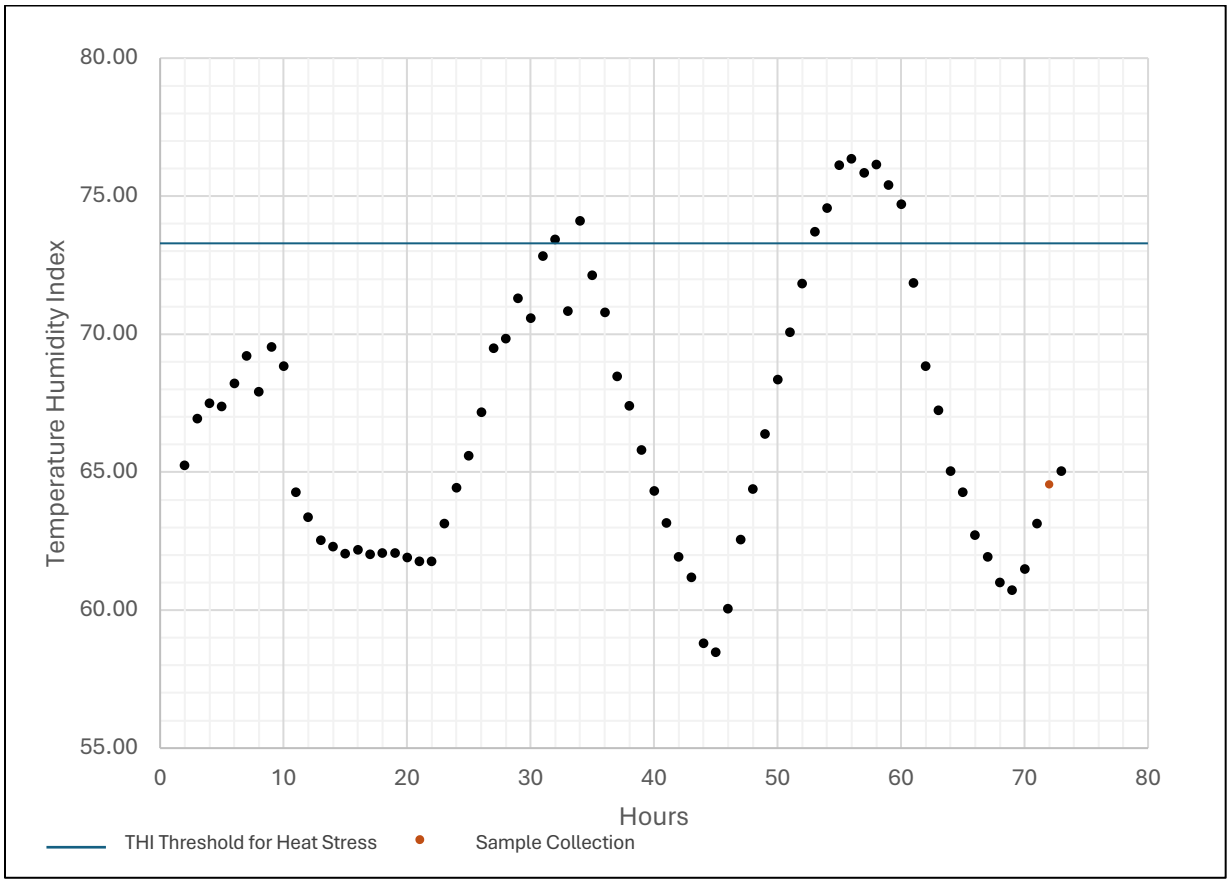

Figure 8.a. Hourly THI for the 72 hours prior to sample collection on June 13th, 2023.

