CLIMATE MASTER CATTLE PROJECT: FIRST STEPS IN DEVELOPING CLIMATE RESILIENT BEEF CATTLE WITH SUPERIOR MEAT QUALITY USING GENETIC SELECTION

by

ROBERT J. WESTER

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Thesis Examining Committee:

John Church (PhD), Professor, Cattle Research Chair and Thesis Supervisor, Natural Resource Science.

Joanna Urban (PhD), Associate Teaching Professor and Co-supervisor, Biology.

Paul Adams (PhD), Biology and Health Science Instructor, Director of The Applied Genomics Centre at Kwantlen Polytechnic University and Committee Member.

Dipesh Prema (PhD), Associate Professor and Committee Member, Chemistry.

Elsa Vasseur (PhD), Associate Professor at McGill University, Innovation and Research Chair in Animal Welfare and Artificial Intelligence (WELL-E) and External Examiner

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Abstract

Global climate change poses significant challenges to the Canadian beef industry, necessitating innovative strategies to mitigate its impacts while ensuring sustainable production and high-quality meat. This work explores the first steps in the development of a novel *Bos taurus* breed, termed Climate Master Composite cattle, aimed at enhancing climate resilience and meat quality traits in response to rising temperatures and changing environmental conditions.

Rapidly increasing global temperatures due to climate change are exerting pressure on the Canadian beef industry, requiring adaptation strategies to maintain productivity to reduce economic losses. Cattle heat stress, the imbalance of heat absorption and dissipation, can have many negative consequences when animals experience it for prolonged periods. Consequences include reduced growth rates, lower fertility, poor meat quality, and increased mortality rates. The introduction of thermotolerant traits, such as the SLICK phenotype, holds promise for improving cattle health and wellbeing in the face of rising global temperatures. Adoption of thermotolerant traits is important for beef consumers and farmers by reducing losses and increasing availability. However, many other cattle traits also shape beef markets, including polledness and marbling. Polledness, or the lack of horns, promotes better cattle temperaments, and reduces risk of injury to farmers and nearby animals. While farmers would benefit from thermotolerant and polled cattle, these traits lack direct importance when consumers are purchasing beef. The tenderness and flavour of beef, roughly estimated by marbling, are attributes that impact consumer purchasing decisions. Therefore, producing a novel composite cattle breed that is thermotolerant, polled, and has superior marbling characteristics would be desired for both producers and consumers.

In Chapter 2, genetic markers associated with the SLICK and polled phenotypes were identified through a literature search and by targeted genotyping methods which were used to assess the predicting capability of these markers on their respective phenotype. One marker for each trait was determined to be the best predictor of the trait, providing a cost-effective and accurate method of tracking these traits in *Bos taurus*

cattle. These markers were then utilized to track the SLICK and polled traits throughout the breeding program implemented in this project.

In Chapter 3, an assessment of the heat tolerance capabilities of the Climate Master Hybrid (3/8 Senepol, 1/8 Red Angus, and 1/2 Galloway) composite breed with SLICK revealed superior performance in mitigating the effects of heat stress. This was characterized by reduced respiration rates and increased activity levels under heat stress conditions. These findings underscore the potential of the SLICK trait to mitigate the adverse effects of climate-induced heat stress on cattle welfare and productivity in temperate environments.

In Chapter 4, an investigation into the genetic markers associated with the marbling traits in Akaushi cattle was conducted using targeted genotyping and a genome-wide association study (GWAS). The GWAS used a mock dataset predicting the marbling characteristics of the cattle. It identified significant SNPs associated with the predicted marbling characteristics of Akaushi cattle. While the genetic markers may not correlate with the true marbling characteristics, they can provide insight on the genetic differences in Akaushi. Further investigations are required to determine the genetic markers associated with Akaushi marbling utilizing actual marbling characteristic data to be able to predict marbling characteristics throughout the breeding program in this project.

In conclusion, the Climate Master Cattle project represents a pioneering effort to address the dual challenge of integrating genetics to create climate resistant animals in the face of increasingly volatile global temperatures, while still maintaining the appropriate genetics to ensure the highest meat quality for the Canadian beef industry. By leveraging genetic selection and innovative breeding strategies, this project aims to improve resilience, sustainability, and profitability in beef production.

Keywords: SLICK, heat stress, SNP, marbling

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GDP: gross domestic product.

DFD: dark, firm, dry.

***HSP70*:** heat shock protein 70 gene.

SNP: single nucleotide polymorphism.

***PRLR*:** prolactin receptor gene.

MUFA: monounsaturated fatty acid.

BMS: beef marbling standard.

BTA: *Bos taurus* autosome.

PCR: polymerase chain reaction.

BLAST: basic local alignment search tool.

FLA: fragment length analysis

THI: temperature-humidity index.

T_R: rumen temperature.

A_I: activity index.

cDNA: complementary DNA.

qPCR: quantitative PCR OR real-time PCR.

FDR: false discovery rate.

ELISA: enzyme-linked immunosorbent assay.

GWAS: genome-wide association study.

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CHAPTER 1: General Introduction

Global temperatures are rising rapidly due to climate change, exerting pressure on the beef industry to maintain their production rate amidst the hardships of hotter climates (Farm Credit Canada, n.d.; IPCC, 2022). Adoption of heat tolerant traits through the introduction of the SLICK genotype into beef cattle could provide superior survivability during higher temperatures (Dikmen et al., 2014). In addition, beef production can also be made easier by selecting for the polled (hornless) trait, which reduces the risk of injury for both cattle and handlers (Thompson et al., 2017). And finally, although the SLICK and polled phenotypes can potentially improve beef production, generating superior carcass characteristics is paramount for the profitability of the beef cattle industry (Grigoletto et al., 2020). Of particular value is the highly popular trait of carcass marbling. Marbling is a staple in luxury beef markets and is characterized by high intramuscular fat content (Motoyama et al., 2016). In the Climate Master Cattle project, we are combining the Red Angus, Senepol, Galloway, and Akaushi *Bos taurus* breeds with the objective of producing a novel synthetic breed called the Climate Master Composite. The overall goal is to produce a polled composite cattle breed that can withstand both hot and cold temperature extremes while exhibiting superior marbling traits.

This introduction aims to explore the importance of the Canadian beef industry, trends and factors in climate change, the impacts of bovine heat stress, the thermotolerant qualities of the SLICK phenotype, the impacts of cold stress, the benefits of polled cattle, and the characteristics of carcass marbling. Additionally, it will cover the goals and plan of the Climate Master Cattle project and outlines how this thesis fits within that framework.

Canadian Beef Industry Economics

The beef cattle sector plays a significant role in Canada's economy, accounting for approximately 21.8 billion dollars of the gross domestic product (GDP) in 2018 – 2020 and approximately 24 billion dollars of the GDP in 2020 – 2022 (Agriculture and Agri-Food Canada, 2022; Canadian Cattle Association, 2024). Throughout 2019 – 2022,

Canada produced an average of 1,335,000 metric tons of beef, imported an average of 219,750 metric tons of beef, and exported an average of 554,500 metric tons of beef (USDA Foreign Agricultural Service, 2023a). Meat consumption generally follows fluctuations in the economy, often reducing the demand of beef and other red meats when prices rise and/or incomes drop (Farm Credit Canada, n.d.). Canada's GDP per capita increased by an average of $3,387 between 2019 and 2021, however, individual median income only increased by an average of $1,630 between 2019 and 2021 (International Monetary Fund, 2024; StatCan, 2023b). While median income is increasing, so are beef prices. For example, sirloin steak prices were an average of $18.57 per kilogram in 2022, which was 11.76% higher than the average price in 2021 (StatCan, 2023c). Throughout 2019 – 2022 Canadians consumed an average of 26.9 kilograms of beef per capita with an average decrease of 0.2 kilograms per year (USDA Foreign Agricultural Service, 2023). This decrease in consumption could be contributed to many factors including increases in beef prices, beef availability, and shifting consumer diets. During 2019 – 2022, the average available volume of beef for consumption was 17.69 kilograms per capita, well below the consumption rate, effectively driving prices up (StatCan, 2023a)). In 2018, 6.76% of Canadians were vegetarian, which has increased to approximately 9% in 2022 (Statista, 2023), and projections are that diets with less beef included in them will continue to increase in Canada. Despite these trends in reduced consumption of beef by Canadians, Canadian beef production still does not meet the consumption demand without imports. Therefore, it is critical that the Canadian beef market increases production to meet consumer demands.

Climate Change and Cattle Industry

As of 2022, the average global surface temperature has increased by 1.01°C since 1880, with an expected further increase to 1.5°C by 2040 (*Climate Change*, n.d.; IPCC, 2022). In Canada, the annual average temperature has increased by 1.7°C between 1948 and 2016 (Zhang et al., 2019). Northern Canada experienced an increase of 2.3°C in that same period (Zhang et al., 2019). These climate changes disrupt weather patterns, escalating the magnitude of the natural climate variabilities to become much more volatile; and increasing the frequency of extreme weather events, such as heat domes,

atmospheric rivers, and polar vortexes (Corringham et al., 2022; NOAA, 2021; Wang et al., 2023; Zhang et al., 2023). Heat domes are an atmospheric circulation anomaly in which high atmospheric pressure traps hot air from heat waves, extending their duration (Zhang et al., 2023). Atmospheric rivers are narrow transport corridors of concentrated water vapour within the lower troposphere, responsible for continental precipitation and flooding events along coastlines (Corringham et al., 2022; Wang et al., 2023). Polar vortexes are periods of severe cold resulting from unstable polar jet streams weakened by rising temperatures, pushing Arctic air to mid-latitude regions (NOAA, 2021).

Consequently, these extreme weather events, and climate change, have generated and intensified burdens on the ecosystem, health and wellbeing, infrastructure, water availability and agriculture in Canada and on a global scale (IPCC, 2022). For agriculture, heat domes and extreme heat waves can cause severe heat stress in livestock, causing various issues, including surges in mortalities and economic losses (IPCC, 2022; Lees et al., 2019). For example, the 2021 Northwest heat dome caused over 650,000 livestock deaths in British Columbia (Environment and Climate Change Canada, 2021). Furthermore, an estimated 10,000 feedlot cattle were reported dead because of heat stress from an extreme heat wave in early June 2022 across Kansas, United States (Myers, 2022). Atmospheric rivers and polar vortexes also pose major detrimental implications to livestock (NOAA, 2021; Wang et al., 2023). The 2021 Pacific Northwest floods, caused by an atmospheric river, led to thousands of livestock deaths, and displaced many cattle across Abbotsford and Sumas Prairie, British Columbia (Kassam, 2021). Additionally, polar vortexes, like the 2019 North American cold wave, led to meat and crop production operations to temporarily shutdown, reducing overall food production (Selby, 2019). In Northern regions such as Canada these cold temperature oscillations can significantly reduce the feed efficiency and subsequent growth rates in cattle (Toghiani et al., 2020).

Climate change itself is caused by a variety of forces including natural (solar and volcanic), greenhouse gas emissions, and other anthropogenic factors (Hegerl et al., 2019). Of these factors, greenhouse gas emissions appear to be a primary driving factor in climate change (Hegerl et al., 2019). In 2020, a total of approximately 48.45 billion tonnes (CO_2 equivalent) of greenhouse gasses were emitted globally; agriculture

accounted for 12.36% of these emissions (Ritchie et al., 2024). When considering just methane and nitrous oxide emissions the actions of the agricultural industry accounts for 43.01% and 81.47% of emissions, respectively (Grossi et al., 2019; Ritchie et al., 2024). Of the global agricultural greenhouse gas emissions, 39.3% are directly from enteric fermentation, while 22% arise from manure management (Arcipowska et al., 2019). As cattle make up approximately 74.5% of global ruminant livestock biomass, it is apparent that cattle farming is a significant factor in climate change (Ritchie, 2022). While it is undeniable that cattle farming is a major cause of climate change, most livestock farmers are either unsure or disagree that livestock farming contributes to climate change (Hyland et al., 2016). Most livestock farmers, however, believe that environmental issues pose risks to global food security (Hyland et al., 2016).

From these perspectives, it is likely that cattle farmers will opt to increase the size of their operations or select for traits related to growing efficiency or survivability to mitigate prospective production losses as climate change progresses. However, if operation sizes are bolstered, greenhouse gas emissions from the agricultural sector would likely increase as well. This, in turn, could create a positive feedback loop where climate change progresses, leading to higher cattle mortality. In response, farmers may further increase cattle production, consequently elevating greenhouse gas emissions and exacerbating the cycle of climate change. It has been estimated that cattle production losses in a high greenhouse gas emission scenario would be between 34.39 and 45.49 billion USD per annum by the end of the century, which would cause farmers to react drastically (Thornton et al., 2022). Cattle survivability due to superior thermotolerant capacity would reduce the impacts of climate change on the cattle industry (Scharf et al., 2010). Most cattle farmers in British Columbia do not believe that global climate change is a major challenge in their operation, and therefore may not consider thermotolerance an important trait to select (Cox et al., 2015). While these perspectives may change, the choice to adopt thermotolerance in cattle will likely be predicated on these animals having sustained or improved meat quality, and other traits naturally within common beef breeds. Larger farming operations tend to have positive attitudes toward innovative strategies which could include selection of thermotolerant cattle, which could encourage smaller operations to follow suit (O'Shea et al., 2018).

Impacts of Bovine Heat Stress

Heat stress results from environmental conditions and metabolic processes that disrupt the balance of heat production and dissipation, exceeding the bovine thermoneutral zone (generally between -5°C to 25°C) and potentially causing adverse effects (Avendaño-Reyes, 2012; Kumar et al., 2011; Lees et al., 2019; Sparke et al., 2001). To mitigate imbalances caused by exceeding the range of the thermoneutral zone, cattle have multiple behavioural and physiological responses to heat stress (Gonzalez-Rivas et al., 2020). However, despite these responses, excessive heat stress negatively affects growth rates, fertility, and in extreme cases can cause increased mortality rates (Bernabucci, 2019; Lees et al., 2019).

Thermoregulation of animals during heat stress requires increased energy usage to mitigate the excessive heat, energy generally required for growth (Ravagnolo & Misztal, 2002). Excessive heat also has been shown to decrease cattle dry matter intake (Brown-Brandl et al., 2005; Curtis et al., 2017; Lees et al., 2019). Furthermore, increasing core body temperatures redistributes blood flow from internal organs, including the gastrointestinal tract, into the extremities (Lees et al., 2019; Baumgard and Rhoads, 2007). Both the reduction in dry matter intake and shifts in blood flow during heat stress reduces the nutrient availability and energy production, further lowering growth rate and ultimately reducing carcass weights (Lees et al., 2019; Bernabucci et al., 2010). Additionally, changes in internal rumen temperatures outside optimal ranges (38°C – 40°C) can disrupt the ruminal microbiome responsible for fermentation, further affecting nutrient absorption (Beatty et al., 2008). Natural thermoregulation efforts during excessive heat stress may not yield sufficient heat dissipation to address heat absorption, especially for cattle breeds adapted for colder environments, magnifying the adverse effects of heat stress (Van laer et al., 2014). Decline in bovine conception rates, fetal growth, follicular growth and development in females, as well as prolonged impairment of spermatogenesis in males, have also been associated with extended periods of heat stress (Casady et al., 1953; Jordan, 2003). During periods of extreme heat stress, metabolic pathways involved in internal thermal regulation cannot function normally (Lees et al., 2019; Mehla et al., 2014). Improper functioning of these processes over

extended periods of time can lead to vascular collapse; and affect the cellular electrolyte balance critical to protein synthesis, likely resulting in bovine mortality (Mehla et al., 2014; Sparke et al., 2001).

Chronic heat stress on cattle also leads to a depletion of glycogen stores, reducing the normal carcass acidification process (Adzitey & Huda, 2011; Gonzalez-Rivas et al., 2020). The resulting highly alkaline carcass pH, through the reduced production of lactic acid, leads to relatively little denaturation of proteins, thus maintaining the myofilament lattice which shrinks during normal post-mortem conditions (Adzitey & Huda, 2011). The changes in the myofilament lattice causes no exudates to be formed and for the refractive index to reduce, leading to higher light absorption (Adzitey & Huda, 2011). Consequently, these physiological changes often result in dark, firm, and dry (DFD) meat (Gonzalez-Rivas et al., 2020). DFD meats are unattractive to consumers due to their unsightly appearance and are more prone to spoilage due to enhanced bacterial deterioration resulting from a pH greater than 5.8 (Pérez-Linares et al., 2015; Van de Water et al., 2003; Viljoen et al., 2002). In Canadian grading systems, DFD meats are referred to as dark cutters and are graded into a lower tier called B4 (Aalhus et al., 2014). According to the National Beef Quality Audit, the estimated cost of B4 beef to the industry in 2016/17 was 10.6 million Canadian dollars, while B grade beef accounted for 2% of the Canadian beef supply during that same year (BCRC, 2020; Canadian Beef Grading Agency, 2019).

Bovine mortality and other adverse effects, like DFD meats, are metrics used to quantify the consequences of heat stress. However, the impact of heat stress can also be assessed by behavioural and physiological indicators (Lees et al., 2019). Behaviourally, cattle can move themselves to shaded areas, if available, and adjust their body alignment in relation to the sun to reduce exposure to solar radiation (Lees et al., 2019). Over prolonged exposure to heat stress, cattle often have reduced activity levels (Heinicke et al., 2019). Involuntary responses to heat stress include alterations to respiration rates, sweating, internal temperatures, and the expression of genes related to heat stress (Gonzalez-Rivas et al., 2020; Lees et al., 2019). Increased respiration and sweating leads to increased heat dissipation which reduces the overall impact of heat stress (Gaughan et

al., 2000; Madar et al., 2010). One of the primary gene families associated with heat stress are heat shock proteins (HSPs) (Gaughan et al., 2013; Kim et al., 2020; Kumar et al., 2015). HSPs are a highly conserved family of molecular chaperones that are activated in response to stressors, such as heat stress, oxygen deprivation, physical distress, and overabundances of reactive oxygen species in cells (Gaughan et al., 2013). Specifically, the HSP70 subfamily expression is higher during heat stress than other HSP subfamilies (Deb et al., 2013; Kishore et al., 2014). HSP70 protects against cellular stress by maintaining proper folding of non-native intracellular proteins that are being denatured due to excess heat (Hassan et al., 2019). Expression of HSP70 and other subfamilies is regulated by the activation of heat shock transcription factor 1 (HSF1), which occurs at ambient temperatures exceeding 35°C (Collier et al., 2008; Hassan et al., 2019). However, levels of HSP expression can vary among breeds due to the phenotypic traits that are involved in thermotolerance (Hassan et al., 2019). Since these behavioural and physiological markers can indicate the onset of heat stress, they can be used to monitor cattle thermotolerance and reduce the impacts or permanent effects of heat stress.

The SLICK Phenotype

From both an economical and animal welfare standpoint, it is desirable to either minimize heat stress or increase the efficacy of thermal regulation processes in beef cattle (Scharf et al., 2010). A breed with traits that could confer better heat tolerance would be an ideal solution for cattle heat stress (Scharf et al., 2010). The slick hair coat (SLICK) phenotype is known to play a role in thermotolerance and is found in a tropical breed of *Bos taurus* cattle called Senepol (Flori et al., 2012; Huson et al., 2014; Littlejohn et al., 2014; Mariasegaram et al., 2007). The Senepol breed is theorized to be a composite breed using Criollo, N'Dama and Red Poll breeds generated in 1918 in the Caribbean Island of St. Croix (Huson et al., 2014; Mariasegaram et al., 2007). Criollo breed was used for their adaptive coat for the tropical environment (Huson et al., 2014). N'Dama breed (also called Gambia Longhorn) was used for their valuable characteristics of resistance to tick-born infestations (Hupp, 1978; Mariasegaram et al., 2007). Red Poll was used for their polled phenotype, gentle temperament, good meat quality and lactation output (Hupp, 1978; Mariasegaram et al., 2007).

The SLICK phenotype is characterized by very short and sleek hair that facilitates better heat loss combined with minimal absorption of heat by solar radiation (Mariasegaram et al., 2007). SLICK also has been shown to have fewer hair follicles and higher sweating capacity for heat stress mitigation (Davis et al., 2017; Dikmen et al., 2008, 2014; Huson et al., 2014; Littlejohn et al., 2014). This phenotype in Senepol has been associated with multiple single nucleotide polymorphisms (SNPs) across *Bos taurus* autosome (BTA) 20 (Huson et al., 2014; Littlejohn et al., 2014; Mariasegaram et al., 2007). These SNPs are in proximity to many genes, including S-Phase Kinase-Associated Protein 2 (*SKP2*), Sperm Flagellar Protein 2 (*SPEF2*), and Prolactin Receptor (*PRLR*) which have all previously been identified as candidate genes for the SLICK phenotype (Casas & Kehrli, 2016; Huson et al., 2014; Littlejohn et al., 2014; Mariasegaram et al., 2007). *SKP2* plays an important role in the degradation of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor, which affects keratinocyte proliferation and hair follicle development (Sharov et al., 2006; Sistrunk et al., 2011). While *SPEF2* plays a crucial role in sperm differentiation and tail development, it has also been associated with the feather growth pathway in male chickens, suggesting that *SPEF2* may also have a role in hair follicle development (Elferink et al., 2008; Sironen et al., 2010). Additionally, *PRLR* has also been associated with hair follicle growth through its interactions with prolactin, which inhibits hair growth, suggesting *PRLR* may also be a causative factor of the SLICK phenotype (Littlejohn et al., 2014; Sosa et al., 2021, 2022b). The superior sweating capacity of the SLICK phenotype is theorized to arise from increased *FOXA1* expression in eccrine sweat glands which is involved in secretory coil development (Sosa et al., 2022a; Zhao et al., 2022). Changes in eccrine sweat gland abundance was not affected by SLICK phenotype, suggesting that it only affects functionality (Sosa et al., 2022a).