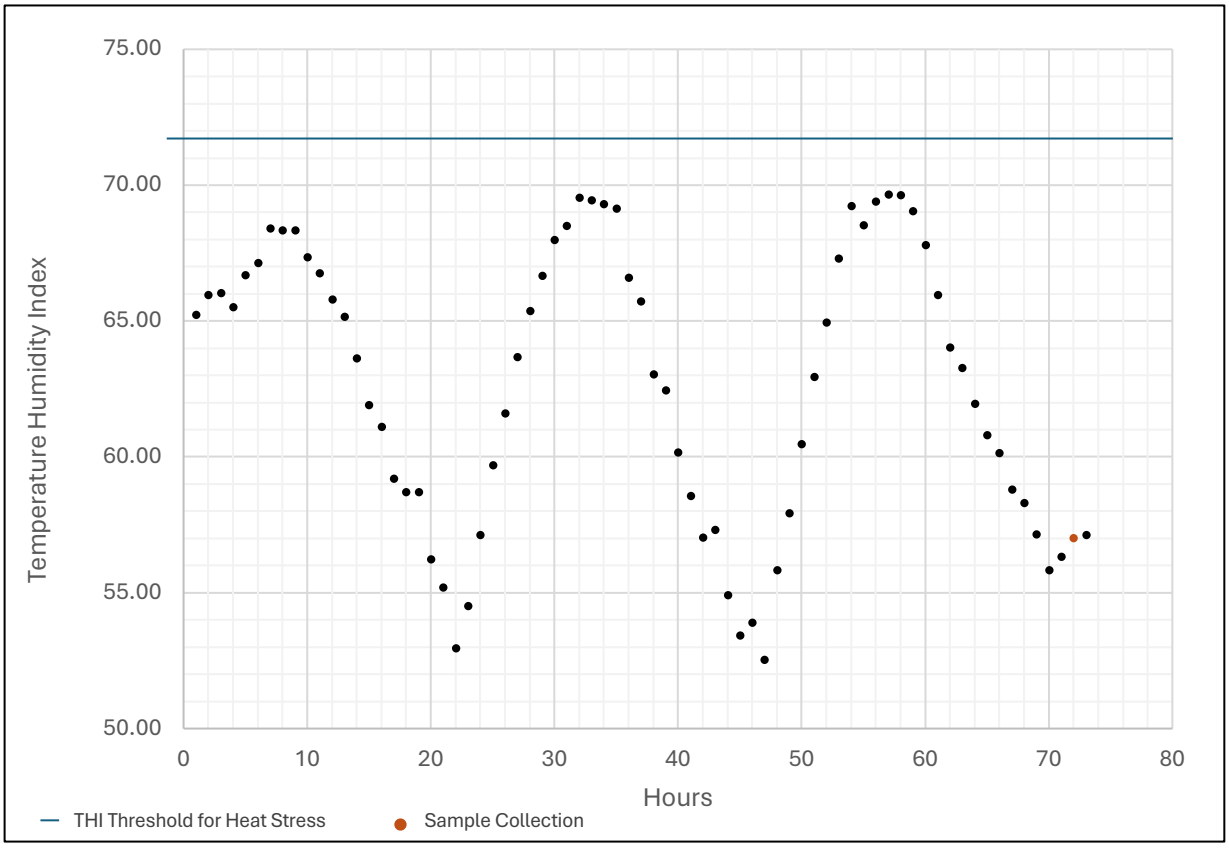

Figure 8.b. Hourly THI for the 72 hours prior to sample collection on August 21st, 2023.

4 DISCUSSION

This study aimed to assess the effectiveness of the SLICK phenotype, caused by the SLICK1 mutation, on the thermotolerant capabilities of cattle. Two groups of cattle, Red Angus (Wild type) and Angus-Senepol hybrids (¼ Angus, ¾ Senepol; SLICK), were used to analyze the haircoat properties of the phenotype, while HSP70 gene expression levels were studied as an indicator of heat stress. Hair coat analysis determined a statistically significant difference between hair lengths. Furthermore, the HSP70 gene showed varying levels of expression between the two groups of cattle during different THI conditions.