Cold Stress and Galloway Cattle

Extreme cold temperatures caused by disrupted polar vortexes also subject beef cattle to a stressful environment, leading to a diversion of energy from cattle growth or fertility to thermoregulatory mechanisms that increase heat production (Howard et al., 2014; Toghiani et al., 2020). Cattle that have adapted to cold climates tend to have thicker and rougher hair coats to decrease heat dissipation, reducing the energy required

for thermoregulatory processes (Scasta, 2021). Galloway cattle have been considered as a winter hardy breed with increased hair density and weight, increasing heat production and reducing heat dissipation (Peters & Slen, 1964; Scasta, 2021). Galloway cattle accounted for 0.21% of Canadian beef registrations in 2021, whereas Angus accounted for 45.7% (Agriculture and Agri-Food, 2021). Contrary to differences in cattle registrations, both Angus and Galloway have similar meat qualities in terms of muscle fiber bundle size and marbling fleck distribution (Albrecht et al., 2006a, 2006b). It has been hypothesized that simultaneous selection of both heat and cold tolerance could be possible, which might be essential for the volatile effects of climate change (Howard et al., 2014; Scasta, 2021). Galloway cattle could contribute to the production of a composite breed that is adapted for both hot and cold environments, without sacrificing the meat quality in comparison to Angus beef.

The Polled Phenotype

Another critical factor in beef cattle production is the presence of horns in animals, which presents a set of negative economical impacts (AVMA, 2014). Conversely, raising cattle without horns has a variety of benefits, including reduced danger to handlers and other animals, reduced carcass waste by bruising and less overall aggressive behaviours (AVMA, 2014; NSW Government, 2007; Schafberg & Swalve, 2015). With the increase of animal welfare concerns by the public, traditional methods of dehorning may need to be phased out; as the process causes significant negative physiological and behavioural responses (AMVA, 2014). The polled (hornless) phenotype could be used to mitigate the negative impacts of removing horns while maintaining the benefits (Thompson et al., 2017; Windig et al., 2015).

The polled phenotype is likely influenced by a complex 202 bp insertion-deletion (InDel) (rs383143898; chr1:2429320-2429335) associated with various breeds of cattle from Celtic nations (Medugorac et al., 2012; Randhawa et al., 2020; Rothammer et al., 2014). This InDel is localized to an intergenic region of BTA 1, between the genes Interferon Alpha and Beta Receptor Subunit 2 (*IFNAR2*) and Oligodendrocyte Transcription Factor 1 and 2 (*OLIG1*/*OLIG2*) (Medugorac et al., 2012). Other SNPs previously associated with these genes may also contribute to the polled phenotype (Randhawa et al., 2020).

IFNAR2 encodes a receptor for interferons alpha and beta, for which the signaling has been associated with osteoclastic bone reabsorption; however, the exact mechanism of action is poorly understood (Stafuzza et al., 2018; Zanin et al., 2020). *OLIG1* and *OLIG2* encode transcription factors involved in oligodendrocyte differentiation and maturation and spinal cord development (Meijer et al., 2012; Stafuzza et al., 2018). However, *OLIG2* has been associated with horn ontogenesis and is highly expressed during early developmental stages in cattle (Allais-Bonnet et al., 2013; Stafuzza et al., 2018; Wiedemar et al., 2014).

Carcass Marbling

While thermotolerance capabilities and polledness confer advantages for sustainability in the face of climate change and safer animal husbandry, respectively, it is important that beef cattle also maintain superior carcass attributes for the North American marketplace (AVMA, 2014; Grigoletto et al., 2020; Thompson et al., 2017). In this regard, incorporating more marbling characteristics of Wagyu, a Japanese breed of cattle, into Canadian beef could help meet the demand of modern consumers for more tender and flavourful beef (Liu et al., 2021; Savell et al., 2016). Consumers consider marbling a major indicator of superior taste and nutritional value, affecting overall carcass value (Liu et al., 2022; Gotoh et al., 2014; Miller, 2020; Savell et al., 2016). The superior marbling traits of Wagyu are commonly characterized by high intramuscular fat and abundant fleck distribution (Hudson et al., 2021; Nguyen et al., 2021). Higher intramuscular fat content and distribution results in a lower overall melting point, contributing to tenderness and flavour in the final product (Gotoh et al., 2014; Taniguchi et al., 2004). Wagyu intramuscular fat has been associated with a fatty acid profile that is significantly rich in monounsaturated fatty acids (MUFAs), specifically C16:1 (palmitoleic acid), C18:1 (oleic acid) and C20:1 (eicosenoic acid) in comparison to other cattle (Gotoh et al., 2014). Oleic acid accounts for the highest percentage of MUFAs in bovine adipose tissues (Abe et al., 2009). MUFAs have been associated with some beneficial health attributes, including no increases in low-density lipoprotein cholesterol and the possible reduction to risk factors of cardiovascular diseases (Gotoh & Joo, 2016; Troy et al., 2016).

In Japan, Wagyu development emphasized quality rather than quantity since beef and other meats are considered side dishes; a different approach to many other cultures leading to highly marbled beef (Gotoh et al., 2018; Gotoh & Joo, 2016). Quality of marbling is measured by the area percentage of carcass fat deposits in the *Longissimus dorsi* (rib eye) between the 12th and 13th ribs, designating the grading to a level using the 12-point Beef Marbling Standard (BMS) scoring system (Gotoh et al., 2014; Polkinghorne & Thompson, 2010). Fat colouration, luster and quality and beef firmness and texture are also considered for the quality scoring of the carcass (Gotoh et al., 2014; Wagyu Authentic, 2018). For the BMS score and the other quality scoring are assigned a meat quality grade level from 1 – 5 which is paired with a yield grade A – C, where A5 is the best possible score (Wagyu Authentic, 2018). Canadian grading follows a similar approach in a stepwise/flowchart fashion, scoring carcass age, fat quality, muscle content, meat colouration, and marbling amount in that order (BCRC, n.d.). Marbling grades from trace to abundant are Canada A, Canada AA, Canada AAA, and Canada Prime, respectively (BCRC, n.d.). In both Japanese and Canadian grading systems, marbling is paramount for the determination of carcass values (Polkinghorne & Thompson, 2010).

To be considered Wagyu beef, the cattle must adhere to two following conditions: it must be from one of the Japanese pure or cross breeds of a Japanese pure breed, and it must be bred and born in Japan confirmed by a traceability system (MAFF, 2008). The Japanese pure breeds are Japanese Black, Japanese Brown (Akaushi), Japanese Shorthorn and Japanese Polled; all four breeds belong to the taxonomic classification *Bos taurus* (Hirooka, 2014; Motoyama et al., 2016). Introduction of the Wagyu breed to Japan is believed to have arisen from an import of the Hanwoo (pronounced “Han-oo”) breed via the Korean Peninsula during the Yayoi period (300 BCE to 300 CE) for farm labour and transportation needs (Motoyama et al., 2016; Smith, 2015). The four lineages of Japanese pure breeds were developed by crossing Hanwoo with foreign breeds of cattle during the Meiji Restoration (1868 – 1912), where restrictions on beef consumption were lifted (Smith, 2015). It is thought that the higher intramuscular fat arose because of selective pressures from vitamin A deficiencies during winters; and the need for additional storage capabilities, which was primarily accomplished in adipose tissues (Harper & Pethick, 2004; Hirooka, 2014; Motoyama et al., 2016). This was hypothesized after experiments

found a negative correlation between vitamin A serum concentrations and marbling scores (Kato et al., 2011; Oka et al., 1998).

Marbling is a complex trait influenced by multiple genes and environmental factors that lead to differences in fatty acid composition within intramuscular adipose tissue (Wang et al., 2019). Many candidate genes have been associated with fatty acid composition, including fatty acid synthase (*FASN*), stearoyl-CoA desaturase (*SCD*), ras-related protein Rab-4A (*RAB4A*) and fatty acid binding protein 4 (*FABP4*) (Abe et al., 2009; Hudson et al., 2021; Michal et al., 2006; Tan and Jiang, 2024; Taniguchi et al., 2004). *FASN* is a cytosolic enzyme that catalyzes the *de novo* biosynthesis of palmitic acid from acetyl-CoA and malonyl-CoA in the presence of NADPH (Ordovás et al., 2008; Roy et al., 2006; Tan and Jiang, 2024). *SCD* is an endoplasmic reticulum enzyme that catalyzes the desaturation of fatty acids for the formation of MUFAs, specifically palmitoleic acid and oleic acid, in mammalian adipocytes (Paton & Ntambi, 2009; Tan and Jiang, 2024; Taniguchi et al., 2004). The fat composition of adipose tissue is indicative of preceding *SCD* function dependant on bioavailability of saturated fatty acids (Paton & Ntambi, 2009). Most saturated fatty acids in adipose tissue are a result of absorbed dietary fatty acids which are chemically reduced in the rumen by microorganisms (Taniguchi et al., 2004). Mammalian *RAB4A* is known to help in the regulation of intracellular trafficking of glucose transport type 4 (GLUT4), an insulin-regulated glucose transporter highly expressed in adipose tissue (Hudson et al., 2021; Tan and Jiang, 2024). Increased cytoplasmic glucose concentrations increase the bioavailability of acetyl-CoA through glycolysis, which can be utilized in many biosynthetic pathways including lipogenesis (Alfarouk et al., 2014; Ophardt, 2003). *FABP4* is an adipocyte-specific cytoplasmic protein that binds to fatty acids and is involved with fatty acid uptake, transport, metabolism, and homeostasis (Michal et al., 2006; Tan and Jiang, 2024). Interaction between *FABP4* and hormone-sensitive lipase catalyzes the hydrolysis of triacylglycerols in adipocytes releasing free fatty acids for mobilization (Michal et al., 2006; Shen et al., 1999).

Climate Master Cattle Project Outline

The primary goal of the Climate Master Cattle project is to develop a novel climate tolerant composite beef cattle breed, the Climate Master Hybrid, using a set of progenitor cattle using *in vitro* fertilization and genetic tools (Figure 1.1, a-b). For adoption of this breed into North American beef markets, it will be necessary that meat quality is similar, or an improvement, to the common breeds currently used in traditional Canadian beef operations. To address this, the Climate Master Hybrid will be crossed with Akaushi (Wagyu) for superior marbling characteristics (Figure 1.1, b). Therefore, the final composite breed of this project, the Climate Master Composite, will have superior climate resistance capabilities and meat qualities (Figure 1.1, c). The combination of these traits would be highly valuable to farmers, offering resilience to climatic extremes and the ability to produce higher-quality beef. Akaushi were chosen instead of Japanese Black cattle, which account for 97% of Wagyu, as Japanese Black have a black coat colour which may interfere with thermotolerant characteristics expected in the Climate Master Hybrid (Lees et al., 2019; Motoyama et al., 2016). Breeding decisions will be guided by genetic selection using the SLICK and polled genotypes.

Once animals are produced, the critical next step is to assess heat and cold tolerances of the Climate Master Hybrid. This will ensure the Climate Master Hybrids resilience to climatic extremes. The next stage is to evaluate marbling characteristics between the Climate Master Hybrid and the progenitor cattle breeds, specifically Akaushi, and then to determine the genetic markers associated with those marbling characteristics. These genetic markers will be essential in guiding the final step of the project breeding the Climate Master Composite (Figure 1.1, b-c).

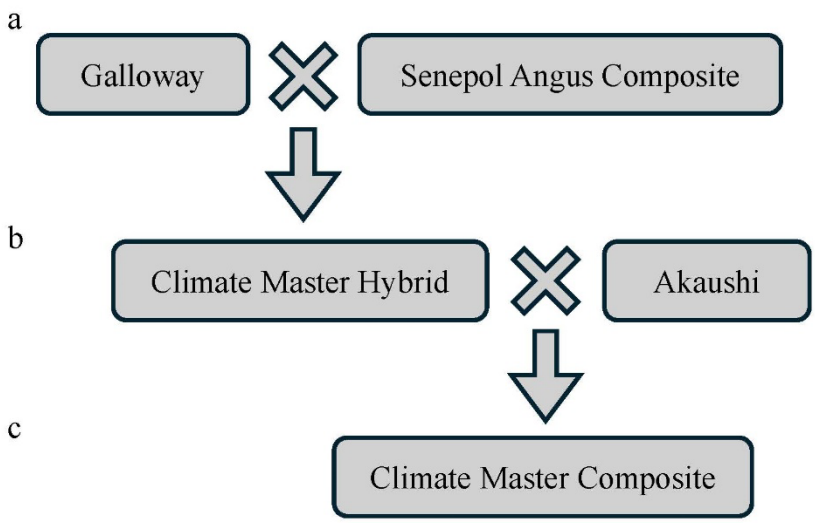

Figure 1.1: Climate Master Cattle breeding plan. Progenitor cattle including pure Galloway and Senepol Angus Composite (1/4 Red Angus x 3/4 Senepol) breeds will be crossed (a). The novel Climate Master Hybrid (1/8 Red Angus x 3/8 Senepol x 1/2 Galloway) will then be crossed with pure Akaushi (Wagyu) cattle (b). The final composite breed called the Climate Master Composite (1/16 Red Angus x 3/16 Senepol x 1/4 Galloway x 1/2 Akaushi) will excel in heat and cold tolerance, while also having superior marbling capabilities (c).

This thesis is divided into three primary objectives, each of which encompass their own chapter. The first objective is genotyping genetic markers previously associated with the SLICK phenotype and polledness to find the best predictors of each, allowing for easy assessment and tracking of these traits across generations during this project. The second objective is to assess the heat stress response of the Climate Master Hybrid with the SLICK genotype. More specifically, this includes the assessment of respiration rates, activity levels, internal rumen temperatures, and HSP70 gene expression and plasma concentrations. These metrics reinforce the benefits of the SLICK phenotype within the Climate Master Hybrid and can be used to help convince producers to adopt the final product of this project. The third objective outlines the genotyping and a genome-wide association study (GWAS) using a mock dataset of marbling characteristics that were completed to determine genetic markers associated with cattle marbling characteristics in Akaushi cattle.

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CHAPTER 2: Pinpointing Reliable Predictors and Genotype Tracking in Progenies for the SLICK and Polled Phenotypes

Abstract

Cattle heat stress is becoming a significant issue for the cattle industry due to trends in climate change, leading to heightened economical losses. Adaptation by the beef industry will be required to mitigate the effects of heat stress. SLICK, a short and sleek hair coat trait found within the tropical breed called Senepol, might be the key to mitigating these effects and improve animal welfare during increased ambient temperatures. Additionally, naturally hornless (polled) cattle are also important for industry to reduce dangerous conditions for both handlers and animals.

In this study, a panel of 12 genetic markers previously shown to be associated with the SLICK and polled traits were evaluated to find the most informative markers for our selection of breeding stock. Sixteen cattle grouped by presence of the SLICK and polled traits were genotyped to determine the markers that accurately predicts each trait. The genetic markers rs517047387 and rs383143898 were identified to be reliable predictors for the SLICK and polled phenotypes, respectively. These markers were then used to genotype the Climate Master Hybrid progeny that were produced in the initial breeding cycle of the Climate Master Cattle project.

Introduction

Cattle (*Bos taurus*) heat stress is described as an imbalance of heat gain and dissipation exceeding the bovine thermoneutral zone, prompting behavioural and physiological changes to mitigate the heat load (Kumar et al., 2011; Lees et al., 2019; Sparke et al., 2001). Despite these reactions, cattle exposed to excessive heat loads can exhibit detrimental consequences to growth, reproductive capabilities, and mortality, producing unfavourable effects within cattle industries (Bernabucci, 2019; Lees et al., 2019). Climate change models predict global temperature increases will continue to persist, further increasing both temperature and duration of heat waves, exerting additional pressures on human health, agriculture, and conservation sectors (Pasqui & Giuseppe, 2019). Severe economical losses have previously been observed in the United States from the effects of heat stress in cattle, accounting for approximately $369 million USD in annual losses for the beef industry (St-Pierre et al., 2003).

Amidst climate change effects, it is important for the cattle industry to adapt accordingly by increasing thermotolerant capacity in cattle (Scharf et al., 2010; Van de Water et al., 2003). The slick hair coat (SLICK) phenotype found in the tropical breed of *Bos taurus* called Senepol has been associated with thermotolerant capabilities (Flori et al., 2012; Huson et al., 2014; Littlejohn et al., 2014; Mariasegaram et al., 2007). SLICK is characterized by very short, sleek hair, while having fewer hair follicles and larger sweat glands, enabling increased mitigation capabilities of the effects of heat stress (Huson et al., 2014; Landaeta-Hernández et al., 2011; Mariasegaram et al., 2007). Mitigating effects of heat stress would generate improvements in cattle health, welfare, and production within industries ultimately minimizing economic losses (Lees et al., 2019; Van de Water et al., 2003).

Another critical factor in animal welfare and cattle husbandry in industries are the presence of horns (AVMA, 2014). Raising cattle without horns poses a variety of benefits, including reduced danger to handlers and other animals, reduced carcass waste, and less overall aggressive behaviours (AVMA, 2014; NSW Government, 2007). Achieving this through traditional methods, like dehorning, are painful procedures that have rightfully become opposed by the public for animal welfare concerns (AVMA,

2014; Sylvester et al., 2004). An alternative method is to breed polled (hornless) cattle that do not require animals to be dehorned, thus maintaining the hornless benefits while negating the harmful impacts of dehorning (Windig et al., 2015).

Reliably predicting the SLICK and polled phenotypes using genetic markers would enable genetic selection, which is paramount for the success of breeding plans (Mrode et al., 2019). SLICK has been previously associated with multiple single nucleotide polymorphisms (SNPs) and microsatellites across the *Bos taurus* autosome (BTA) 20 (Huson et al., 2014; Littlejohn et al., 2014; Mariasegaram et al., 2007). Similarly, the polled phenotype has been associated with multiple SNPs within BTA 1 (Randhawa et al., 2020). Therefore, our objective was to determine the genetic markers that act as the best predictors of the SLICK and polled phenotypes, respectively, and to track these genotypes within progenies bred using *in vitro* fertilization. The ability to track these genotypes would be invaluable in developing our unique Climate Master Hybrid and Composite.

Materials and Methods

All procedures outlined in this section were conducted in accordance with the Canadian Council of Animal Care guidelines and was approved by the Animal Care Committee of Thompson Rivers University (Kamloops, B.C., Canada) (File number: 102823).

Initial Animal Inclusion

Cattle were chosen for this study based on their phenotypic profile. Primarily animals were selected based on presence or absence of the SLICK and polled phenotypes. These cattle were used as progenitors for breeding the proposed Climate Master Hybrid and Composite. Phenotypes were determined by an experienced farmer with proficiency in recognizing coat characteristics and horn structures. All progenitor cattle were also included in subsequent genotyping to validate the ability to track these phenotypic trait genetically and to predict the passing of those traits to progenies.

Site Information

Buck Lake Ranch is located in Beaverdell, British Columbia (49.4342°N, 119.0883°W) which is approximately 60 kilometers from Kelowna. The property is composed of 260 hectares, along with additional leased acres for summer grazing, dedicated to forage production and beef cattle raising. Cattle were able to graze freely, but study animals were generally held in large feedlot pens during phenotypic evaluation.

Sampling and DNA Extraction

Individuals within the study were tagged using a specialized ear tag system developed and gifted by the Southern Alberta Institute of Technology (SAIT). The system tags the animal with an identification number and deposits a sample of ear tissue into a specialized receptacle. Tags were applied between the two ridges of auricular cartilage, approximately one-third the distance between the base and tip of the ear. Ear tissues were stored and transported in ambient conditions until DNA was extracted.

DNA extractions were completed using a modified protocol of the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Markham, Ontario, Canada) (Qiagen, 2023). After

the addition of Buffer ATL and Proteinase K, the ear tags were incubated overnight in a MaxQ™ 420 HP Incubated Tabletop Oribital Shaker (Thermo Fisher Scientific, Burnaby, B.C., Canada) at 56°C set to 100rpm. Following the extraction, the purity and quantity of each sample was determined using the NanoDrop One instrument (Thermo Fisher Scientific, Burnaby, B.C., Canada).

Primer Design

SNPs and microsatellites identified previously in the literature to be associated with either SLICK or the polled traits were acquired (Huson et al., 2014; Littlejohn et al., 2014; Mariasegaram et al., 2007; Randhawa et al., 2020). The genomic coordinates of each SNP were converted to the ARS-UCD1.3 *Bos taurus* bovine genome assembly using the BLAST-like alignment tool (Kent, 2002); and then used to retrieve the consensus sequences from Ensembl (Cunningham et al., 2022). The resulting files were viewed using A Plasmid Editor (ApE) software (Davis & Jorgensen, 2022). One to two primer sets consisting of both a forward and reverse primer were designed using the primer designing tool within ApE. Each primer was designed to have a predicted melting temperature between (60°C and 64°C). Primer set amplicons were designed to be between 100 and 1,000 base pairs in length, with the SNP or microsatellite locus (or loci) in the middle of the amplicon. Each primer set was submitted to the National Centre for Biotechnology Information's (NCBI) Primer-BLAST (Ye et el., 2012) to determine whether there would be off-target amplification. Primer sets were ordered from Integrated DNA Technologies (Coralville, Iowa, United States).

PCR Optimization

All designed primer sets underwent a polymerase chain reaction (PCR) optimization step. The general reaction recipe was: 0.1 µL Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Burnaby, B.C., Canada), 2 µL 5X buffer, 1 µL 0.4 mM dNTPs, 1 µL of each primer at 5 pmol, 2 µL Betaine, 2 µL of 1.5 ng/µL to 20 ng/µL of template DNA, and 0.9 µL dH_2O. All PCRs were carried out in a SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific, Burnaby, B.C., Canada) with the following reaction conditions: 1 cycle of 98°C for 30 seconds, 35 cycles of 98°C for 10 seconds,

X°C for 30 seconds, 72°C for 30 seconds, then 1 cycle of 72°C for 5 minutes. X°C represents either 58°C, 60°C, or 62°C. All optimization reaction arrays were completed using stock *Bos taurus* DNA extracted from bovine blood that tested negative for *Mycobacterium bovis*. This DNA was extracted using the GeneJet Genomic DNA Purification Kit following the manufacturer's protocol (Thermo Fisher Scientific, 2024) by Michael Ke of the Applied Genomics Centre. Each optimization array was accompanied by a PCR assay targeting DNA Methyltransferase 3 Beta (*DMNT3B*) gene previously optimized using a 60°C annealing temperature as a positive control and no primers additives as a negative control.

Success of each PCR was determined by visualization using gel electrophoresis. A 2% gel was made using Ultrapure™ Agarose, 1X TAE Buffer and SYBR™ Safe DNA Gel Stain. Loading recipes were made on Parafilm using 3 µL of the PCR product, 3 µL of DNA Gel 6X Loading Dye, and 3 µL dH_2O, then 6.5 µL of mixture was loaded into its respective well alongside the GeneRuler 1kb Plus DNA Ladder. The gel was run at 120V for 35 minutes in a Sub-Cell GT Horizontal Electrophoresis System (Bio-Rad Laboratories, Woodinville, Washington, United States) powered by a PowerPac™ Basic Power Supply (Bio-Rad Laboratories, Woodinville, Washington, United States). The gel was visualized using the Molecular Imager Gel Doc XR+ System (Bio-Rad Laboratories, Woodinville, Washington, United States).

For each SNP or microsatellite locus, one primer set was selected based on specificity, amplicon size, and annealing temperature. After each primer set was chosen, PCRs were carried out on all samples. Loci that contained SNPs were sequenced using Sanger Sequencing and microsatellites were assessed using fragment length analysis.

Sanger Sequencing

After successful PCR amplification of each locus of interest across all samples, the nucleotide sequence of the amplicons was determined using Sanger Sequencing. This method can determine the genotype for each of the SNPs. PCR products were subjected to an ExoSAP-IT™ *Express* (Thermo Fisher Scientific, Burnaby, B.C., Canada) cleanup step to purify the PCR product from leftover reaction reagents, following the

manufacturer's protocol (Thermo Fisher Scientific, 2017). 5 µL of the PCR product was mixed with 2 µL of equal parts ExoSAP-IT™ and dH_2O. The reaction was then carried out in a SimpliAmp™ Thermal Cycler, with the following conditions: 37°C for 4 minutes, 80°C for 1 minute, and then a 4°C hold.

The purified PCR product was then quantified using a Qubit 4 Fluorometer (Thermo Fisher Scientific, Burnaby, B.C., Canada). The sequencing reaction was completed using the BigDye™ Terminator V3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Burnaby, B.C., Canada) following the manufacturer's protocol (Thermo Fisher Scientific, 2010). The reaction recipe was: 1 µL of BigDye™, 1.5 µL 5X buffer, 1 µL of one primer from the set, 12 ng of DNA and dH_2O to bring the reaction volume to 10 µL. The reaction was run in a SimpliAmp™ Thermal Cycler under the following conditions: 1 cycle of 96°C for 1 minute, 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes.

The BigDye™ product was then purified using the BigDye Xterminator™ Purification Kit (Thermo Fisher Scientific, Burnaby, B.C., Canada). 5 µL of the BigDye™ product, 22.5 µL of SAM Solution, and 5 µL BigDye Xterminator™ were vortexed for 30 minutes in a PCR plate. The plate was then centrifuged and 15 µL of the supernatant was transferred to either a MicroAmp 0.2 mL 8-well optical strip or 96-well optical plate. SeqStudio™ Septa (Thermo Fisher Scientific, Burnaby, B.C., Canada) was then placed over the wells immediately before placing the samples into a SeqStudio™ instrument (Thermo Fisher Scientific, Burnaby, B.C., Canada) to be sequenced. All sequencing runs were specified using SeqStudio™ Plate Manager software and loaded into the SeqStudio™. Depending on size of the PCR amplicon size produced by each primer set, run modules were either MediumSeq (~500bp) or LongSeq (~800bp).

Fragment Length Analysis

For loci containing microsatellites, amplicon length was measured using fragment length analysis (FLA) instead of Sanger Sequencing. Modified PCR reaction were ran with the same reaction conditions determined during optimization, however, the forward primer was replaced with a new primer of the same sequence fluorescently labelled with

a 6FAM 5' reporter dye (Thermo Fisher Scientific, Burnaby, B.C., Canada). The resulting PCR product was then visualized using gel electrophoresis as previously outlined in the PCR Optimization subsection. After confirmation of band presence, the PCR product was diluted by a factor of 100. A 1 µL aliquot of the diluted PCR product was then mixed with 0.15 µL of 600 LIZ size standard (Thermo Fisher Scientific, Burnaby, B.C., Canada) and 8.85 µL HiDi Formamide (Thermo Fisher Scientific, Burnaby, B.C., Canada) in a MicroAmp Optical Strip. The strip was then covered with septa and loaded into a SeqStudio™ instrument, programed using SeqStudio™ Plate Manager software. Dye set was set to G5(DS-33) and the LongFragAnalysis run module was used for all microsatellite assays.

Analysis

Sequencing results in the form of electropherograms were extracted from the SeqStudio™. Electropherograms with Phred scores below 30 were discarded. Those sequencing reactions were redone until a electropherogram passed quality control. Nucleotide sequences were then trimmed using Mott's algorithm (Chen et al., 2014). The trimmed sequences were then aligned against the *Bos taurus* reference genome obtained from Ensembl during primer design to obtain the genotype of each individual sample at each locus.

Analysis of FLA samples was performed using the GeneMapper™ 5 software (Thermo Fisher Scientific, Burnaby, B.C., Canada). Fragment lengths were than compared to the 600 LIZ size standard to get accurate measurements of the sizes of each amplicon containing the microsatellite.

Progeny Genotyping

After a locus was identified to be directly associated with either the SLICK or polled, that information was then used to make breeding decisions. Genotypes of all progenitor cattle and Climate Master Hybrid cattle were determined using the same experimental pipeline. This allowed the tracking of the SLICK and polled genotypes across generations. Genotyping and crossing information were used to generate pedigrees using CeGaT's Pedigree Chart Designer program (Tübingen, Germany).

Results

Climate Master Progenitor Cattle

Sixteen initial animals belonging to one of three progenitor breeds proposed to be used for the Climate Master Composite breed were genotyped to determine the best predictor of the SLICK and polled phenotype. Of these 16 cattle, 6 were Senepol Angus composite, 6 were pure Galloway, and 4 were pure Akaushi. The Senepol Angus composite cattle were 3/4 Senepol and 1/4 Red Angus and displayed both the SLICK and polled phenotype. Pure Galloway cattle only had the polled phenotype, and Akaushi cattle did not display either phenotype, acting as negative controls. Seven of the cattle were male and nine were female. All cattle were healthy and capable of producing offspring.

Genetic Markers

Through literature review, 10 SNPs and 2 Microsatellites were identified. Seven of the genetic markers (5 SNPs and 2 Microsatellites) were reported to be associated with the SLICK phenotype and subsequent hair development (Huson et al., 2014; Littlejohn et al., 2014; Mariasegaram et al., 2007), whereas 5 SNPs were associated with the polled phenotype (Randhawa et al., 2020). All genetic markers are outlined in Table 2.1.

Table 2.1: Genetic markers associated with the SLICK and polled phenotypes identified during literature review.

Marker Name	Type[1]	Phenotype	Chr[2]	Position[2]	Allele[3]	Source	Closest Gene
rs42343907	SNP	SLICK	20	38084918	G/A	Huson et al., 2014	*SKP2*
rs132939689	SNP	SLICK	20	38509507	T/C	Huson et al., 2014	*SPEF2*
rs135282100	SNP	SLICK	20	38215638	A/G	Huson et al., 2014	*UGT3A2*
rs517047387	SNP	SLICK	20	39099214	C/N	Littlejohn et al., 2014	*PRLR*
rs520582588	SNP	SLICK	23	35332871	A/C	Littlejohn et al., 2014	*PRL*
DIK2416	MS	SLICK	20	37748162 - 37748201	AC (20)	Mariasegaram et al., 2007	*SLC1A3*
NRDIKM023	MS	SLICK	20	38280106 - 38280145	AC (20)	Mariasegaram et al., 2007	*CAPSL*
rs210350155	SNP	Polled	1	2491161	C/A	Randhawa et al., 2020	*OLIG1*
rs383143898	SNP	Polled	1	2429320 - 2429335	X[a]/I[b]	Randhawa et al., 2020	*IFNAR2*
rs797088784	SNP	Polled	1	2578598	G/A	Randhawa et al., 2020	*OLIG2*
rs799403053	SNP	Polled	1	2486811	T/C	Randhawa et al., 2020	*OLIG1*
rs801127025	SNP	Polled	1	2372456 - 2372457	N/X[c]	Randhawa et al., 2020	*IFNAR2*

[1]Either single nucleotide polymorphism (SNP) or microsatellite (MS).
[2]Chromosome (Chr) and position refer to the ARS-UCD1.3 genome assembly.
[3]SNP shows nucleotide substitution and MS shows nucleotide repeat and number of repeats in reference genome.
[a] Represents a nucleotide string: GAATAG.
[b] Represents an insertion-deletion of 202 base pairs.
[c] Represents a nucleotide string: TGGTAGGCTGGTATTC.
*Deletions are denoted by 'N'.

Primer Design and PCR Optimization

For each genetic marker (Table 2.1), 1 to 2 primer sets were designed, generating a total of 22 primer sets to be optimized. The optimization phase identified the most optimal primer set for each genetic marker (Figure 2.1). Genetic markers, primer design, and optimization information is outlined for the SLICK and polled phenotypes in Tables 2.2 and 2.3, respectively.

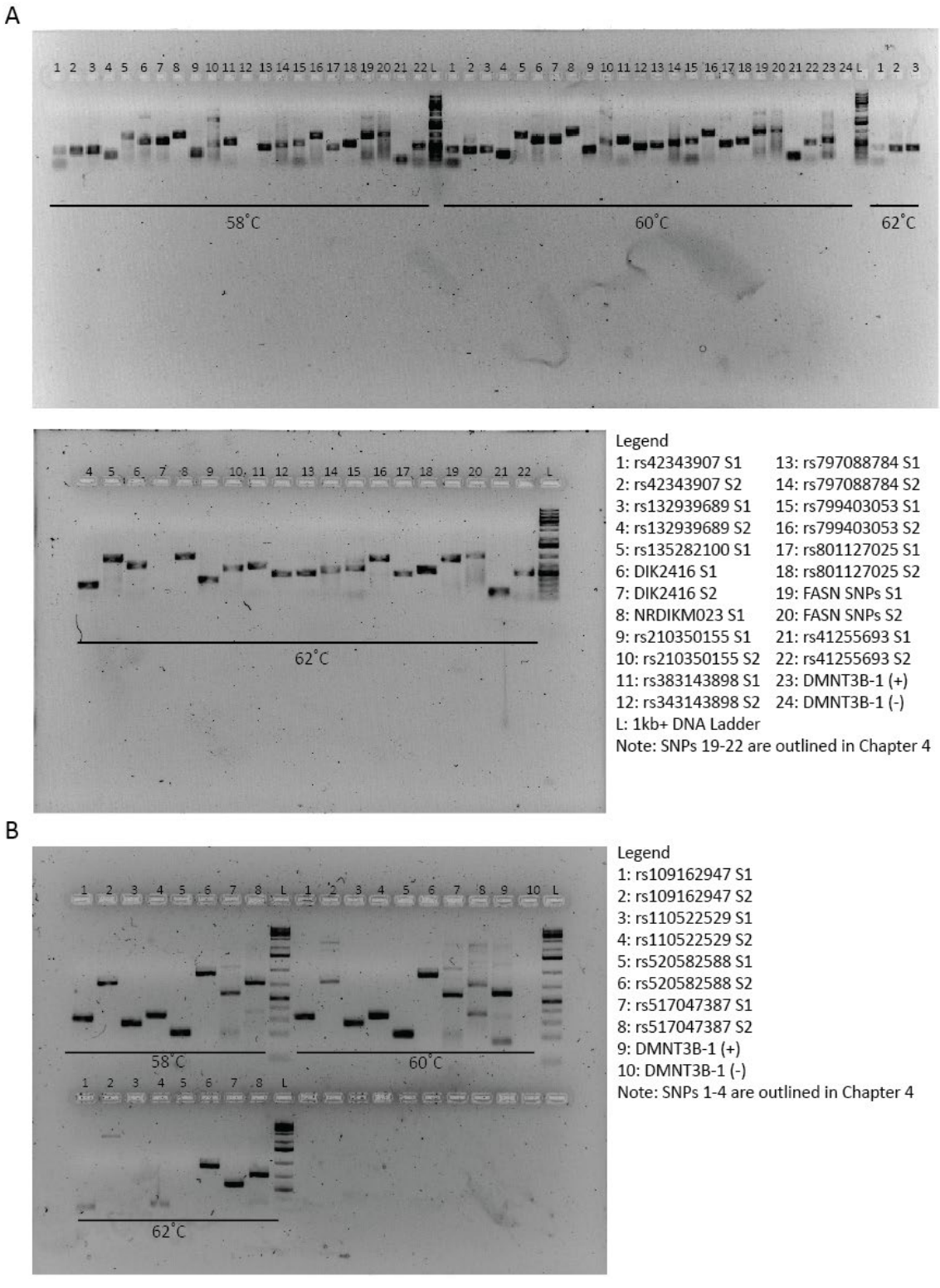

Figure 2.1: Gel electrophoresis results of PCR optimization step of designed primer sets outlined in Tables 2.1 and 2.2.

Table 2.2: SNPs and microsatellites identified to be associated with the SLICK phenotype. Displays primer set designing information, closest gene, and literature source.

Marker Name	Set	Primer	Sequence (5´-3´)	Length (bp)	T_m[1]	GC%	Amplicon	Analysis[2]	Final T_a[3]
rs42343907	1	F1	CTTTCCAGGTCCTTCTCTCTTCCAG	25	62.31	52.00	395 bp	N	-
		R1	TGCTGATAGCATCTGACGTGGAG	23	62.35	52.17			
	2	F2	CTTCCCTCTGGCCTCCTACA	20	60.33	60.00	374 bp	SEQ	62°C
		R2	AACGTCCCTGCTATTTTTAGTGTC	24	59.31	41.67			
rs132939689	1	F1	CTGGAGCTAGTTCTCATGGCTGAC	24	62.76	54.17	399 bp	SEQ	62°C
		R1	CCAGTCTACATGAGCTGAATTTCC	24	59.43	45.83			
	2	F2	GTCCTGTCTCCTACCAGAGG	20	58.23	60.00	258 bp	N	-
		R2	CAAACATAAGCAGTTCCTCCAG	22	57.31	45.45			
rs135282100	1	F1	GGAATGAGAGGGCAAGAACAAA	22	58.84	45.45	895 bp	SEQ	62°C
		R1	AGCCACTGAAATTAGCCAACTGAG	24	61.34	45.83			
rs517047387	1	F1	CCTGGATCTTGACAGTGACTCTG	23	60.37	52.17	561 bp	SEQ	62°C
		R1	TTTGGGAACAGAGCCAGCAC	20	60.82	55.00			
	2	F2	GTAGATGACTGTGAGGACCAGCAG	24	62.46	54.17	719 bp	N	-
		R2	ACCAGGATGTTGCTATCTGTC	21	57.45	47.62			
rs520582588	1	F1	GTTTGCCAGGGAATGGATCATT	22	59.23	45.45	204 bp	SEQ	60°C
		R1	ACTCCCGTCCCTGCAAACTAAG	22	62.25	54.55			
	2	F2	CCTCATGCTCTTTGAGTACACT	22	57.80	45.45	917 bp	N	-
		R2	TCTCCTCAAACCACAGGATG	20	56.84	50.00			
DIK2416	1	F1	GGAGCGAGCAGAAAGAAGCAAG	22	62.06	54.55	669 bp	FLA	62°C
		R1	AAGACGTGTCTGCTCACTTAG	21	57.71	47.62			
	2	F2	CACTGGAAGTCTCTTTACCACACAC	25	61.48	48.00	655 bp	N	-
		R2	GGAATGGGACTCAAAGACAGAAAG	24	59.54	45.83			
NRDIKM023	1	F1	CACCCAAAGAGTCCCAGTATAAACC	25	61.09	48.00	941 bp	FLA	62°C
		R1	AATCCACTTCTGGCACTAACTACC	24	60.32	45.83			

[1]T_m: Melting temperature in °C.

[2]Each primer set was either used for Sanger sequencing (SEQ), fragment length analysis (FLA), or not at all (N).

[3]T_a: Annealing temperature.

Table 2.3: SNPs identified to be associated with the polled phenotype. Displays primer set designing information, genomic coordinates, closest gene, and literature source.

Marker Name	Set	Primer	Sequence (5′-3′)	Length	T_m[1]	GC%	Amplicon	Analysis[2]	Final T_a[3]
rs210350155	1	F1	CCTTTATGTCACGTTGGGTCTTTG	24	60.32	45.83	303 bp	SEQ	62°C
		R1	TGGTTTCGTTTGCTTGCTTGTCAG	24	62.96	45.83			
	2	F2	GGATGGTTTGTGTTTCAGAGCC	22	60.03	50	562 bp	N	-
		R2	ACCTTAGTGGCCCTGGTCTTTG	22	62.22	54.55			
rs383143898	1	F1	GTCTGCGCCTTTCTTGTTATAC	22	57.74	45.45	649 bp	N	-
		R1	CACTGCTCGGAAACCGTAAC	20	59.21	55			
	2	F2	GCTTAGAAGTGTGGCCGGTAGAA	23	62.49	52.17	443 bp	SEQ	62°C
		R2	CAAAGGCAGAGATGTTGGTC	20	56.71	50			
rs797088784	1	F1	CTGGCCTTCCTCTAGTAGTG	20	56.44	55	464 bp	SEQ	62°C
		R1	TCACACCTGCCTCCACTTTATCAC	24	62.61	50			
	2	F2	CTGTACGTTGGAGGAGTGGTGG	22	62.55	59.09	527 bp	N	-
		R2	ACAAACATCTGTGGACTTGACACG	24	61.74	45.83			
rs799403053	1	F1	CATACTGAACAACGCAGGCTTAGA	24	61.15	45.83	593 bp	SEQ	62°C
		R1	ATATGCCTCTGCGTGTGTGTAG	22	60.48	50			
	2	F2	CCACTTTCTCTGCTACACACAT	22	58.33	45.45	894 bp	N	-
		R2	CTTTGCTCACATGGTGGTCTTC	22	59.77	50			
rs801127025	1	F1	CAAAGCAGACTGGAGCTTCTAA	22	58.33	45.45	472 bp	N	-
		R1	AGCTTGCTACTGCACAACTGG	21	61.15	52.38			
	2	F2	GACTCCCTTCCCAGACACTAGG	22	61.21	59.09	568 bp	SEQ	62°C
		R2	TCAAGTTCACTAGCCTTATGGTGC	24	60.86	45.83			

[1]T_m: Melting temperature in °C.

[2]Each primer set was either used for Sanger sequencing (SEQ), fragment length analysis (FLA), or not at all (N).

[3]T_a: Annealing temperature.

Genotyping

All samples from the progenitor cattle for the Climate Master Cattle project were genotyped for all SNPs associated with the SLICK and polled phenotypes. All results of this genotyping for the SLICK and polled phenotypes are outlined in Table 2.4 and Table 2.5, respectively.

Genotyping of the genetic markers associated with the SLICK phenotype revealed that only rs517047387 had a deletion exclusive to Senepol Angus Composite cattle which was not present in Galloway and Akaushi cattle. Additionally, rs42343907 exhibits an allele exclusive to both Senepol Angus Composite and Galloway cattle which was not present in Akaushi cattle. The other SNPs (rs132939689, rs135282100, and rs520582588) exhibited no unique trends between the cattle breeds. The fragment length of the microsatellites DIK2416 and NRDIKM023 were determined after the genotyping on at least one sample from each breed and found no correlations between breeds in preliminary analyses. Therefore, further analysis of DIK2416 and NRDIKM023 was not conducted at this point.

Genotyping of the markers associated with the polled phenotype revealed that only rs383143898 had an allele exclusive to Senepol Angus Composite and Galloway cattle, which was not present in Akaushi cattle. All other SNPs (rs42343907, rs132939689, rs135282100, and rs520582588) shared the same genotype which matched the reference genome.

Table 2.4: Genotyping results for the 5 SNP and 2 microsatellite loci associated with the SLICK phenotype.

Cattle				SNPs					Microsatellites	
Breed	SLICK	Polled	ID	rs42343907	rs132939689	rs135282100	rs517047387[a]	rs520582588	DIK2416	NRDIKM023
				G/A	T/C	A/G	C/N	A/C	-	-
Senepol Angus Composite	Yes	Yes	Wrangler	AG	-	-	NC	AA	660.69	772.22
			J11	AA	CC	CC	NN	AA		
			J12	AG	CT	AA	NC	AA		
			R4	AA	CC	-	NN	AA		
			R1	AA	CC	GG	NN	AA		
			Sabre	AG	CT	AA	NC	AA		
Galloway	No	Yes	Ferrari	AG	CT	AA	CC	AA	653.33	770.34
			Earthquake	AA	CT	AA	CC	AA	656.03	774.62
			11H	AA	CT	AA	CC	AA		
			64X	AA	CT	-	CC	AA		
			131W	AG	CT	AA	CC	AA		
			64H	AG	TT	AA	CC	AA		
Akaushi	No	No	Raymond	GG	CC	AA	CC	AA	653.19	772.08
			2009H	GG	CC	AA	CC	AA		
			2001H	GG	CC	AA	CC	AA		
			2018H	GG	CT	AA	CC	AA		

[a]All cattle with SLICK have an 'N' in their rs51747387 genotype and all cattle without SLICK do not have an 'N'.

*A deletion allele is denoted by 'N'.

Table 2.5: Genotyping results for the 5 SNP loci associated with the polled phenotype.

Cattle				SNPs				
Breed	SLICK	Polled	ID	rs210350155	rs383143898[a]	rs797088784	rs799403053	rs801127025
				C/A	X/I	G/A	T/C	N/X
Senepol Angus Composite	Yes	Yes	Wrangler	CC	II	GG	TT	NN
			J11	CC	XI	GG	TT	NN
			J12	CC	XI	GG	TT	NN
			R4	CC	XI	GG	TT	NN
			R1	CC	XI	GG	TT	NN
			Sabre	CC	II	GG	TT	NN
Galloway	No	Yes	Ferrari	CC	II	GG	TT	NN
			Earthquake	CC	II	GG	TT	NN
			11H	CC	XI	GG	TT	-
			64X	CC	XI	GG	TT	NN
			131W	CC	XI	GG	TT	-
			64H	CC	XI	GG	TT	NN
Akaushi	No	No	Raymond	CC	XX	GG	TT	NN
			2009H	CC	XX	GG	TT	NN
			2001H	CC	XX	GG	TT	NN
			2018H	CC	XX	GG	TT	NN

[a]All polled cattle have an 'I' in their rs383143898 genotype and all horned cattle do not have an 'I'.