4.1 Hair Coat Measurements

Hair strands were collected form cattle in order to confirm that the Angus-Senepol hybrids that have inherited the SLICK1 mutation do exhibit a noticeably different hair coat physiology than the purebred Red Angus group. As expected, the SLICK cattle presented with a significantly shorter haircoats, averaging 3.7 cm, compared to Angus' 5.15 cm ($p = 0.0287$). Hair length is an important factor that can impact the efficacy of evaporative cooling from the skin. Because cattle dissipate excess heat through cutaneous EVHL, a longer hair coat becomes an obstruction to free evaporation of sweat from the skin surface and reducing the animals 'ability to regulate body temperature. Confirmation that the two cattle groups present with different hair coat lengths suggests that any observed differences in heat stress could be attributed to variation in their EVHL capacity.

Hair diameter has also been found to affect EVHL as it can impact the airflow at the skins surface (Collier et al, 2014). The diameter of hair pieces was also measured but did not report a significant difference between the two cattle groups ($P = 0.841$). Although this does not allow us to confirm that hair diameter is a major factor in differentiating thermoregulatory abilities between the two cattle groups, it is still possible that hair diameter and hair density combined can affect airflow regulation at the skins surface.

Another reason that SLICK cattle are better able to regulate their body temperature is due to their increased sweating rate, as demonstrated by Dikmen at al. (2008). This trait is affected by properties such as sweat gland density and size. As explained by Collier et al. (2008), cattle have apocrine sweat glands – there is one sweat gland associated with each hair fiber – therefore hair density directly affects the number of sweat glands. Unfortunately, the exact area from which cattle were shaved for hair samples was not recorded and some hair breakage occurred. As a result, it

was not possible to accurately calculate the number of hair strands per unit area or determine hair coat density for each group.

Furthermore, although hair diameter measurements were taken, they are not indicative of sweat gland size. According to Hernandez et al. (2024) and Mateescu et al. (2023), skin biopsies are required to measure sweat gland size in cattle. Both of these studies explored the effect of sweat glad characteristics for heat tolerance on beef cattle. Although they did not investigate the same breed of SLICK cattle, both reported a difference in sweat gland area between Angus and Brahman cattle, another heat tolerant breed (Mateescu et al, 2023; Hernandez et al, 2024).

4.2 Genotypes

The genotyping results allowed us to confirm which cattle carried the SLICK1 mutation in order to confidently split the herd into two groups based on genotype and not phenotype alone. The genotypes align with the observed phenotypic differences and corresponding hair lengths. Although the genotypes themselves do not warrant an extensive discussion, they successfully provided a genetic basis needed to organize the other results from this study.

4.3 RNA Extractions and cDNA Generations

Individual RNA extractions were required to analyze HSP70 gene expression levels in each animal studied. Although the RNA extractions themselves are a routine part to quantify gene expression, the successful extracts validate the modified RNA extraction method that was used. RNA extractions that were not successful and yielded a poor-quality sample, came from hair follicles that were not correctly stored and ended up degrading. The successful cDNA generations also indicated that the RNA was intact and suitable for further applications. Results from the qPCR

reactions confirmed that cDNA generations were successful and intact to yield gene expression results.

4.4 HSP70 Gene Expression Analysis

To evaluate the efficacy of the SLICK phenotype on thermotolerance, HSP70 gene expression levels were measured as an indicator of a heat stress response. We predicted that because the Angus-Senepol hybrids (SLICK) presented with a shorter haircoat, they would have an advantage in heat dissipation and consequently experience less heat stress, resulting in lower HSP70 expression compared to the Angus (Wild type) group under the same conditions. The relative expression of HSP70 ($2^{-\Delta Ct}$) between SLICK and Angus cattle were compared for each individual sampling condition.

Overall, we found inconsistent HSP70 gene expression results in relation to the respective THI conditions. Both groups of cattle demonstrated an under expression of HSP70 in relation to the housekeeping genes (positive ΔCt values) for June and August sampling times. Although the expression levels of HSP70 genes were low—falling below those of the housekeeping genes—expression was still detectable, indicating that some activation of HSP70 occurred in both cattle groups.