*A deletion is denoted by 'N'.

†A string of nucleotides is denoted by 'X'. Table 2.1 outlines the exact nucleotide sequence.

‡An insertion-deletion is denoted by 'I'.

Progeny Genotype Tracking

After the best predictor from the array of genetic markers for both the SLICK and polled phenotypes were determined, the markers were used to track genotypes of the progenies from each of six crosses produced from select progenitor cattle. All genotypes are outlined in the pedigrees (Figure 2.2).

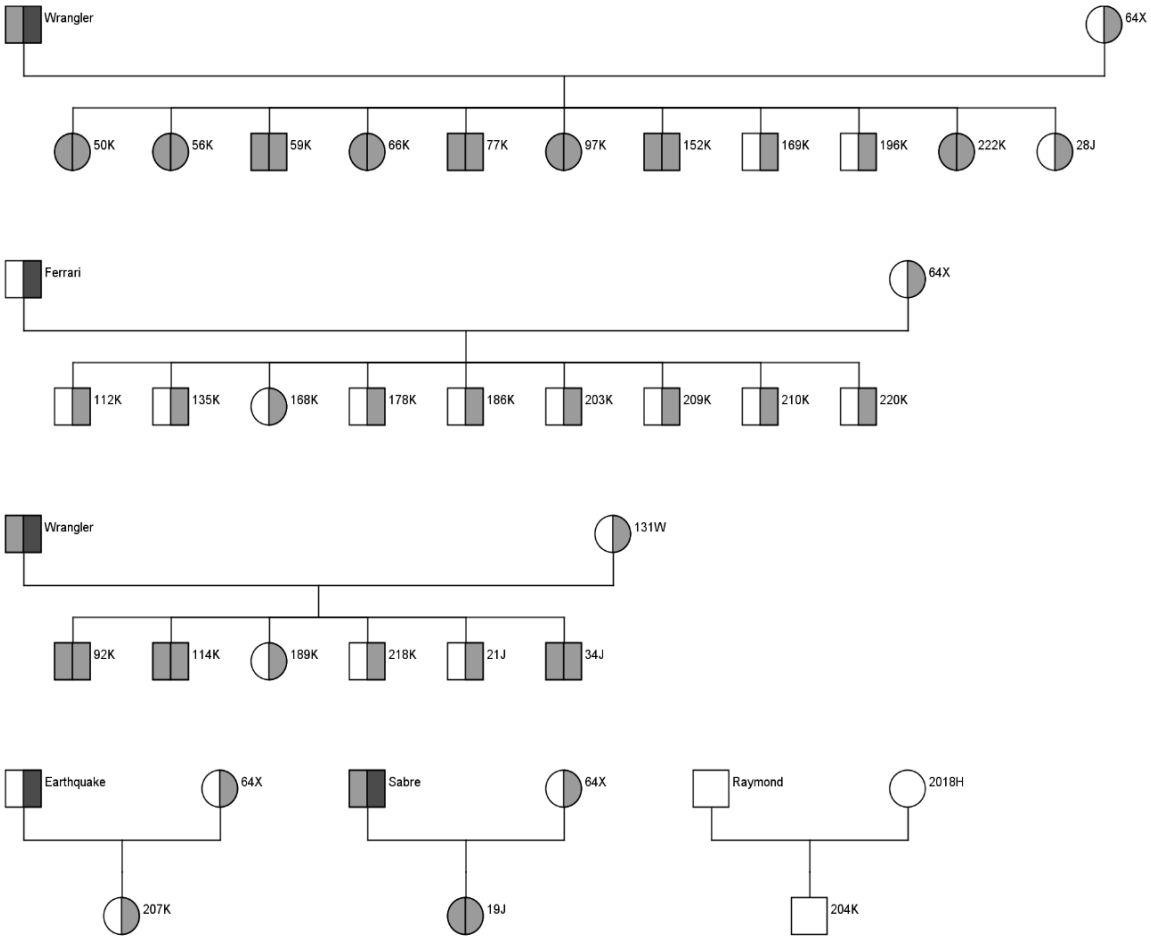

Figure 2.2: Pedigrees showing the breeding results of the cattle. Squares indicate males and circles indicate female. Two phenotype statuses are displayed within the symbol for each individual, SLICK (left) and polled (right). White indicates no allele for the phenotype, light grey indicates one allele for the phenotype (heterozygous), and dark grey indicates two alleles for the phenotype (homozygous).

Discussion

This study aimed to determine reliable genetic predictors of the SLICK and polled phenotypes of the cattle used in the Climate Master Cattle project from an array of genetic markers that have been previously associated with these respective traits. For the SLICK phenotype, the rs517047387 deletion was consistently found in all Senepol Angus Composite cattle in our study exhibiting the SLICK phenotype (Table 2.4). Additionally, the rs517047387 deletion was absent in all animals that did not have the SLICK phenotype (Table 2.4). Therefore, rs517047387 appeared to be a reliable predictor of the SLICK phenotype within our study. On the other hand, the markers rs42343907, rs132939689, rs135282100, DIK2416, and NRDIKM023 did not have consistent alleles or fragment lengths only present in animals with the SLICK phenotype and were therefore not considered as reliable predictors of the SLICK phenotype (Table 2.4). Genetically, rs517047387 (chr20:39099214GC>G) causes a p.Leu462* frameshift mutation in the prolactin receptor gene (*PRLR*), truncating 120 C-terminal amino acids from the long isoform of the receptor (Littlejohn et al., 2014). The deletion was present in all SLICK cattle, indicating the short isoform of *PRLR* is likely a causal factor of the SLICK phenotype. This phenotype appears to follow an autosomal dominant inheritance pattern, as only one deletion in the rs517047387 genotype is required for the expression of the SLICK phenotype (Table 2.4) which is consistent with previous literature (Huson et al., 2014; Littlejohn et al., 2014). A similar mutation in the chicken homolog of *PRLR* was described to cause a reduction in feather growth and suggests altered JAK-STAT signalling pathways (Bu et al., 2013). Furthermore, in murine models, prolactin receptor knockout mice exhibit longer and coarser hair (Craven et al., 2001), and prolactin has been suggested to act directly in follicle epithelia to modulate hair cycle activity (Foitzik et al., 2003). Additionally, the SNP rs520582588 (chr23:35332871A>C) was previously linked with a hairy coat phenotype that is functionally reciprocal to rs517047387, therefore genotyping this locus in the foundation stock was used to determine whether the SLICK phenotype can be blocked in progenies (Littlejohn et al., 2014). While previous literature and the confirmation that rs517047387 is a reliable predictor of SLICK within our population points towards prolactin signalling pathway being involved in hair growth, the exact mechanism of prolactin and its receptor that generates the SLICK phenotype

remains unknown. Fragment length analysis (FLA) of DIK2416 and NRDIKM023 between the samples yielded inconsistent results that suggests variability within cattle groups which is not a trait generally exhibited by reliable predictors. Furthermore, the preliminary FLA was conducted after the Sanger sequencing workflow and the analysis of the predicting capability of rs517047387. For these reasons and the separate workflow required for FLA, this workflow was not conducted on the remaining samples of the progenitor stock of the Climate Master Cattle project.

A total of 5 SNPs were identified in BTA 1, across a 206,141bp range to be associated with the polled phenotype (Table 2.5) (Randhawa et al., 2020). Only rs383143898 appears to have an insertion-deletion allelic variant that is consistent in all cattle with the polled phenotype and absent in all horned cattle in our sample set. As such, rs383173898 was used as a reliable predictor for the polled phenotype within our sample group. Similarly, to the SLICK predictor, rs383143898, appears to follow an autosomal dominant inheritance pattern. This is evident as only one insertion-deletion allele seems to result in the polled phenotype. No differences in the SNPs rs210350155, rs797088784, rs799403053, and rs801127025 across all samples were observed eliminating them as possible predictors of polledness. The SNP rs383143898 is a complex insertion-deletion replacing 6bp with a 202bp insert (Medugorac et al., 2012; Randhawa et al., 2020). Three genes surround rs383143898: oligodendrocyte transcription factor 1 and 2 (*OLIG1*/*OLIG2*) and interferon alpha and beta receptor subunit 2 (*IFNAR2*) (Medugorac et al., 2012). *OLIG1* and *OLIG2* are involved in spinal cord development and oligodendrocyte differentiation and maturation (Meijer et al., 2012; Stafuzza et al., 2018). Interestingly, *OLIG2* has been associated with horn ontogenesis in cattle during developmental stages (Allais-Bonnet et al., 2013; Stafuzza et al., 2018; Wiedemar et al., 2014). Signalling pathways of *IFNAR2* have also been associated with osteoclastic bone reabsorption (Stafuzza et al., 2018; Zanin et al., 2020). While the mechanism of this is unconfirmed, rs383143898 could be a cis-regulatory element to *IFNAR2* or more likely *OLIG2* due to its role in cattle horn ontogenesis.

While mechanisms of the rs517047387 and rs383143898 markers for the SLICK and polled phenotypes, respectively, are unconfirmed, these genetic markers act as

predictors for their phenotypes in our cattle population. This allows for a simple, rapid, and cost-effective method of predicting these traits throughout the breeding conducted in this project. SLICK and polled phenotypes following autosomal dominant inheritance patterns also enables a high success rate for passing these traits onto offspring. Since rs517047387 and rs383143898 have been previously associated with the SLICK and polled phenotypes, respectively, this study further supports their suitability to act as genetic predictors (Littlejohn et al., 2014; Randhawa et al., 2020). Additional research is required to determine the molecular mechanisms of PRLR, OLIG2, and IFNAR2 involved in the generation of the SLICK and polled phenotypes; however, having reliable predictors for these phenotypes will be invaluable for the cattle industry in the development of new synthetic composite cattle breeds like the one we have proposed in the future.

Chapter Contributions

Initial experimental design and NSERC funding acquisition was completed by Dr. John Church and Dr. Paul J. Adams. SNP targets associated with the SLICK and polled phenotypes were identified through literature search completed by Dr. John Church, Dr. Paul J. Adams, and Robert J. Wester. Assays were constructed and optimized by Robert J. Wester using a previously validated assay optimized by Michael Ke. Samples were acquired and sent to the Applied Genomics Centre by Dr. John Church and Wyatt Cook. DNA extraction of samples and the subsequent Sanger sequencing and fragment length analysis was completed by Robert J. Wester. Analysis and pedigree construction was completed by Robert J. Wester.

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CHAPTER 3: Assessment of Heat Tolerant Capabilities in Climate Master Hybrids with the SLICK Genotype

Abstract

Beef cattle (*Bos taurus*) heat stress is an emerging issue amidst the effects of climate change, causing a multitude of negative outcomes to the quality of life for animals within temperate environments, such as North America. A trait called SLICK found in Senepol cattle is a promising asset for heat tolerant capabilities. As such, we developed a new Climate Master Hybrid breed using Senepol, Angus, and Galloway to withstand extreme temperature ranges in Canada. It is proposed to cross Akaushi with the Climate Master Hybrid to enhance carcass characteristics in the future, called the Climate Master Composite. A key aspect of the development of the Climate Master Hybrid after the inclusion of the alleles of interest has been introduced into the cattle, is to assess if the introduced allele is sufficient to produce the heat tolerant phenotype in these hybrid animals. To do this we compared our Climate Master Hybrid cattle with the SLICK genotype using an array of metrics associated with heat tolerant capabilities, against a panel of different control groups.

A total of 33 cattle, separated into 4 groups: Akaushi (Wagyu), Angus, Climate Master Hybrid crosses without the SLICK genotype, and Climate Master Hybrid crosses with the SLICK genotype, were used to measure respiration rate, internal rumen temperatures, activity levels, HSP70 gene expression and HSP70 plasma concentrations to characterize the effects of heat stress on each cattle group. Our Climate Master Hybrid with the SLICK genotype displayed evidence of superior heat tolerant capabilities as determined by lower respiration rates ($p < 0.001$) and increased activity levels ($p < 0.01$) when exposed to elevated temperature humidity index (THI) levels. However, we found no difference between SLICK and our other cattle groups when comparing rumen temperature ($p > 0.05$), HSP70 gene expression ($p = 0.367$), and HSP70 plasma concentrations ($p = 0.421$). It can be inferred that SLICK likely provides superior heat tolerant capacity, however, the initial THI threshold for heat stress is the same through HSP70 results. Therefore, it suggests that SLICK thermotolerant capabilities arise through adjustments in heat dissipation rather than heat absorption or production.

Introduction

Cattle (*Bos taurus*) heat stress is a growing issue for the beef cattle industry in North America. Heat stress is characterized by an imbalance of thermal heat production and dissipation, resulting in an internal temperature that exceeds the thermoneutral zone (Lees et al., 2019). This has been known to cause a plethora of detrimental effects to growth, fertility, meat quality, and overall wellbeing (Bernabucci, 2019; Lees et al., 2019). To offset these effects, cattle have multiple behavioural and physiological responses to heat stress, which are generally observable around the temperature-humidity index (THI) of 72 (Gonzalez-Rivas et al., 2020). These responses include increased respiration rates, reduced movement, increased internal temperatures, and increased expression of genes dedicated to cellular homeostasis during stress, such as heat shock protein 70 (HSP70) (Deb et al., 2013; Kishore et al., 2014; Peana et al., 2007). With escalating prevalence and magnitude of elevated temperatures due to climate change, it is apparent that the adverse effects of heat stress will become increasingly harmful and pervasive (Zhang et al., 2019). Common North American beef cattle breeds, like Angus and Galloway, are adapted to colder temperatures, however, with changes in the climate, these breeds are struggling throughout periods of hotter temperatures (Peters & Slen, 1964; Scasta, 2021).

A tropical beef breed of cattle called Senepol, might present a solution for cattle heat stress with its heat tolerant capabilities (Huson et al., 2014; Littlejohn et al., 2014). Senepol's higher heat tolerance has been attributed to its short and sleek hair (SLICK) trait which is coupled with an increased capacity for sweating, which reduces heat absorption by reflecting ultraviolet radiation and increases heat loss through sweating (Huson et al., 2014; Littlejohn et al., 2014). This autosomal dominant trait is localized to chromosome 20 (BTA20) within an exon of the prolactin receptor (*PRLR*) gene (Littlejohn et al., 2014; Porto-Neto et al., 2018). The phenotype is encoded by a deletion that causes a frameshift introducing a premature stop codon, truncating the cytosolic tail of the receptor (Littlejohn et al., 2014; Porto-Neto et al., 2018). Due to its autosomal dominant inheritance pattern, the SLICK phenotype can be readily introduced into other cattle breeds through controlled breeding (Porto-Neto et al., 2018).

While the SLICK phenotype found in Senepol presents superior thermotolerant capacity, implementation of this breed into Canadian beef markets would not by easy in terms of farmer/consumer reception, as well as the highly variable weather patterns in Canada (Liu et al., 2022). Angus cattle has been a pillar of Canadian beef markets and introducing a novel and unfamiliar breed into this system would likely face farmer resistance and/or consumer reluctancy (Agriculture and Agri-Food Canada, 2022; Liu et al., 2022). Therefore, a 3/8 Senepol, 1/8 Red Angus, and 1/2 Galloway composite breed, aptly called the Climate Master Hybrid, was generated to potentially mitigate these concerns. Ideally, this composite breed should have both resistance to extreme temperature ranges resulting from the hot and cold climates in Canada, through the capabilities of the SLICK phenotype and the natural cold tolerance of Galloway, respectively. Quantification of this Climate Master Hybrid's heat-tolerant capabilities were assessed using multiple heat stress-associated metrics against Angus and Akaushi controls. Akaushi cattle, a specific breed of Wagyu, were included in this study as Wagyu is becoming increasingly popular in luxury Canadian beef markets (Horner, 2015). Akaushi cattle were chosen over Japanese Black cattle, which account for 97% of the Wagyu in Japan, due to their black coat which may interfere with the heat tolerant characteristics expected in our Climate Master Hybrid (Lees et al., 2019; Motoyama et al., 2016). Additionally, Akaushi were originally developed to be productively viable in temperate environments of the Kumamoto and Kochi prefectures (Kimura et al., 2021). The proposed final goal of this project is to produce a Climate Master Composite, which will potentially have improved carcass characteristics through breeding of the Climate Master Hybrid with Akaushi cattle. Ultimately, this research aims to further the understanding of the SLICK phenotype in a novel composite breed amid hotter temperatures to determine its suitability for implementation into the Canadian beef industry in the future.

Materials and Methods

This section includes three separate experimental subsections containing the materials and methods used to assess the thermotolerant capabilities of the Climate Master Hybrid with the SLICK genotype. These three subsections include respiration rate testing, bolus monitoring, and HSP70 expression analyses. Briefly, respiration rate testing involves breathing measurements in response to changes in temperature conditions. Bolus monitoring includes the observation of changes in internal rumen temperatures and relative activity levels. HSP70 expression analyses include the gene expression and blood plasma concentrations of the molecular chaperone HSP70. A total of 33 cattle were separated into four distinct groups: Akaushi, Angus, Climate Master Hybrids without the SLICK genotype (non-SLICK), and Climate Master Hybrids with the SLICK genotype (SLICK). To reduce genetic variation in the Climate Master Hybrid offspring, the progeny was generated by only two crosses. Eggs from two Galloway female donors (64X and 131W) were bred using semen from a single 3/4 Senepol 1/4 Red Angus Composite bull (Wrangler) heterozygous for the SLICK genotype. Crosses were generated using *in vitro* fertilization, with viable embryos successfully implanted into various Angus cross surrogates. The resulting Climate Master Hybrid progeny were either full or half siblings to each other as they were sired by the same bull, differing by the presence or absence of the SLICK genotype. The presence of the SLICK phenotype was confirmed using single nucleotide polymorphism (SNP) genotyping as outlined in Chapter 2. The individual cattle used for each of experiments are outlined in Table 3.1.

All procedures outlined in this section were conducted in accordance with the Canadian Council of Animal Care guidelines and was approved by the Animal Care Committee of Thompson Rivers University (Kamloops, B.C., Canada) (File number: 102823).

Table 3.1. Animals used in the assessment of the SLICK phenotype experiments. All animals were kept on Buck Lake Ranch over the course of the experiments.

Animal Information				Respiration Rate Testing	Bolus Monitoring	HSP70 Expression Analyses
ID	Group	Sex	SLICK[1]			
204K	Akaushi	M	CC	Yes	Yes	Yes
13J	Akaushi	F	CC	Yes	Yes	Yes
14J	Akaushi	F	CC	Yes	Yes	Yes
15J	Akaushi	F	CC	Yes	Yes	Yes
40J	Akaushi	F	CC	Yes	Yes	Yes
43J	Akaushi	F	CC	Yes	Yes	Yes
4839	Akaushi	M	CC	Yes	Yes	No
4932	Akaushi	M	CC	No	Yes	No
319	Angus	F	CC	Yes	Yes	Yes
317	Angus	F	CC	Yes	Yes	Yes
316	Angus	F	CC	Yes	Yes	Yes
313	Angus	F	CC	Yes	Yes	Yes
318	Angus	F	CC	Yes	Yes	Yes
315	Angus	F	CC	Yes	Yes	Yes
94K	Angus	M	CC	No	Yes	Yes
169K	non-SLICK	M	CC	Yes	Yes	Yes
196K	non-SLICK	F	CC	Yes	Yes	Yes
218K	non-SLICK	M	CC	Yes	Yes	Yes
21J	non-SLICK	M	CC	Yes	Yes	Yes
189K	non-SLICK	F	CC	Yes	Yes	Yes
312	non-SLICK	F	CC	Yes	Yes	Yes
77K	SLICK	M	NC	Yes	Yes	Yes
92K	SLICK	M	NC	Yes	Yes	Yes
114K	SLICK	M	NC	Yes	Yes	Yes
152K	SLICK	M	NC	Yes	Yes	Yes
34J	SLICK	M	NC	Yes	Yes	Yes
50K	SLICK	F	NC	Yes	Yes	Yes
66K	SLICK	F	NC	Yes	Yes	Yes
97K	SLICK	F	NC	Yes	Yes	Yes
222K	SLICK	F	NC	Yes	Yes	Yes
19J	SLICK	F	NC	Yes	Yes	Yes
59K	SLICK	M	NC	Yes	Yes	Yes
56K	SLICK	F	NC	Yes	Yes	No

[1]SLICK genotype: 'N' represents deletion that is associated with the SLICK phenotype.

Site Information

Buck Lake Ranch is located in Beaverdell, British Columbia (49°28'51"N 119°02'40"W) which is approximately 60 kilometers from Kelowna. The property is 260 hectare deeded, is 822 m above sea level, and has been developed for high-capacity beef cattle raising with the inclusion of summer grazing from vast amounts of surrounding leased land. During periods between sampling, cattle were able to graze freely on the property. Additionally, Beaverdell has one of the highest diurnal temperature variations in Canada with especially cool nights.

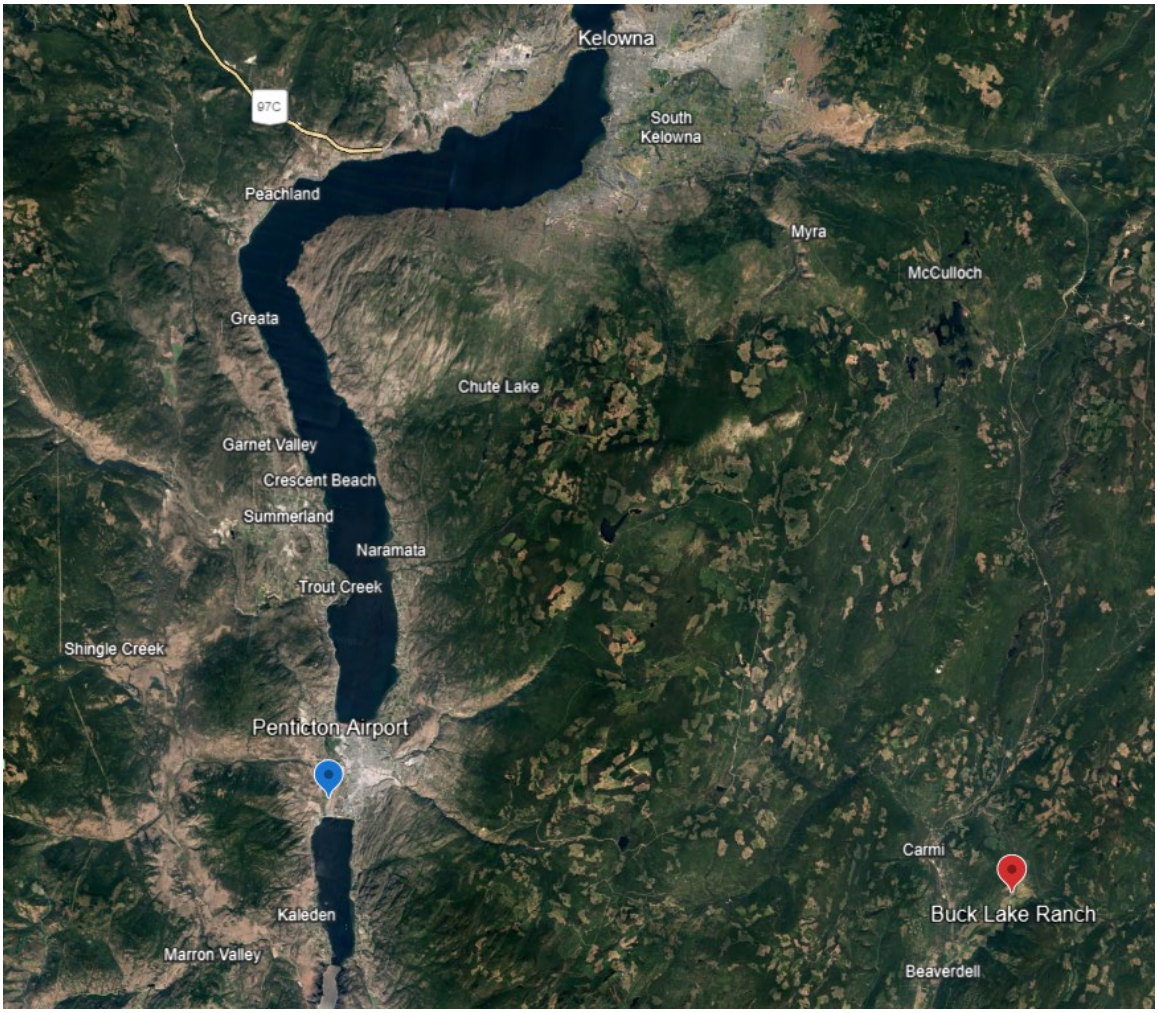

Figure 3.1: Site location outlining the position of Buck Lake Ranch (red) and the weather station at Penticton Airport (blue) used for collecting climate data for the Bolus Monitoring and HSP70 Analyses sections. Image captured using Google Earth (Google, Mountain View, California, United States).

Respiration Rate

Respiration rate was collected for the animals outlined in Table 3.1 using a novel technique developed by Mufford et al., involving drones to minimize the observer effects on measurement (2021). All animals were tagged with a unique Penning Tag (Wheel'n'Tree Enterprises, Edmonton, Alberta, Canada) glued to their backs for aerial identification. Data collection occurred during four different time periods: July 15, 2023, at 1400 hours, and July 16, 2023, at 0700, 1400, and 1900 hours. Both a DJI Mavic 3T Quadcopter (Dà-Jiāng Innovations Science and Technology Co., Ltd., Shenzhen, China) and a DJI Mavic Enterprise Advanced Quadcopter (Dà-Jiāng Innovations Science and Technology Co., Ltd., Shenzhen, China) were used to record video of cattle at an altitude of 3-5 meters above ground. When in position above a group of cattle, the quadcopters recorded video for a minimum of three minutes of stable footage for each animal. Cattle identities were confirmed visually using a Vortex® Viper® HD 3000 Laser Rangefinder (Vortex Optics, Barneveld, Wisconsin, USA). During these collection periods, ambient temperature and relative humidity were recorded using a Kestrel 5400AG Portable Weather Station (Neilsen-Kellerman Company, Boothwyn, Pennsylvania, USA). This data was then used to calculate the temperature humidity index (THI), using the following equation (García-Ispierto et al., 2006):

$$THI = (0.8 \times T_a) + RH \times (T_a - 14.4) + 46.4$$

where T_a is ambient temperature (°C), RH is relative humidity (%).

Captured videos from the UAVs were processed using Observer XT Software (Noldus, Information Technology Wageningen, The Netherlands) to quantify respiration rate on each individual animal. Respiration rate was calculated by counting flank movements, which were time stamped as behavioural events. Other behavioural events that were recorded included: changing positions, skin twitching, grooming, and regurgitating. The positional state: standing, lying, and walking, of the animals was also recorded. Only flank movements exceeding a two-minute duration were included in the respiration rate calculation. Flank movement counts were divided by recording time to

calculate breaths per minute (bpm). Data collection and UAV piloting were carried out by Matthew Francis and Mathis Gegout, undergraduate summer research assistants.

Respiration rate data was grouped by collection period and each period was subjected to normality testing for downstream analysis using the Shapiro-Wilk test (Shapiro & Wilk, 1965). When the data was non-parametric, the Kruskal-Wallis test (Kruskal & Wallis, 1952) and the pairwise Wilcoxon rank sum test (Wilcoxon, 1945) were used to determine differences between sample groups at each period. However, if the data followed a normal distribution, an ANOVA (Fisher, 1921) was performed, followed by a Student's T-test (Student, 1908) for pairwise group comparisons if the ANOVA was statistically significant. The Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995) of adjustment was used to control for false discovery rates (FDR). All analyses and visualizations of the data was conducted in R 4.2.3 Statistical Software (R Core Team, 2023). Data manipulation was performed using the dplyr package (Wickham et al., 2023) and data visualization was performed using the ggplot2 (Wickham, 2016), ggpubr (Kassambara, 2023), tidyverse (Wickham et al., 2019), and patchwork packages (Pedersen, 2022).

Bolus Monitoring

On May 15, 2023, all animals outlined in Table 3.1 had a Moonsyst bolus (Moonsyst International Ltd., Kinsale, Country Cork, Republic of Ireland) inserted down their esophagus using a 33 mm balling gun. The unique identifier of each of the boluses were recorded and assigned to each of the respective animals in the Moonsyst system. The Moonsyst system regularly records internal rumen temperatures (T_R) and the movements of each animal using an accelerometer. The bolus records T_R and activity each hour, which is promptly sent to a Moonsyst Gateway Receiver (Moonsyst International Ltd., Kinsale, Country Cork, Republic of Ireland) when in range. If cattle are out of range of the receiver, then the bolus saves the data until it is within range. The movement data automatically marks the number of times the movements pass activity thresholds, as determined by a three-axis accelerometer contained within the Moonsyst bolus, at three different preset levels: low, high, and very high. All data received by the Moonsyst Gateway receiver is automatically uploaded to the Mooncloud (Moonsyst

International Ltd., Kinsale, Country Cork, Republic of Ireland) and data from June 1 to August 31, 2023 was collected for analysis. From the activity threshold values, an Activity Index (A_I) was calculated using the following equation:

$$A_I = A_L + 2A_H + 3A_{VH}$$

where A_L is low activity, A_H is high activity, and A_{VH} is very high activity.

Relative humidity and temperature were collected by from a weather station by Penticton Airport during the same time frame of bolus data collection which was downloaded from a publicly available database called Weather Underground (Weather Underground Inc., 1995). The Penticton Airport Station (49°27'26"N 119°36'25"W) is at an elevation of 345 m and is the closest available weather station to Beaverdell, BC (49°28'51"N 119°02'40"W) with 37.38 km between locations (See Figure 3.1) and with a difference of 477 m elevation. The relative humidity and temperature measures were then used to calculate the THI, using the equation outlined above (García-Ispierto et al., 2006).

The normality of the T_R and A_I data was determined using density plots and the Shapiro-Wilk test (Shapiro & Wilk, 1965). Both T_R and A_I were then subjected to a Kendall's tau rank correlation test (Kendall, 1938), a robust non-parametric version of Pearson's Correlation, against THI to identify associations between the variables. A LOESS regression was applied to T_R and A_I to visualize trends of the data (Jacoby, 2000). The Kruskal-Wallis test (Kruskal & Wallis, 1952) and the pairwise Wilcoxon rank sum test (Wilcoxon, 1945) were used to determine differences between sample groups. The control of FDR in all statistical tests was achieved through the application of the Benjamini-Hochberg adjustment procedure (Benjamini & Hochberg, 1995). All statistical analyses and visualizations were completed using R 4.2.3 Statistical Software (R Core Team, 2023). Data manipulation and organization was performed using the dplyr package (Wickham et al., 2023) and final visualizations were generated using the ggplot2 (Wickham, 2016), ggpubr (Kassambara, 2023), tidyverse (Wickham et al., 2019), and patchwork packages (Pedersen, 2022).

HSP70 Analyses - Gene Expression

Hair samples were collected from the 30 cattle identified in Table 3.1 at two time points, May 15, 2023, and August 3, 2023. The hair was pulled from the tails of the cattle ensuring the root follicle was present and placed, follicle first, in a falcon tube with 5 mL RNA*later* (Thermo Fisher Scientific, Burnaby, B.C., Canada). Samples were kept on ice until being stored in a -20°C freezer until RNA extraction.

Total RNA was extracted using the PureLink™ RNA Mini Kit (Thermo Fisher Scientific, Burnaby, B.C., Canada) and TRIzol reagent (Thermo Fisher Scientific, Burnaby, B.C., Canada). Follicles from the hair samples were thawed on ice and then cut to size and placed into 2 mL Geno/Grinder™ tubes preloaded with 3mm zirconia beads (Cole-Parmer, Québec City, Québec, Canada). The tubes were seated in a Geno/Grinder™ cryogenic block (Cole-Parmer, Québec City, Québec, Canada) that was placed in a -80°C freezer for at least 60 minutes. Once all the samples were placed in their respective tubes, the cryogenic blocks were placed in the SPEX™ SamplePrep Geno/Grinder™ instrument (Fisher Scientific, Burnaby, B.C., Canada). Samples were then homogenized at 1300 rpm for 30 seconds for 3 cycles, with a 30 second rest between each cycle. After homogenization, the PureLink™ RNA Mini Kit protocol using TRIzol was followed with the on-column PureLink™ DNase Treatment (Thermo Fisher Scientific, Burnaby, B.C., Canada) protocol (Thermo Fisher Scientific, 2012). RNA was eluted with 39 µL of RNase-free water and 1 µL of RiboLock (Thermo Fisher Scientific, Burnaby, B.C., Canada) was added. Purity and concentration of the RNA was quantified using the NanoDrop One (Thermo Fisher Scientific, Burnaby, B.C., Canada) instrument. Immediately following the RNA extraction, the samples were subjected to a cDNA conversion using the High-Capacity cDNA Reverse Transcription Kit. The cDNA was then quantified using the NanoDrop One instrument.

Relative gene expression of HSP70 was measured using Real-Time PCR (qPCR). Custom TaqMan assays for both HSPA1A (HSP70 gene) and the two endogenous controls, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-tubulin isoform 5, which were designed using the 3.0.1 Primer Express software (Life Technologies, Carlsbad, California, United States). Primer pairs and probe sequences for each assay are

outlined in Table 3.2. All qPCR reactions were prepared using 5 µL of Fast Advanced Master Mix (Thermo Fisher Scientific, Burnaby, B.C., Canada), 0.5 µL of 20X TaqMan assay, 3.5 µL dH_2O, and 1 µL of template cDNA. The 20X assays were custom mixed to have the primer concentration at 900 nM and the probe concentration at 250 nM. Each assay was optimized for annealing temperature using a gBlock designed containing binding sites for each assay constructed by Integrated DNA Technologies (Coralville, Iowa, United States). See Figure A.1 in Appendix for the annotated sequence. All reactions after optimization were completed using the following thermocycler conditions: initial denaturation of 95°C for 60 seconds, followed by the 45 cycles of 95°C for 30 seconds and 63°C for 60 seconds. All qPCR reactions were done in triplicate and carried out in a QuantStudio 7 instrument (Thermo Fisher Scientific, Burnaby, B.C., Canada).

Table 3.2. TaqMan assays used for gene expression analysis.

Assay	Component	Sequence (5′-3′)	Quencher	Fluorophore
HSP70	Forward	CCGGTGCCCTGCCTTT		
	Reverse	GGCCGTTTTCAGGTTTGAAG		
	Probe	CGGCTCCGAGATAA	MGB	6FAM
GAPDH	Forward	CCCTCCACGATGCCAAAGT		
	Reverse	GGCGTGAACCACGAGAAGTATAA		
	Probe	AGGAGGCATTGCTGACAATCTTGAGGGT	TAMRA	VIC
β-tubulin	Forward	GCTGTTCTTATTCTGCACGTTGA		
	Reverse	CGTGGGCGGATGTCCAT		
	Probe	CATCTGCTCATCTACC	MGB	6FAM

Cycle threshold (Ct) values were collected from the QuantStudio 7 instrument and were used in downstream analysis if at least two of the three replicates were successful as two consistent replicates are likely reliable. Analysis of the gene expression data was completed by calculating fold change between the experimental and baseline data using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), using the following equations:

$$\Delta\Delta Ct = \Delta Ct_E - \Delta Ct_B = (Ct_{TE} - Ct_{CE}) - (Ct_{TB} - Ct_{CB})$$

$$Fold\ Change = 2^{-\Delta\Delta Ct}$$

where Ct_{TE} is the mean cycle threshold for experimental HSP70, Ct_{CE} is the average mean cycle threshold for experimental endogenous controls (GAPDH and β-tubulin), Ct_{TB} is the mean cycle threshold for baseline HSP70, and Ct_{CB} is the average mean cycle threshold for baseline endogenous controls (GAPDH and β-tubulin).

Fold change values were tested for normality using the Shapiro-Wilk test (Shapiro & Wilk, 1965). The Kruskal-Wallis test (Kruskal & Wallis, 1952) and pairwise Wilcoxon rank sum test (Wilcoxon, 1945) were used to compare sample groups. We applied the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995) to mitigate FDR in all statistical tests. All statistical analysis and plotting were completed using R 4.2.3 Statistical Software (R Core Team, 2023). Data transformation was performed using the dplyr (Wickham et al., 2023) and qpcr (Spiess, 2018) packages. Final graphical representations of the data were generated using the ggplot2 (Wickham, 2016), ggpubr (Kassambara, 2023), tidyverse (Wickham et al., 2019), and patchwork packages (Pedersen, 2022). Temperature humidity index (THI) values were collected for seven days prior to each hair collection periods, as outlined in the Bolus Monitoring subsection, to compare the daily average peak temperatures leading up to each collection period.

HSP70 Analyses - Blood Plasma Concentrations

Blood samples were collected from the 30 cattle outlined in Table 3.1 on August 3, 2023. Approximately 2 mL of blood was collected from the coccygeal vein using sterile needles and syringes and placed into 3 mL BD Vacutainer™ tubes coated with K_2 EDTA anticoagulant (Fisher Scientific, Burnaby, B.C., Canada). Blood samples were kept on ice for at least 30 minutes before centrifugation at 1300 RCF at 2°C for 10 minutes to fractionate the blood. Approximately 500 µL of blood plasma was transferred to 2 mL cryogenic tubes (VWR International, Radnor, Pennsylvania, United States) and kept on ice until placed in a -80°C freezer.

Plasma HSP70 concentrations were quantified using the Invitrogen HSP70 Human ELISA Kit (Thermo Fisher Scientific, Burnaby, B.C., Canada). Human HSP70 is analogous to bovine HSP70 and anti-human HSP70 antibodies have previously been successfully to measure HSP70 in bovine studies (Gaughan et al., 2013). A 25 µL aliquot

of each plasma sample was diluted using 225 µL of the provided sample diluent prior to the ELISA (1:10 dilution factor). The lyophilized human HSP70 standard was reconstituted in distilled water to a concentration of 20 ng/mL, then diluted using the sample diluent to a concentration of 2.5 ng/mL. A 2-fold serial dilution was used, producing a standard curve with concentrations ranging from 2.5 ng/mL to 0.156 ng/mL. Sample diluent was used as a blank. All samples and standards were prepared in duplicate as outlined in the manufacturer's protocol (Thermo Fisher Scientific, 2018). Absorbance at 450 nm and 620 nm (reference) was measured for each sample using the SpectraMax M2 Microplate Reader (Molecular Devices, San Jose, California, United States).

The 620 nm absorbance was used to correct the 450 nm absorbances of all samples and standards. The corrected 450 nm absorbances of the standard curve and its respective concentrations were used to generate a linear regression model. This model was then used to calculate HSP70 concentrations of the plasma samples based on their respective corrected absorbance at 450 nm. Concentration values were then tested for normality using the Shapiro-Wilk test (Shapiro & Wilk, 1965). The Kruskal-Wallis test (Kruskal & Wallis, 1952) and pairwise Wilcoxon rank sum test (Wilcoxon, 1945) were used to compare sample groups. We applied the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995) to mitigate FDR in all statistical tests. All statistical analysis and plotting were completed using R 4.2.3 Statistical Software (R Core Team, 2023). Data transformation was performed using the dplyr (Wickham et al., 2023) package. Final graphical representations of the data were generated using the ggplot2 (Wickham, 2016), tidyverse (Wickham et al., 2019), superb (Cousineau, 2021) and patchwork packages (Pedersen, 2022).

Results

Respiration Rate

A total of 32 cattle were monitored for changes in respiration rate at four different time points, with each time point having a different THI value. The THI on July 15 at 13:00 – 15:00 (afternoon), and July 16 at 07:00 – 09:00 (morning), 13:00 – 15:00 (afternoon), and 19:00 – 21:00 (evening), was 74.41 ± 1.21, 67.11 ± 3.38, 75.55 ± 1.49, and 70.62 ± 1.61, respectively. The collected respiration rates for these time periods are all outlined in Figure 3.2 and Table 3.3.

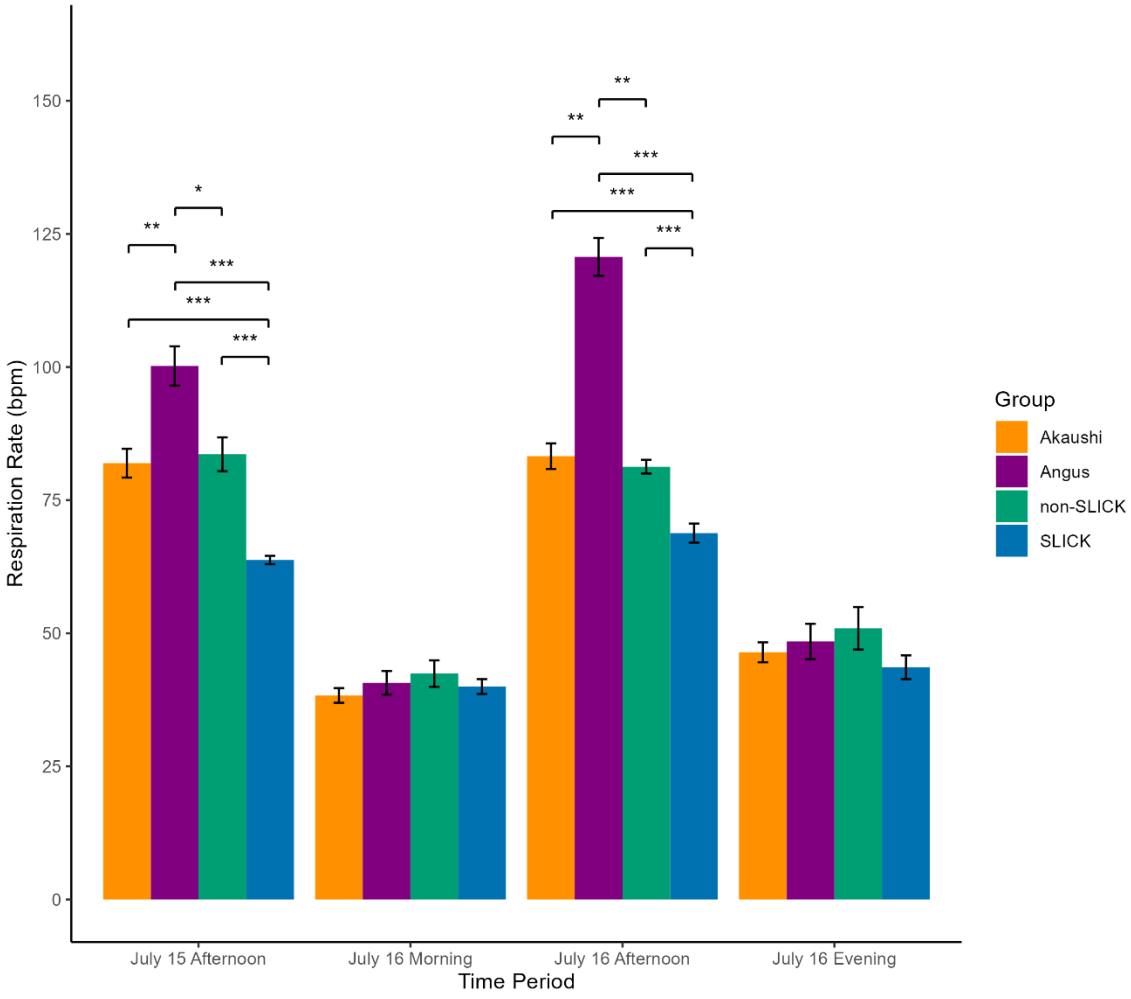

Figure 3.2: Bar plot of mean respiration rate in breaths per minute (bpm) of each group at each collection period. The pairwise Wilcoxon rank sum test was used to compare groups and the Benjamini-Hochberg procedure was used to adjust for FDR. Significance is shown as * which indicates $p < 0.05$, ** which indicates $p < 0.01$, and *** which indicates $p < 0.001$.