Data collected from the June sampling period indicates that there was no significant difference in HSP70 gene expression between the two cattle groups ($p=0.210$). When comparing the THI conditions that the cattle would have experiences for 3 days prior to collection, it is notable that the THI does pass the threshold for heat stress (72), reaching 76.4. As no significant differences were found between the two groups, we can speculate that similar HSP70 responses were induced in both groups. Although there was no significant difference between ΔCt values,

the $2^{-\Delta Ct}$ values presented in Figure 7 display a higher expression of HSP70 in Red Angus cattle than in SLICK cattle, which aligns with our hypothesis that SLICK cattle experience less heat stress.

As for data collected in August, the difference in HSP70 expression between the cattle groups is significant ($P = 0.048$). This is unexpected given the THI for the 3 days prior to collection of these samples did not cross the threshold for heat stress. It would be expected that in environmental conditions where the THI does not reach a point high enough to induce heat stress, there should be little to no HSP70 gene expression. When examining the $2^{(-\Delta Ct)}$ values for the SLICK group between June and August, the decrease in HSP70 expression aligns with expectations. However, the Angus group displayed an unexpected increase in HSP70 expression despite the lower THI conditions, which may be reflective of the cumulative heat stress experienced by the Angus (Wild type) throughout the summer.

Because the sampling date was later in the summer, it is important to consider not only the intensity of the THI, but also the duration and the heat stress response it may elicit. A study performed by Ouellet et al. (2019), showed that prolonged exposure to THI above 65 negatively impacted the production of dairy cattle in Quebec, Canada. Given that the cattle in the present study consistently experienced conditions where the THI exceeded 65 throughout the summer – reaching a high of 69 in the days before sampling – this prolonged exposure may have contributed to the unexpected increase in HSP70 expression observed in the Angus group. Although the THI recorded in the days prior to sample collection in August did not cross the heat stress threshold, the cumulative effects of sustained, lower-level heat stress over the course of hotter months, may have resulted in a delayed or prolonged stress response. This persistent environmental pressure

could explain why the Angus cattle group exhibited higher HSP70 expression, compared to the SLICK cattle, even in the absence of high THI conditions.

It is also possible a major heat stress event occurred earlier than the THI conditions displayed in Figure 8b. Previous research conducted by Kim et al. (2020) indicates that HSP70 expression levels increase, and peak at 3 days following a heat stress event, before returning to baseline within 6 days, when the cattle remain in the same environmental conditions. This expression pattern may align with the data collected, suggesting that the increase in HSP70 expression observed in the Angus (Wild type) group could be linked to a previous heat stress event. However, it should be noted that Kim et al. (2020) observed this pattern in cattle that experienced THI conditions of 88.

There is also the speculation that the age of animals during sampling could play a role in HSP70 gene expression, as many genes are affected by age (Kim et al, 2020). A study presented by Kaushik et al. (2022) investigated the differential expression of HSP70 in ruminants during a growth phase in response to heat stress. Although they recorded a noticeable difference in HSP70 gene expression between heat-stress tolerant versus heat-stress susceptible goats, they also noticed significantly higher mRNA expression in animals at 9 months of age compared to all other age groups. In addition to a protective role, HSP70 can also be involved in cell growth and proliferation (Kaushik et al, 2022). Given that the animals used in this study were less than a year old, it is possible we had sampled during a growth phase which may provide an explanation for the jump in relative HSP70 gene expression seen in the Angus type group in August. Because the two groups of cattle are composed of different lineages, growth phases may not be identical. However, these hypotheses would need to be further investigated in respect to calf development stages.

Another explanation for this discrepancy may be due to the increase in cattle sampled in August versus in June. It should also be considered that the additional cattle did not experience the same conditions as the original cattle for the previous months. As mentioned before, 9 of the cattle available in August were previously kept at Tod Mountain during earlier sampling dates. The addition of new cattle, majority of which were SLICK, altered the sample sizes and increased the standard deviation values for both groups. The sample sizes changed from n = 6 to n = 12 for the SLICK group and from n = 6 to n = 7 for the Angus (Wild type) group. The standard deviations increased from 1.07 and 0.88, to 2.72 and 4.15 for the SLICK and Angus (Wild type) groups, respectively. These cattle may have had an increased HSP70 gene expression for reasons aside from heat stress, as mentioned above. This is reflected in Figure 7, as the error bars for the Angus (Wild type) group are noticeably larger than the rest, indicating the presence of an outlier.