Normality testing using the Shapiro-Wilks test indicated three of the four collection periods were non-parametric ($p < 0.05$), the collection period on July 16 at 19:00 – 21:00, however, was determined to be parametric ($p = 0.332$). In order of collection period, the Kruskal-Wallis test, identified differences within time periods July 15 at 13:00 – 15:00 ($p = 4.24\times10^{-5}$) and July 16 at 13:00 – 15:00 ($p = 4.24\times10^{-5}$). Collection times, July 16 at 07:00 – 09:00 and 19:00 – 21:00, did not have any difference between groups (Kruskal-Wallis p-value = 0.515, and ANOVA p-value = 0.279, respectively). On July 15 at 13:00 – 15:00, all pairwise comparisons, except between Akaushi and non-SLICK cattle ($p = 0.731$) were statistically significant. The p-values for all comparisons are as follows: Akaushi and Angus cattle ($p = 1.74\times10^{-3}$), Akaushi and SLICK cattle ($p = 2.15\times10^{-4}$), Angus and non-SLICK cattle ($p = 0.0104$), Angus and SLICK cattle ($p = 2.15\times10^{-4}$), and non-SLICK and SLICK cattle ($p = 2.15\times10^{-4}$). Similarly, all comparisons, except between Akaushi and non-SLICK cattle ($p = 0.252$), were statistically significant on July 16 at 13:00 – 15:00. The p-values for the remaining comparisons are as shown: Akaushi and Angus cattle ($p = 1.75\times10^{-3}$), Akaushi and SLICK cattle ($p = 9.52\times10^{-4}$), Angus and non-SLICK cattle ($p = 2.59\times10^{-3}$), Angus and SLICK cattle ($p = 6.46\times10^{-4}$), and non-SLICK and SLICK cattle ($p = 9.53\times10^{-4}$).

Table 3.3: Respiration rate results for each collection periods outlining bpm for each cattle group and overall rates.

Date/Time	2023/07/15 13:00 – 15:00	2023/07/16 07:00 – 09:00	2023/07/16 13:00 – 15:00	2023/07/16 19:00 – 21:00
Overall	75.67 ± 25.42[a]	39.39 ± 6.29[a]	80.38 ± 16.05[a]	46.65 ± 1.36[b]
Akaushi	84.32 ± 10.93[a]	37.41 ± 4.92[a]	85.12 ± 3.71[a]	46.42 ± 1.86[b]
Angus	99.75 ± 10.04[a]	40.36 ± 5.90[a]	119.79 ± 7.76[a]	48.45 ± 3.33[b]
non-SLICK	82.84 ± 12.06[a]	42.73 ± 5.46[a]	81.38 ± 2.68[a]	50.93 ± 3.97[b]
SLICK	62.75 ± 2.74[a]	39.66 ± 5.33[a]	67.98 ± 7.52[a]	43.61 ± 2.22[b]

[a] median ± interquartile range

[b] mean ± standard error

*bpm: breaths per minute.

Bolus Monitoring - Internal Rumen Temperature and Activity Index

Internal rumen temperatures (T_R) and activity indicies (A_I) were tracked using Moonsyst boluses from June 1 to August 31, 2023. A total of 20,718 data points were collected for both measures over the course of the summer data collection period. Both T_R and A_I indicated non-parametric distribution using the Shapiro-Wilks test ($p = 4.37\times10^{-8}$, and $p = 6.46\times10^{-15}$, respectively). See Figure A.2 in the Appendix for distribution plots.

Overall comparisons using Kruskal-Wallis testing determined that there were differences between groups in both T_R ($p = 3.18\times10^{-50}$) and A_I ($p = 1.82\times10^{-119}$). All data points were visualized against THI and a LOESS regression was used to outline trends as presented in Figure 3.3 for T_R and Figure 3.4 for A_I.

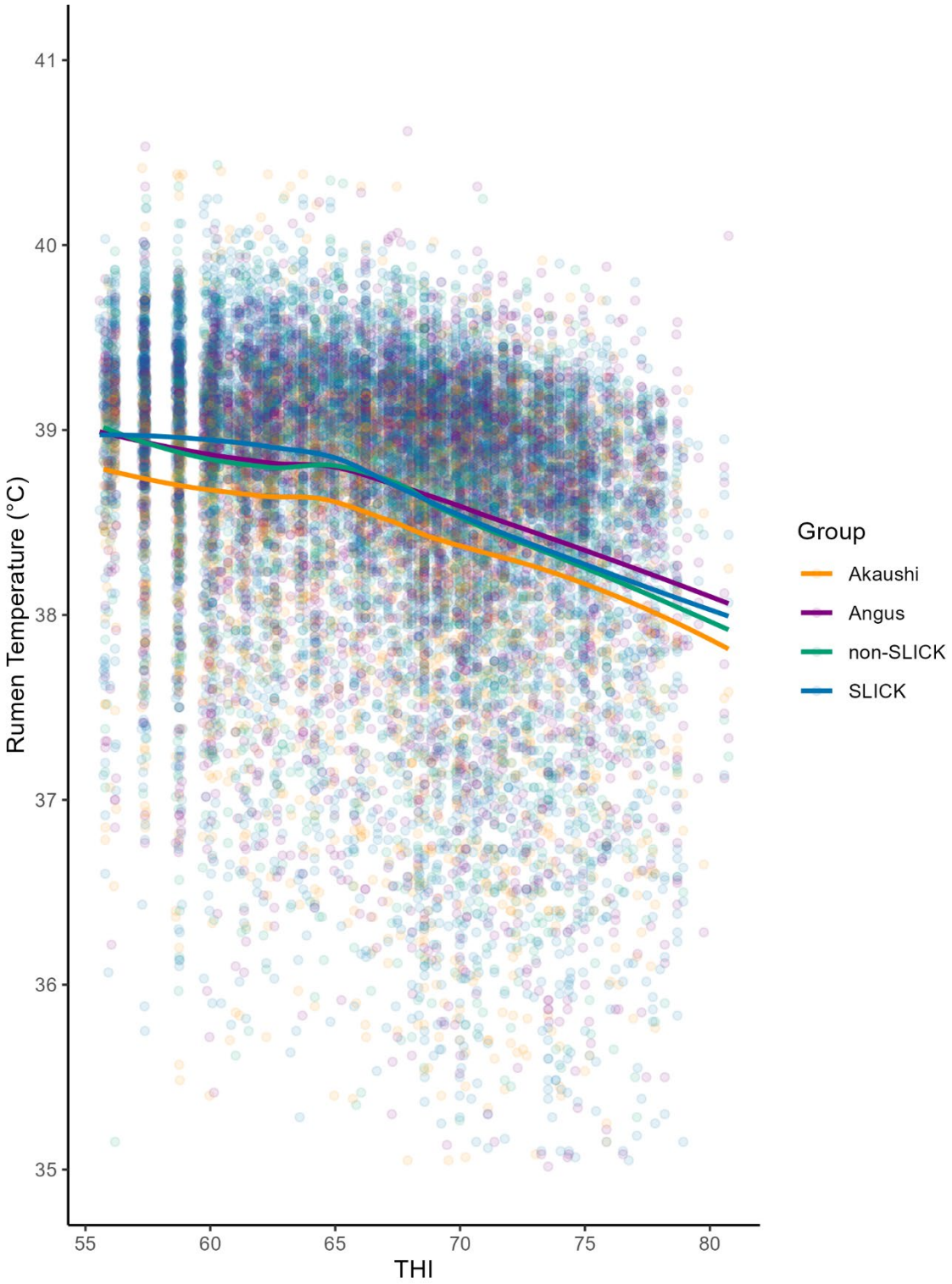

Figure 3.3: Scatter plot of internal rumen temperature (T_R) against THI. Trendlines for each cattle group are overlayed on the plot and were generated using the LOESS method of regression.

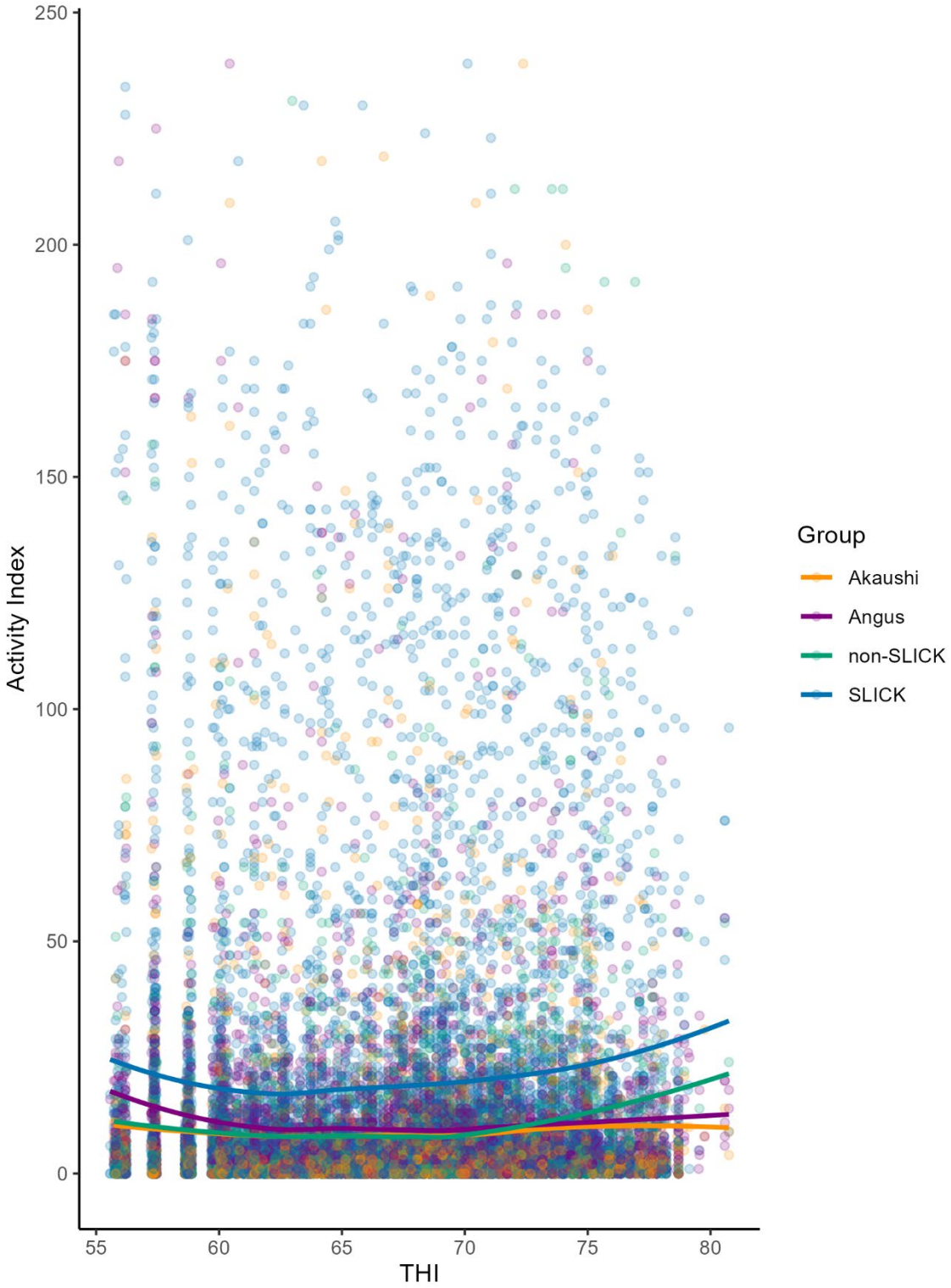

Figure 3.4: Scatter plot of activity index (A_I) against THI. Trendlines for each cattle group are overlayed on the plot and were generated using the LOESS method of regression.

The Wilcoxon rank sum test was then used to do pairwise comparisons between groups. Akaushi cattle appeared to have a statistically significant different T_R in comparison to Angus ($p = 2.13\times10^{-38}$), non-SLICK ($p = 3.30\times10^{-24}$), and SLICK ($p = 1.41\times10^{-46}$) cattle. Additionally, statistical differences between T_R in non-SLICK and SLICK cattle were found ($p = 0.0366$). No differences were found in T_R between Angus and non-SLICK or SLICK ($p > 0.05$) cattle (Table 3.4). In terms of A_I, statistically significant differences were determined between each comparison ($p < 0.05$) as outlined in Table 3.4.

Table 3.4. Wilcoxon rank sum p-values adjusted using the Benjamini-Hochberg procedure for pairwise comparisons in cattle groups for both T_R and A_I across all THI values.

Group 1	Group 2	T_R p-value	A_I p-value
Akaushi	Angus	2.13×10^{-38}	5.03×10^{-71}
Akaushi	non-SLICK	3.30×10^{-24}	7.88×10^{-29}
Akaushi	SLICK	1.41×10^{-46}	6.92×10^{-104}
Angus	non-SLICK	0.247	1.96×10^{-8}
Angus	SLICK	0.247	9.37×10^{-11}
non-SLICK	SLICK	0.0366	3.94×10^{-27}

*T_R: Internal Rumen Temperature, A_I: Activity Index.

Correlation of both T_R and A_I against THI was also determined using Kendall's τ coefficient. An overall weak negative correlation ($\tau = -0.190$, $p < 2.20\times10^{-16}$) was observed between T_R and THI with similar results for each cattle group as displayed in Table 3.4. Conversely, a very weak positive correlation ($\tau = 0.037$, $p = 3.04\times10^{-13}$) was determined between A_I and THI with similar results for each cattle group (Table 3.5), except Angus cattle which had no correlation ($\tau = 0.005$, $p = 0.636$).

Table 3.5. Correlation results for T_R and A_I against THI using Kendall's τ coefficient.

	T_R Correlation			A_I Correlation		
	τ	z	p-value	τ	z	p-value
Overall	-0.190	-40.80	$< 2.20\times10^{-16}$	0.037	7.29	3.04×10^{-13}
Akaushi	-0.156	-13.40	$< 2.20\times10^{-16}$	0.037	3.04	2.40×10^{-3}
Angus	-0.193	-20.30	$< 2.20\times10^{-16}$	0.005	0.47	0.636
non-SLICK	-0.196	-17.01	$< 2.20\times10^{-16}$	0.042	3.55	3.84×10^{-4}
SLICK	-0.236	-29.87	$< 2.20\times10^{-16}$	0.046	5.67	1.47×10^{-8}

*T_R: Internal Rumen Temperature, A_I: Activity Index, z: z-statistic.

To better assess differences between cattle groups as the THI increased, data was binned into five ranges of varying THI values (THI < 60, 60 ≤ THI < 65, 65 ≤ THI < 70, 70 ≤ THI < 75, and 75 ≤ THI). Distribution of data for each group at these THI ranges are presented in Figure 3.5a (T_R) and Figure 3.6a (A_I). Overall comparisons between sample groups using the Kruskal-Wallis test identified statistically significant differences for all THI ranges ($p < 0.05$) as outlined in Table A.1 in the Appendix. Pairwise Wilcoxon rank sum tests were then used to identify differences between groups at each of the THI ranges after adjustment using the Benjamini-Hochberg procedure as visualized in Figure 3.5b (T_R) and Figure 3.6b (A_I). Statistically significant differences ($p < 0.05$) were visualized between many cattle groups at these THI ranges for both T_R and A_I. Akuashi cattle had statistically significant lower T_R against each other cattle group below a THI of 75 (Figure 3.5b). At THI ranges equal to and higher than 75, Akaushi cattle had significantly lower T_R only when compared to Angus cattle (Figure 3.5b). Additionally, SLICK cattle had a statistically significant higher A_I ($p < 0.05$) in comparison to each other cattle group at THI ranges equal to or greater than 70 (Figure 3.6b). At THI ranges below 70 SLICK cattle had statistically significant higher A_I ($p < 0.05$) in comparison to Akaushi and non-SLICK cattle, however, differences in A_I between SLICK and Angus cattle were not statistically significant (Figure 3.6b). For individual p-values from pairwise Wilcoxon rank sum tests at these ranges please refer to Table A.2 in the Appendix.

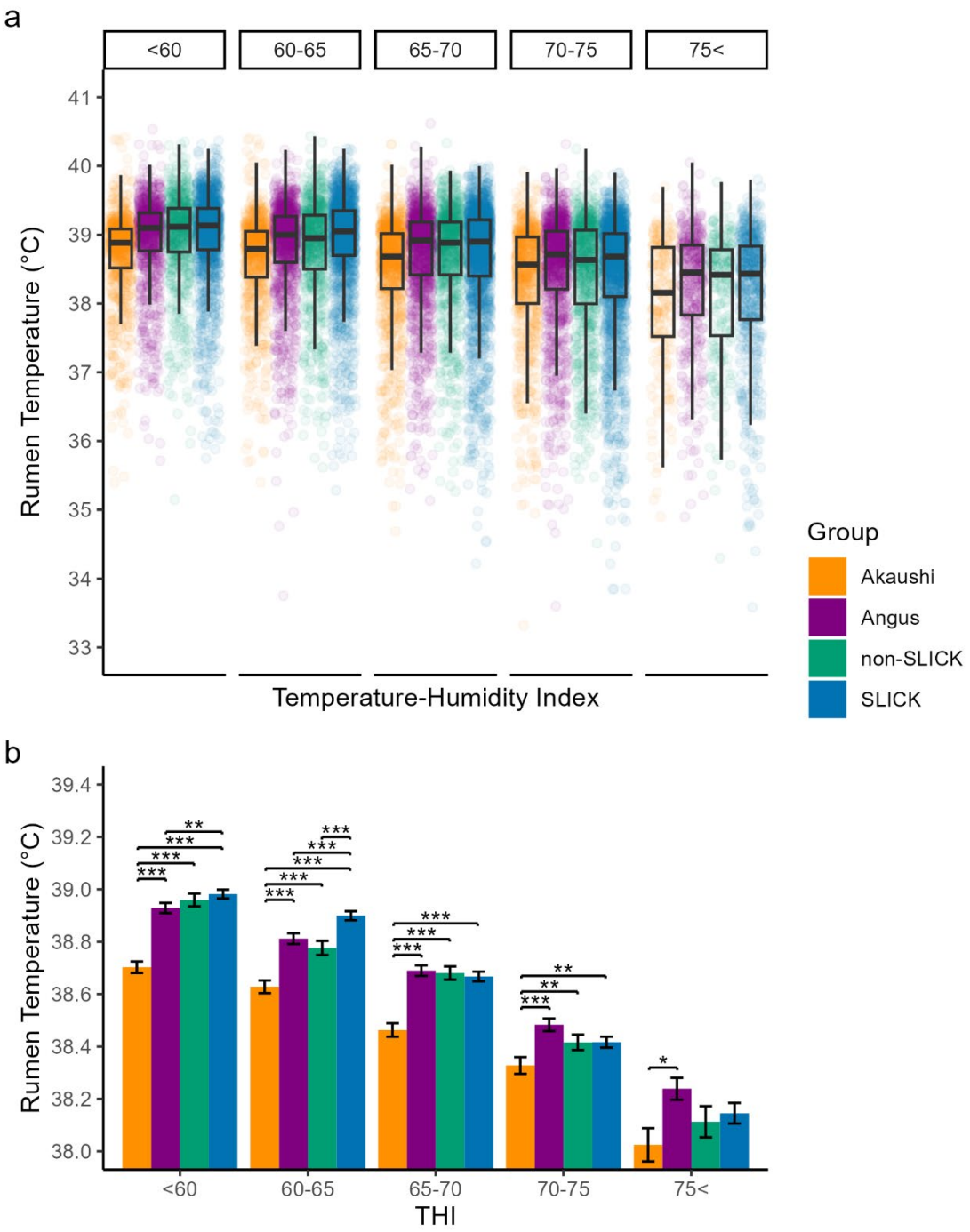

Figure 3.5: Distribution of internal rumen temperature (T_R) data overlayed with boxplots for each cattle group among THI ranges (a). Bar plot of mean T_R for each cattle group at each THI range (b). Pairwise Wilcoxon rank sum test was used to compare groups and was adjusted for FDR using the Benjamini-Hochberg procedure. Significance is shown as * which indicates $p < 0.05$, ** which indicates $p < 0.01$, and *** which indicates $p < 0.001$.

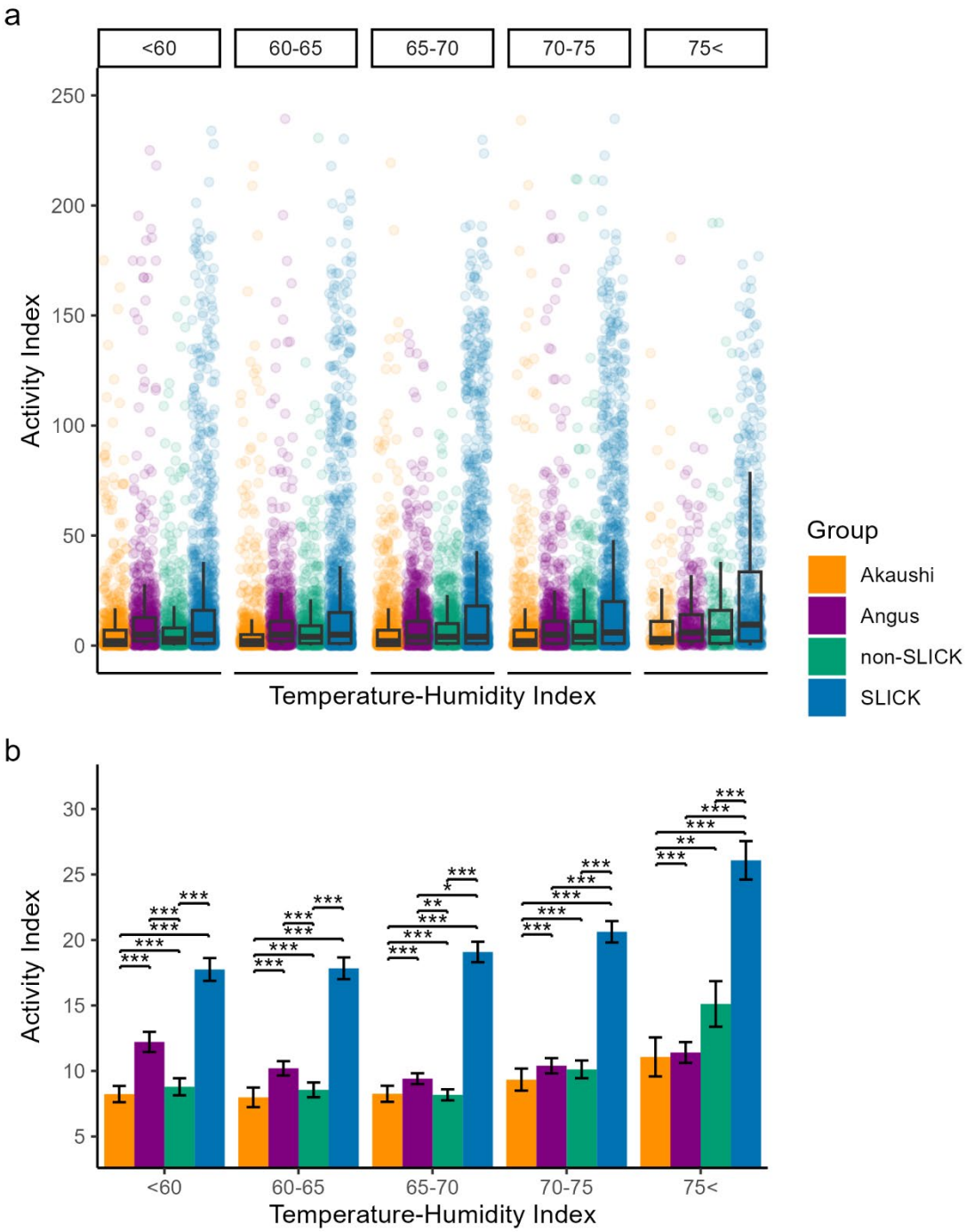

Figure 3.6: Distribution of activity index (A_I) data overlayed with boxplots for each cattle group among THI ranges (a). Bar plot of mean A_I for each cattle group at each THI range (b). Pairwise Wilcoxon rank sum test was used to compare groups and was adjusted for FDR using the Benjamini-Hochberg procedure. Significance is shown as * which indicates $p < 0.05$, ** which indicates $p < 0.01$, and *** which indicates $p < 0.001$.

HSP70 Gene Expression

HSP70 gene expression was determined using qPCR and the fold change ($2^{-\Delta\Delta Ct}$) of each sample was calculated allowing for the comparison between HSP70 expression between collections. Akaushi observed a median fold change of 0.401 ± 0.862, while Angus had a median fold change of 2.852 ± 5.833, non-SLICK had a median fold change of 1.775 ± 4.084, and SLICK had a median fold change of 1.223 ± 6.154 (see Figure 3.7). Kruskal-Wallis testing identified no statistically significant differences between groups ($p = 0.367$), which was confirmed through pairwise Wilcoxon signed-rank testing ($p > 0.05$). The average peak THI for the seven days leading up to the first and second collection periods were 70.45 ± 4.11 and 74.72 ± 1.15, respectively.

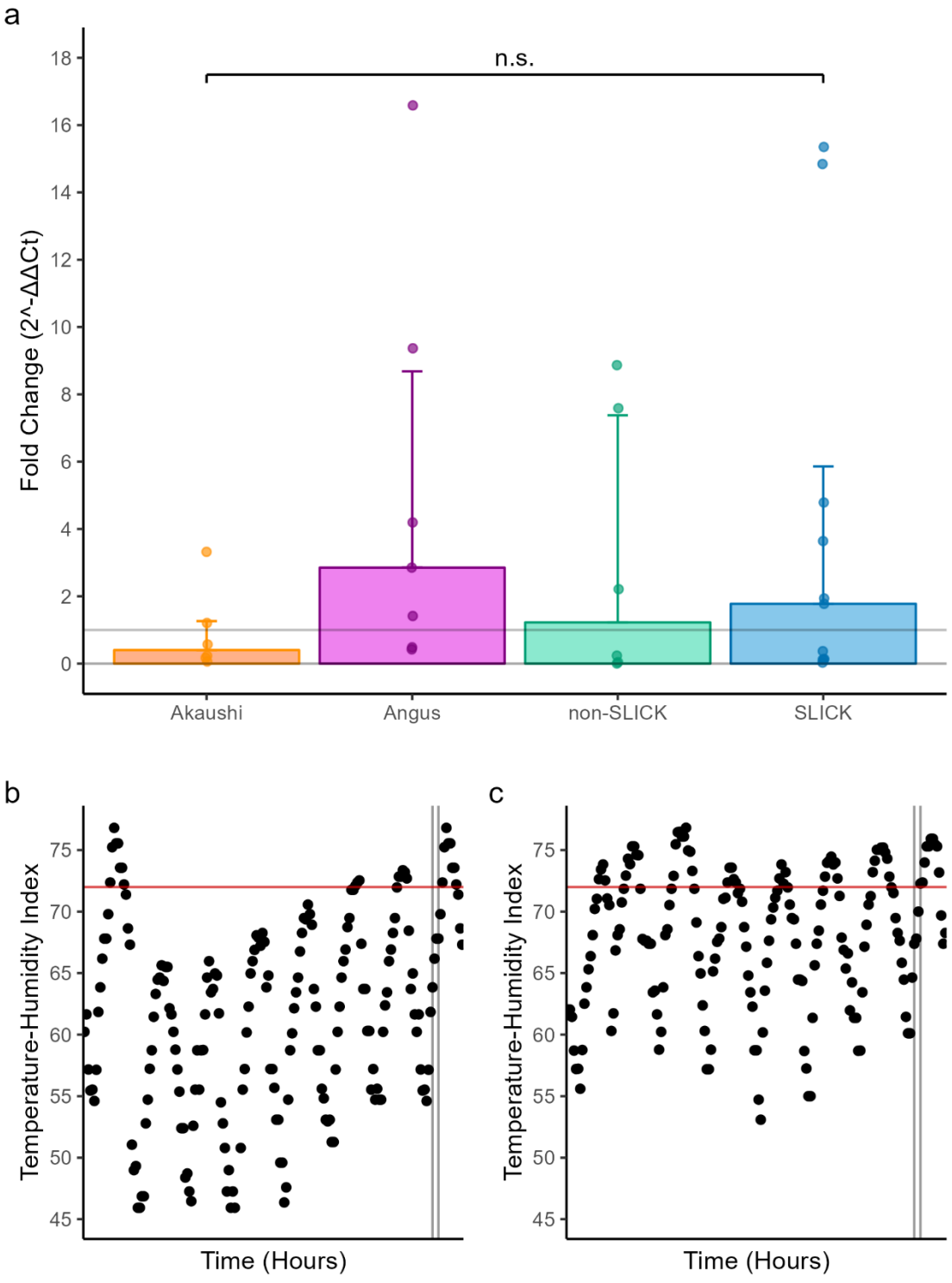

Figure 3.7: Bar plot of median HSP70 gene expression fold change ± SD of each cattle group depicted the change in gene expression between collection periods (a). Scatter plots showing the THI for every hour of the seven days leading up to the May 15, 2023 (b) and Aug 3, 2023 (c) collection periods.

HSP70 Plasma Analysis

HSP70 was quantified using an ELISA kit and HSP70 protein concentrations within the plasma samples collected on August 3, 2023. The absorbances of the standard curve were used to generate a linear regression model ($y = 0.196x + 0.099$, $R^2 = 0.997001$) which was then used to calculate HSP70 concentrations of the plasma samples. The median HSP70 concentration for Akaushi cattle was determined to be 1.38 ± 2.18 ng/ml, while Angus cattle showed a median concentration of 1.28 ± 1.12 ng/ml (see Figure 3.8). Additionally, non-SLICK cattle had a median concentration of 0.25 ± 1.55 ng/ml, and finally SLICK cattle exhibited a median concentration of 1.38 ± 1.48 ng/ml (see Figure 3.8). Kruskal-Wallis testing identified no statistically significant differences between groups ($p = 0.421$), which was confirmed through pairwise Wilcoxon signed-rank testing ($p > 0.05$).

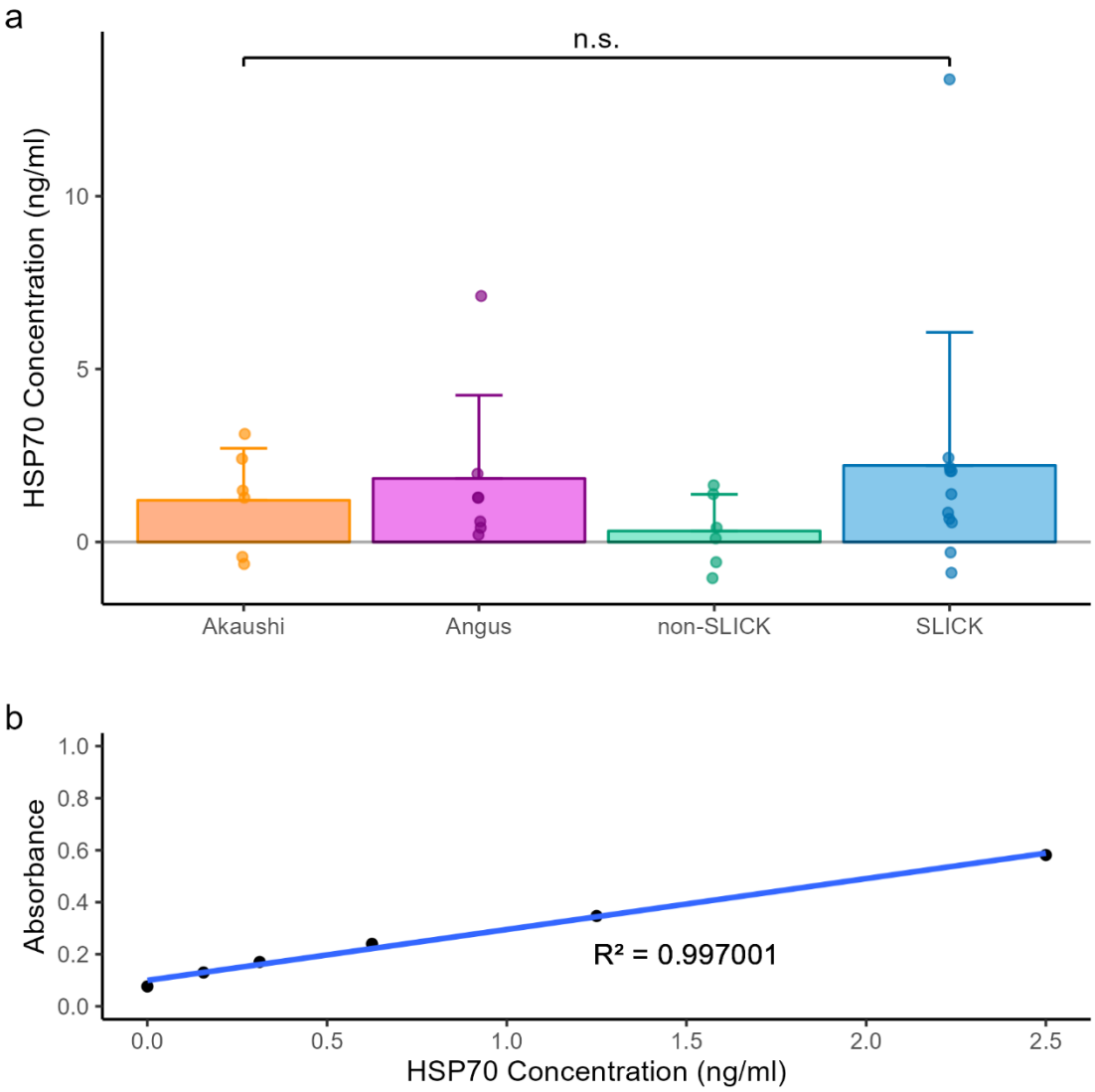

Figure 3.8: Bar plot of median HSP70 concentrations in blood plasma calculated determined using ELISA (a). HSP70 standard curve from ELISA for absorbance λ=450nm (b).

Discussion

To assess the heat tolerant capabilities of our Climate Master Hybrid, we measured respiration rate, internal rumen temperatures, activity levels, HSP70 gene expression, and HSP70 protein concentrations under different THI levels. It was found that our Climate Master Hybrid with the SLICK genotype experienced statistically significant differences in respiration rate and activity levels during elevated THI levels in comparison to the other cattle groups that lack the SLICK genotype. Internal rumen temperatures and the HSP70 assays did not yield significant results between SLICK cattle and other groups.

Increased respiration rates are a known biological response to heat stress in cattle (Lees et al., 2019). Respiration rates in SLICK cattle were statistically lower than other groups during THI levels above 72 (Figure 3.2), indicating lower heat stress response in animals with the SLICK genotype. Notably, Angus cattle had the highest respiration rates indicative of elevated heat stress, which could be attributed to their black coat colour. Coat colour has been known to influence heat load in cattle due to increased solar absorption (Lees et al., 2019). Furthermore, the Akaushi and our Climate Master Hybrid both have lighter coat colours (light brown and red) compared to the black coat colour of Angus cattle, and the respiration rates between Akaushi and non-SLICK cattle were statistically similar to each other. However, both Akaushi and non-SLICK cattle had statistically higher respiration rates than animals with the SLICK genotype. The Climate Master Hybrids with and without the SLICK genotype had observably longer hair coats compared to the Akaushi and Angus cattle, which might be attributed to the Galloway genetic influence (see Figure A.3). Longer coats generally increase heat retention (Lees et al., 2019; Peters & Slen, 1964), however, the Climate Master Hybrids had statistically lower respiration rates than Angus. Climate Master Hybrids with the SLICK genotype had respiration rates that were statistically lower than cattle without the SLICK genotype. From these results, we can conclude that the lower respiration rate observed in the cattle with the SLICK genotype is attributed to both coat colour and the traits inherent to animals with the SLICK genotype. Additionally, we have shown that incorporating Galloway cattle in crosses with animals possessing the SLICK genotype did not inhibit heat resistant qualities inherent to the SLICK genotype. The combination of Galloway

genetics and the SLICK genotype may even provide our Climate Master Hybrid with a novel coat characteristic that can withstand both extremely hot and cold climates. Tolerance to both climatic extremes would highly beneficial in the Canadian beef industry as it would allow cattle to thrive in both summer and winter conditions.

Cattle suffering from extended heat stress generally remain sedentary to reduce the internal heat production caused by metabolomic processes that occur during increased activity (Aharoni et al., 2013; Heinicke et al., 2019). Across all THI levels, SLICK cattle had high activity index (A_I) levels overall in comparison to other cattle (Figure 3.4), which was confirmed through statistical analysis (Table 3.4). This was further confirmed when binning the data into smaller chunks, indicating that SLICK cattle were much more active compared to other cattle groups when the THI was above 70 (Figure 3.6b). Interestingly, when the THI was below this point, SLICK cattle were statistically more active then Akaushi and non-SLICK cattle, but not Angus cattle. This indicates that below a THI of 70, Angus and SLICK cattle had comparable activity levels.

Overall differences were observed in internal rumen temperatures (T_R) between Akuashi cattle and all our other cattle breeds (Table 3.4). Furthermore, an overall difference in T_R between SLICK and non-SLICK was also observed (Table 3.4). A statistically significant weak negative correlation was observed between T_R and THI for each cattle group (Table 3.5). This shows that T_R decreases as THI increases, which contradicts previous research that report an increase in rumen temperatures with increased ambient temperatures (Gengler et al., 1970). However, it should be noted that this was reported from data collected in a controlled environment (Gengler et al., 1970) and that the bolus measurements were recorded over hour-long periods, which could mask immediate changes to T_R. It could be true that T_R and THI may have a positive correlation, however, other factors correlated to THI could be decreasing T_R to offset the changes initially incurred by increases in THI. Water intake is known to increase during increased THI (Arias & Mader, 2011; Gengler et al., 1970) which may reduce T_R to offset the effects of increased THI, leading to the negative correlation observed. The strongest correlation between T_R and THI (τ = -0.238) was observed in SLICK cattle. This is presumably due to the statistically significant higher spike in T_R between SLICK

and our other cattle groups when the THI ranged from 60-65 (Figure 3.5b). Similar T_R medians between SLICK, Angus, and non-SLICK cattle were observed when the THI was at or above 65 (Figure 3.5b). Interestingly, Akaushi cattle had a significantly lower T_R at all THI ranges below 75 against the other cattle groups as displayed in Figure 3.5. This is consistent with the LOESS trendlines as the Akaushi trendline was visibly distinct compared to other cattle group trendlines (Figure 3.3). Akaushi median T_R, alongside other groups, remained within optimal rumen temperature ranges for fermentation (Beatty et al., 2008). However, rumen temperature and heat stress indicators have been associated with changes in the gut microbial compositions (Czech et al., 2022; Kim et al., 2020). Furthermore, marbling and carcass weight in Japanese Black cattle have been associated with differences in microbial composition (Sato et al., 2024). Therefore, the statistically lower rumen temperature may present associations consistent with the marbling characteristics inherent to Akaushi and other Wagyu breeds.

For both HSP70 gene expression change and plasma concentration analyses we found no significant differences between cattle groups (Kruskal-Wallis: $p = 0.367$ and $p = 0.336$, respectively). As no significant differences were found in both assays, we can infer that the multiple days exposed to elevated THI values (Figure 3.7c) likely initiated similar HSP70 responses across all cattle groups. We observed an overall increase in HSP70 expression during increased THI, indicating that all cattle groups were experiencing the onset of heat stress. However, SLICK cattle appear to tolerate the effects of heat stress more efficiently, as indicated by their activity levels and respiration rates. Initial responses to heat stress occurring at similar conditions for all groups may indicate that the heat absorption characteristics are also similar. This would suggest that the SLICK cattle may have initiated a physiological pathway which ultimately resulted in increased heat loss capabilities. This speculation is consistent with the increased sweating rates observed in other populations of SLICK cattle which can contribute to superior heat loss capacity (Dikmen et al., 2014; Littlejohn et al., 2014). Since HSP70 is expressed at comparable rates in all our cattle groups, it suggests that responses to heat stress are initiated at similar THI values.

The multiple measures collected throughout this project can be used to estimate the THI threshold for heat stress in our cattle. Respiration rates across our cattle groups had a noticeable overall increase between THI values of 70.62 ± 1.61 (July 16 at 19:00 – 21:00) and 74.41 ± 1.21 (July 15 at 13:00 – 15:00) as indicated in Table 3.3. From these THI values we can infer that the THI threshold for our cattle is between the bounds of both THI values (72.23 – 73.20). Furthermore, SLICK cattle had statistically significant different A_I levels in comparison to all other cattle groups within the THI ranges of 70 – 75 and above 75. Both in beef and dairy cattle, the THI range for mild to moderate heat stress is generally accepted to be between 72 – 79 (Kim et al., 2023; Mbuthia et al., 2022). This THI threshold of 72 matches the results which found differences in SLICK cattle at THI ranges of 72.23 – 73.20 and 70 – 75 for respiration rates and activity levels, respectively.

Limitations of this study are primarily resulting from limited environmental impacts where we only observed a maximum THI of 80.75 throughout the course of the experiment, which is considered slightly above the bounds of moderate heat stress (Kim et al., 2023; Mbuthia et al., 2022). Furthermore, having an increased sample size would substantiate the findings reported in this study. Another factor that could have contributed to other limitations is the location of the closest public weather station to the study site used for the bolus monitoring and HSP70 assay experiments. The Penticton Airport weather station is the closest to the Buck Lake Ranch site, with a distance of 37.38 km between locations. Variations in wind speed, atmospheric pressure, and differences in elevation (477m difference) could have affected the relevant accuracy of the ambient temperature and relative humidity recorded by the weather station in relation to our study site. It should be noted; however, that the Penticton Airport weather station and the Kestrel 5400AG Portable Weather Station used in the respiration rate data collection showed statistically similar ($p > 0.05$) THI values at precisely the same times, except during the July 16 evening collection ($p = 0.0359$). While there was a slightly higher THI in Penticton, it was evident that the weather patterns in both locations followed the same trends which serves to lessen the potential impacts of this factor. Another limitation for the boluses, was that Moonsyst, the manufacturer, was not forthcoming with information regarding how the accelerometer determines between low, high, and very high activity

thresholds. Without explicit information on how this is calculated, or the accelerometer data itself, unintentional biases could have been introduced into the analyses. While the Moonsyst bolus offers a low maintenance and long-term collection method for both rumen temperatures and activity, validation of its suitability to replace other methods, such as ear tag accelerometers, remains to be completed. For the HSP70 assays, a high interquartile range in both gene expression and plasma concentrations was observed which could have masked differences, if any. HSP70 gene expression and protein concentration are good indicators of heat stress and spikes in expression can be apparent within three days of exposure to heat stress inducing conditions (Kim et al., 2020). As such, increasing sampling to weekly collection periods may potentially yield differences between cattle with and without the SLICK genotype over a temporal period long enough to observe whether the spikes in HSP70 gene expression is different between our cattle groups, and how prolonged exposure to increased THI ultimately affects HSP70 expression. Additionally, increasing sample size as mentioned before would likely decrease these large interquartile ranges observed in the dataset. These changes to the experimental design may demonstrate whether responses to the onset of heat stress are truly equivalent between our cattle groups.

While SLICK cattle exhibit superior heat tolerant capabilities, it is important to fully characterize this capability with further investigations. The heat stress indicators observed in this study imply that cattle with the SLICK genotype are not experiencing heat stress effects to the same degree as our other cattle. Investigations regarding heat absorption and heat dissipation would be required to understand how the SLICK phenotype equips cattle with improved thermotolerant traits. Specifically, recording skin and rectal temperatures could estimate heat absorption and sweating rates and its association with sweat gland functionality, and dermal abundance could estimate heat dissipation (Dikmen et al., 2014; Littlejohn et al., 2014). These measures could help better characterize how SLICK cattle reduce the impacts of heat stress. Additionally, as internal rumen temperatures showed a weak negative correlation with THI, it would be interesting to record water intake to determine the associated relationship with rumen temperatures and THI. In the event that water intake, internal rumen temperature and THI

are associated, it could potentially support using internal rumen temperature data as an indicator of heat stress.

Finally, as the Climate Master Hybrid was conceived to be tolerant to the wide range of temperature extremes in Canada, a follow-up investigation on the cold tolerant capabilities hypothesized to be contributed by the Galloway breed need to be assessed. Assessment of cold tolerance could use metrics such as hair coat density and length, infrared surface temperatures, rectal temperatures, feed efficiency, water consumption, respiration rate, heartrates, and biochemical markers in the blood associated with metabolism and cellular stress, all of which have been associated with cold stress in beef cattle (Wang et al., 2023). A comprehensive assessment of the heat and cold tolerant capabilities of this new composite would further support its applicability to the Canadian beef industry in the future.