Methodological explanations for the unexpected HSP70 expression levels observed from the Angus type group may be due to non-specific amplification or primer dimers detected during qPCR analysis. The gene expression assay was changed from TaqMan to SYBR Green chemistries due to problems experienced with the TaqMan assay probes. However, forward and reverse primers were not complementary to each other and melt curves obtained from qPCR analysis suggests that this did not happen. It is also unlikely that these errors would have occurred among one set on samples and not the others.

Overall, HSP70 gene expression levels obtained from the two cattle groups did not correlate with the THI conditions. Given that other studies with similar objectives have consistently reported significant findings, suggests that the environment where these cattle were raised may be beneficial for mitigating heat stress. For instance, Kim et al. (2020) observed a significant difference in HSP70 expression ($P = 0.0120$) in beef calves under different sampling

conditions in Korea, where the THI reached 83.04. Similarly, Taborda-Charris et al. (2023) reported a significant change in HSP70 expression ($P = 0.0011$) in cattle (from Northern Colombia) as the THI increased from 76 to 83.

Due to our sampling site being located just 20 minutes from Sun Peaks Mountain, the local geography may have limited the occurrence and duration of high THI values experienced by cattle. While this may have prevented us from observing significant and consistent changes in HSP70 expression, it also suggests that moderate environments like Heffley Creek offer optimal conditions to raise beef cattle and limit heat stress over temperate or tropical conditions.

5 CONCLUSION AND FUTURE WORK

The increasing risk that climate change and rising temperatures poses for cattle production, met with the increasing global demand for high quality meat and dairy products – particularly Canadian beef – highlights the need for effective strategies to mitigate heat stress in livestock. A promising solution to alleviate the heat stress experienced by cattle is the introduction of the SLICK1 mutation, and corresponding SLICK phenotype. To determine if the SLICK phenotype was effective at increasing the thermotolerance of Canada's top beef producing breed, Red Angus cattle, we investigated the expression levels of the HSP70 gene.

While we did confirm that the Angus (Wild type) cattle and Angus-Senepol hybrid (SLICK) cattle differed in haircoat morphology, HSP70 gene expression results were inconsistent. The identification of the SLICK1 mutation, along with its associated shorter hair length and the variability in other haircoat characteristics observed in supporting research, suggests that there is an opportunity to improve cutaneous EVHL in production cattle. Moreover, selecting for these

traits in beef cattle could improve resilience to heat stress without compromising production performance.

In order to really determine if the SLICK1 mutation is beneficial to Red Angus beef cattle, future research would have to include more consistent sampling times before, during, and after known heat stress events, where the THI exceeds the stress threshold for longer amounts of time. It would be ideal to extract samples from the same herd over multiple hours and days in order to more thoroughly investigate the fold changes in HSP70 expression within each group of cattle. In order to better characterize the hair coat morphology of each cattle group, future work may include skin biopsies where hair follicle and sweat gland density could be measured. The difference in pore size for sweat excretion could be determined by subtracting the diameter of hair from the area to provide an idea of how much room there is for sweat dissipation at the skins surface. If future studies identify differences in haircoat traits between Wild type and SLICK cattle, and establish a relationship with EVHL rates, these may be traits that could be selected for in the future to improve heat tolerance.

5.1 LIMITATIONS

The major limitation in this study was herd access. Sampling times had to work within the availability of the farm owner which limited when samples from calves could be taken. Furthermore, although the hair tail extractions are not considered invasive, the actual process was timely to separate each calf from the herd and could be stressful, which we did not want to subject young calves to repeatedly. Another limitation that could have supported our results was that we did not consider other physiological parameters that could indicate heat stress such as core body temperature and respiration rates.

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