Our results suggest that our Climate Master Hybrids with the SLICK genotype were affected by heat stress, however, these animals initiated responses in order to mitigate these effects in ways divergent to our other cattle groups. SLICK Climate Master Hybrids appeared to possess superior capabilities in the mitigation of these effects as demonstrated by decreased respiration rates and increased activity levels observed during this study. This study was among the first in which activity levels in cattle was quantified using Moonsyst boluses, which presents a novel long-term method of collecting activity data, alongside rumen temperature data. However, comparisons to other methods of quantifying activity data that have been previously validated is required to determine the suitability of the Moonsyst bolus in quantifying activity. Additionally, we have demonstrated that the longer hair coat inherent to the Galloway breed is not incompatible with the heat tolerant capabilities observed in animals with the SLICK genotype. Consequently, the SLICK phenotype presents itself as a potential solution for cattle industries based in temperate climates facing escalating climate change concerns by utilizing the trait in more cold tolerant breeds like Galloway.

Chapter Contributions

Initial experimental design and NSERC funding acquisition was completed by Dr. John Church and Dr. Paul J. Adams. Respiration rate data was collected and handled by

Matthew Francis and Mathis Gegout. Moonsyst boluses were administered and HSP70 sampling (hair and blood) were collected by Dr. John Church, Dr. Joanna Urban, and Robert J. Wester. Processing of blood samples was completed by Robert J. Wester and Dr. Joanna Urban. RNA extractions from hair samples were completed by Robert J. Wester. ELISA to determine HSP70 plasma concentrations was completed by Robert J. Wester. Bolus and HSP70 assay data handling was managed by Robert J. Wester. All statistical analyses for all measures were completed by Robert J. Wester.

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CHAPTER 4: Exploration of Genetic Markers Associated with Akaushi Marbling Using Targeted Genotyping and GWAS

Abstract

Marbling, the deposition of intramuscular fat in cattle, can considerably impact beef quality and consumer preference. Akaushi cattle, like other Wagyu breeds are renowned for their exceptional marbling characteristics which can be bred into Western cattle breeds. While genetic markers have previously been associated with marbling characteristics of other Wagyu breeds, genetic markers associated with the marbling of Akaushi cattle remains elusive. A comprehensive investigation to identify genetic markers of Akaushi marbling was conducted to develop molecular tools for tracking marbling in crossbreeding programs. Genotyping assays targeting seven previously identified marbling-associated single nucleotide polymorphisms (SNPs) were completed to determine differences between Akaushi and other cattle in our study. Additionally, a genome-wide association study (GWAS) was conducted using a BovineSNP50 v3.0 Infinium BeadChip to identify potential genomic regions with SNPs that are unique to Akaushi cattle. While no distinct genotype was found specific to Akaushi using seven marbling-associated SNPs, the GWAS revealed 12 SNPs unique to the Akaushi genome that could be associated with the superior marbling in Akaushi. Surrounding these unique SNPs were a total of 18 genes of varying functions. Notably, the SNP rs29013454 ($p = 3.37 \times 10^{-5}$) is located upstream of the gene *PDHA2*, which plays a role in the conversion of pyruvate to acetyl-CoA and may contribute to marbling in Akaushi through regulatory elements. Many of the other surrounding genes of the 12 SNPs are involved in neural functions and cellular development, which may pose neurological and developmental implications between Akaushi and other cattle breeds used in the GWAS.

Introduction

Marbling in cattle has become a widely popular trait in the beef industry and is a major factor that affects consumer perception of beef quality (Liu et al., 2022). It is characterized by an increase in visible intramuscular fat deposits between bundles of muscle (Nguyen et al., 2021). This increase in intramuscular fat is associated with superior flavour, colour, and tenderness that lends it to being a major factor increasing carcass value (Liu et al., 2021; Savell et al., 2016; Beak et al., 2021; Harper and Pethick, 2004). Marbling itself is a complex trait that could be influenced by multiple genes associated with lipid metabolism, as well as diet and environmental factors that collectively contribute to differences in the abundance and distribution of the intramuscular fat deposits (Wang et al., 2019; Gotoh et al., 2018; Krusinski et al., 2022).

Akaushi cattle, a *Bos taurus* breed of Wagyu, was initially developed to be reared in the humid subtropical environment of the Kumamoto and Kochi prefectures of Japan, while maintaining its superior marbling (Kimura et al., 2021). Like other breeds of Wagyu, Akaushi marbling is characterized by an elevated intramuscular fat content that is enriched in monounsaturated fatty acids (Gotoh et al., 2014). Some genetic variations have been previously associated with Wagyu marbling. However, most markers have been identified using Japanese Black cattle or through a combination of different Wagyu breeds (Abe et al., 2009; Taniguchi et al., 2004; Hudson et al., 2021). Suggested candidate genes for marbling include *FASN*, *SCD*, and *RAB4A*, which play unique roles in fatty acid synthesis, fatty acid elongation, and glycolysis, respectively (Abe et al., 2009; Taniguchi et al., 2004; Hudson et al., 2021; Tan and Jiang, 2024). We attempted to determine genetic markers unique to Akaushi cattle compared to our non-Akaushi study breeds through targeted genotyping and a genome-wide association study (GWAS). It was expected that SNPs unique to Akaushi might be linked to their superior marbling trait. If found, these SNPs could be used to develop an inexpensive and rapid genotyping assay to track the marbling associated genotype in future breeding strategies between our Climate Master Hybrid and Akaushi cattle (Figure 1.1).

Materials and Methods

All procedures outlined in this section were conducted in accordance with the Canadian Council of Animal Care guidelines and was approved by the Animal Care Committee of Thompson Rivers University (Kamloops, B.C., Canada) (File number: 102823).

Site Information

The animals used in the study were located in Beaverdell, British Columbia at Buck Lake Ranch (49.4342°N, 119.0883°W) roughly 60 kilometers from Kelowna. The primary focus of the 260 hectare ranch is the raising of beef cattle, with all cattle having the opportunity to graze freely on the property during the study.

Animal Selection

Animals selected for use in this study were included based primarily on simple availability. Each animal was assigned to one of four groups denoting breed: Akaushi, Galloway, Senepol Angus Composite, and Cross. Senepol Angus Composite cattle (3/4 Senepol and 1/4 Red Angus), referred to as ‘Senepol’, were crossed with pure Galloway to form the Climate Master Hybrid (3/8 Senepol, 1/8 Red Angus, and 1/2 Galloway), referred to as ‘Cross’. The cattle used for both genotyping and GWAS is outlined in Table 4.1. Genotyping was conducted immediately alongside genotyping covered in Chapter 2 using the same sample group. The GWAS includes the Climate Master Hybrid and more Akaushi cattle.

Table 4.1 Animals used for genotyping and the GWAS.

Animal Information			Genotyping	GWAS
ID	Sex	Breed		
Wrangler	M	Senepol	Y	Y[a]
J11	M	Senepol	Y	Y
J12	M	Senepol	Y	Y
R4	F	Senepol	Y	Y
R1	F	Senepol	Y	Y
Ferrari	M	Galloway	Y	Y
Earthquake	M	Galloway	Y	N
Sabre	M	Galloway	Y	N
11H	F	Galloway	Y	Y
64X	F	Galloway	Y	N
131W	F	Galloway	Y	Y
64H	F	Galloway	Y	N
Raymond	M	Akaushi	Y	N
2009H	F	Akaushi	Y	Y
2001H	F	Akaushi	Y	Y
2018H	F	Akaushi	Y	Y[a]
56K	F	Cross	N	Y
77K	M	Cross	N	Y
112K	M	Cross	N	Y[a]
135K	M	Cross	N	Y
152K	M	Cross	N	Y
169K	M	Cross	N	Y
204K	M	Akaushi	N	Y
207K	F	Cross	N	Y
209K	M	Cross	N	Y
4839	M	Akaushi	N	Y

[a]Sample was run in duplicate on a BovineSNP50 v3.0 Infinium BeadChip, using two sections of the microarray instead of one.

DNA Extraction

Individuals within the study were tagged using a specialized ear tag system developed and gifted by the Southern Alberta Institute of Technology (SAIT). The system tags the animal with an identification number and deposits a sample of ear tissue

into a specialized receptacle. Tags were applied between the two ridges of auricular cartilage, approximately one-third the distance of the ear from the head. Samples were then stored and transported in ambient conditions until DNA was extracted.

DNA extractions were completed using a modified protocol of the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Markham, Ontario, Canada). After addition of Buffer ATL and Proteinase K, the ear tags were incubated overnight in a MaxQ™ 420 HP Incubated Tabletop Oribital Shaker (Thermo Fisher Scientific, Burnaby, B.C., Canada) at 56°C set to 100rpm. All other steps of the DNA extraction protocol were followed as outlined in the Qiagen handbook (Qiagen, 2023). Following extraction, the purity and quantity of each sample was determined using a NanoDrop One instrument (Thermo Fisher Scientific, Burnaby, B.C., Canada).

Primer Design

SNPs and microsatellites identified previously in the literature to be associated with marbling characteristics in Wagyu breeds were acquired (Abe et al., 2009; Taniguchi et al., 2004, Hudson et al., 2021; Ishii et al., 2013; Uemoto et al., 2011). The genomic coordinates of each SNP were converted to the ARS-UCD1.3 *Bos taurus* bovine genome assembly using the BLAST-like alignment tool (Kent, 2002) and then used to retrieve the consensus sequences from Ensembl (Cunningham et al., 2022). The resulting files were then viewed using A Plasmid Editor (ApE) software (Davis & Jorgensen, 2022). One to two primer sets consisting of both a forward and reverse primer were designed using the primer designing tool within ApE. Each primer was designed to have a predicted melting temperature between (60°C and 64°C). Primer set amplicons were designed to be between 100 and 1,000 base pairs in length, with the SNP or microsatellite locus (or loci) in the middle of the amplicon. Each primer set was submitted to the National Centre for Biotechnology Information's (NCBI) Primer-BLAST (Ye et el., 2012) to determine whether there would be off-target amplification. Primer sets were ordered from Integrated DNA Technologies (Coralville, Iowa, United States).

Genotyping

Following optimization, one single assay for each SNP location was used to genotype the cattle identified in Table 4.1. DNA samples were used in PCR with the following recipe: 0.1 µL Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Burnaby, B.C., Canada), 2 µL 5X buffer, 1 µL 0.4 mM dNTPs (1 µL of each primer at 5 pmol, 2 µL Betaine, 2 µL of 1.5 ng/µL to 20 ng/µL of template DNA, and 0.9 µL dH_2O. All PCRs were ran in a SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific, Burnaby, B.C., Canada) with the following reaction conditions: 1 cycle of 98°C for 30 seconds, 35 cycles of 98°C for 10 seconds, X°C for 30 seconds, 72°C for 30 seconds, then 1 cycle of 72°C for 5 minutes, where X°C represents the Final T_a. Gel electrophoresis was used to confirm the success of each PCR.

SNP identity of each successful amplification was determined through Sanger Sequencing. PCR products were subjected to an ExoSAP-IT™ *Express* (Thermo Fisher Scientific, Burnaby, B.C., Canada) cleanup step, following the user manual (Thermo Fisher Scientific, 2017). This purifies the PCR product from the remaining PCR components. A total of 12ng of the purified PCR product were used in a BigDye™ sequencing reaction using and following the protocol outlined in the BigDye™ Terminator V3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Burnaby, B.C., Canada) (Thermo Fisher Scientific, 2010). The sequencing reaction was then purified using the BigDye Xterminator™ Purification Kit (Thermo Fisher Scientific, Burnaby, B.C., Canada). The purified sequencing reaction was then placed in a 0.2 mL MicroAmp optical strip and covered by a SeqStudio™ Septa (Thermo Fisher Scientific, Burnaby, B.C., Canada). A SeqStudio™ instrument (Thermo Fisher Scientific, Burnaby, B.C., Canada) was used to sequence the samples and output the resulting electropherograms. The electropherograms were then aligned to the *Bos taurus* consensus sequence to determine the identity of each SNP.

SNP Array

A total of 24 cattle samples as outlined in Table 4.1, were used for the BovineSNP50 v3.0 Infinium BeadChip (Illumina, San Diego, California, United States)

assay, which determines the SNP identity of 53,218 SNPs. The procedure was carried out following the manufacturer's protocol (Illumina, 2019). Briefly, DNA samples were amplified, fragmented, and hybridized to the BeadChip, followed by an extension and staining step, prior to imaging. Stained BeadChips were then visualized using an iScan System (Illumina, San Diego, California, United States). The iScan System uses two high powered fluorescent lasers which measures the fluorescent emissions of the extended and stained beads. Overlaying this information with GenomeStudio 2.0 Software (Illumina, San Diego, California, United States) with a unique map file containing all the SNP locations on the BeadChip allows the software to determine the identity of each SNP within the array for each of the samples.

Genome-Wide Association Study (GWAS) Pipeline

GenomeStudio 2.0 software was used to export a SNP array genotyping results file (.ped) and a SNP marker information file (.map) in the PLINK format (Purcell et al., 2007). Both files for the pipeline were imported into R 4.2.3 Statistical Software, which was used to compute the entire GWAS pipeline (R Core Team, 2023; Wickham et al., 2023). The genotyping results were imported using the BGLR package, as it is in the specialized PLINK format (Pérez and de los Campos, 2014). Quality control of the genotyping results was carried out by mean imputation (Donders et al., 2006) and then filtered by minor allele frequency (MAF). All MAFs below 5% were dropped. A hierarchical clustering dendrogram and Principal Component Analysis (PCA) was used to visualize population structure using the SNPRelate package (Zheng et al., 2012).

Alongside the SNP array outputs, a phenotyping file was generated from mock data of marbling characteristics consistent with the breeds used in the SNP array using the Japanese beef marbling standard (BMS) grading system as a framework (Gotoh et al., 2014; Motoyama et al., 2016). Mock data represents possible meat quality characteristics based on breed, since the actual beef quality was not measured as part of the scope of the current project. Akaushi cattle are known to exhibit marbling characteristics that are superior to prime quality beef indicating that the breed will likely exhibit high (BMS 7-9) to very high (BMS 10-12) marbling (Weinheimer, 2016). Galloway cattle exhibit similar marbling characteristics as Angus cattle which typically exhibit slight (BMS 4-5)

marbling (Albrecht et al., 2006a, 2006b). Tropical breeds of cattle, such as Senepol, often have lower eating qualities than typical beef, suggesting inferior marbling characteristics (Meat & Livestock Australia, 2024). However, since the Senepol Angus Composites have a large degree of Red Angus genetic influence, this group of animals likely have meat qualities slightly lower (BMS 3-5) than what is typically characterized in pure Angus cattle (Albrecht et al., 2006a). Finally, the Cross cattle should have a BMS score between what is likely exhibited by both Galloway and Senepol Angus Composite breeds as it has genetic influence from both breeds. The mock data was constructed, assigning BMS scores to each DNA sample, producing mean BMS scores consistent of the marbling assumptions for each breed. The values used in this mock dataset are outlined in the results section.

A GWAS was performed using the rrBLUP package, which uses an additive relationship matrix with variance components calculated once using Restrictive Estimation Maximum Likelihood (REML) methods (Kang et al., 2010; Yu et al., 2006; Zhang et al., 2010). Correction for multiple testing was completed using the Li and Ji method to control the false discovery rate at $\alpha = 0.05$ (Cinar & Viechtbauer, 2022; Li & Ji, 2005; Benjamini & Hochberg, 1995). A Manhattan plot was generated using the Tidyverse package, displaying the SNPs that pass the significance threshold (Wickham et al., 2019). Significant SNPs were extracted, and their genomic coordinates were converted from the UMD3.1 genome assembly to the ARS-UCD1.3 genome assembly using the BLAST-like alignment tool (Kent, 2002). Closest genes within a maximum of 300kb of the identified SNPs were determined using Ensembl (Cunningham et al., 2022). Further information regarding the gene function to determine whether it has functionality in lipid metabolism pathways was collected using NCBI (Sayers et al., 2022).

Results

SNP Identification and Primer Design

Seven SNPs were selected as targets for marbling genotyping based on their previously reported associations with marbling in beef cattle (Abe et al., 2009; Taniguchi et al., 2004, Hudson et al., 2021; Ishii et al., 2013; Uemoto et al., 2011) (Table 4.2). Notably, two of the SNPs were within 14bp of each other, requiring only six genotyping assays. Two primer sets were designed and optimized using a polymerase chain reaction (PCR) for genotyping assay. The surrounding region of the SNP rs110644506 had many possible nucleotide variations in the reference genome which resulted in only one primer set being designed and optimized. All seven SNPs and their primer sets tested are outlined in Table 4.3.

Table 4.2: SNPs associated with fat marbling in Wagyu cattle identified through literature review.

Marker Name	Chr[1]	Position[1]	Allele	Source	Closest Gene
16024A>G and 16039T>C	19	50791465 and 50791480	A/G[a] and T/C[a]	Abe et al., 2009	*FASN*
rs41255693	26	21272422	C/T[a]	Taniguchi et al., 2004	*SCD*
rs110522529[b]	28	1233653	T/C	Hudson et al., 2021	*RAB4A*
rs109162947[b]	28	1236655	G/A	Hudson et al., 2021	*RAB4A*
rs109863510[b]	19	50879654	C/T	Ishii et al., 2013	*LRRC45*
rs110644506[b]	19	51232973	C/T	Uemoto et al., 2011	*FAAP100*

[1]Chromosome (Chr) and position refer to the ARS-UCD1.3 genome assembly.
[a]Allele associated with marbling.
[b]SNP identified in GWAS study and marbling associated allele was not specificied.

Table 4.3: Primers that were designed and optimized for genotyping SNPs identified to be associated with marbling.

Marker Name	Gene	Set	Primer	Sequence (5´-3´)	Length	T_m[1]	GC%	Amplicon	Final T_a[2]
16024A>G and 16039T>C	*FASN*	1	F1	CCTTGTGCAAGACCTTCTGCC	21	61.75	57.14	877bp	62°C
			R1	CACGGAGGAGAAGACCACGAAG	22	62.59	59.09		
		2	F2	CCTACTGGACTCTCTCTTTGAAG	23	57.68	47.83	981bp	N/A
			R2	AGAAGGCGGGAGAGGAGAAG	20	60.69	60.00		
rs41255693	*SCD*	1	F1	ATGTATGGATACCGCCCTTATGAC	24	60.08	45.83	144bp	N/A
			R1	TTCTGGCACGTAACCTAATACCCTAAGC	28	64.41	46.43		
		2	F2	GGTGTCCTGTTGTTGTGCTT	20	58.90	50.00	502bp	62°C
			R2	ATGACTGGGAAGAGAACAGC	20	57.22	50.00		
rs110522529	*RAB4A*	1	F1	CATTCTGCACTTACTGTGCC	20	57.09	50.00	270bp	N/A
			R1	GACCCAACAACTCCATTTCTG	21	57.08	47.62		
		2	F2	CTGTGCCTACCCATCATTACAA	22	58.12	45.45	334bp	60°C
			R2	TGGGATGATAAACTGCCTCAAG	22	58.11	45.45		
rs109162947	*RAB4A*	1	F1	CGAAAGAATCCACAAACTCCCA	22	58.85	45.45	324bp	62°C
			R1	AGTGCTTTGAAGCTGGGTAGTG	22	60.81	50.00		
		2	F2	GATTGTGCCCTGTCAGCACT	20	60.61	55.00	748bp	N/A
			R2	TTCTATGAGTTTTATGATTTTG	22	48.26	22.73		
rs109863510	*LRRC45*	1	F1	GCTTTGCAGAGAAACCTGGTGA	22	61.33	50.00	422bp	62°C
			R1	TTTCTCCCACATCAGTCTGTGCTC	24	62.61	50.00		
		2	F2	CACACACTTGATGGCCTGCAT	21	61.49	52.38	824bp	N/A
			R2	GGGAGTGACATGCCTGAGAAC	21	60.68	57.14		
rs110644506	*FAAP100*	1	F1	GGTCTCTTCTCAGTTCTTTGCCC	23	61.12	52.17	800bp	62°C
			R1	GTCTACAGGAAGGGAAGGGTGG	22	61.75	59.09		

[1]Predicted melting temperature of primer in °C.

[2]Presence of final T_a indicates primer set was utilized for genotyping.

Genotyping

All progenitor cattle from the Climate Master Cattle project were genotyped for the seven SNP markers. The FASN genotyping assay contains two SNP markers with 14bp between them, g.16024A>G and g.16039T>C. No single SNP or combination of SNPs showed obvious differences between Akaushi and non-Akaushi cattle. Genotyping results are outlined in Table 4.4.

Table 4.4: Marbling SNP genotyping results.

Cattle		**Marbling SNPs**					
Breed	**ID**	**FASN[1]**	**SCD[2]**	**RAB4A[3]**	**RAB4A[4]**	**LRRC45[5]**	**FAAP100[6]**
		A/G,T/C	**C/T**	**T/C**	**G/A**	**A/G**	**A/G**
Senepol	Wrangler	GG,CC	CC	TC	GG	AA	-
	J11	GG,CC	CC	TC	AG	AG	AA
	J12	AG,TC	CC	TT	AG	GG	GG
	R4	GG,CC	TC	TC	AG	GG	GG
	R1	GG,CC	CC	TC	AG	AG	AG
Galloway	Ferrari	-	CC	TC	GG	GG	-
	Earthquake	GG,CC	CT	CC	AA	-	-
	Sabre	AG,CT	CT	CC	AA	GG	-
	11H	GG,CC	CC	TC	AG	GG	GG
	64X	GG,CC	TT	TC	AG	GG	GG
	131W	GG,CC	TC	TT	GG	-	AG
	64H	GG,CC	TT	TT	GG	GG	-
Akaushi	Raymond	-	CC	CC	AA	AG	-
	2009H	AG,CT	TT	TC	AG	AG	AG
	2001H	AG,CT	TT	TC	AG	GG	GG
	2018H	GG,CC	TC	TC	AG	GG	GG

[1]Represents g.16024A>G and g.16039T>C SNPs.
[2]rs14255693, [3]rs110522529, [4]rs109162947, [5]rs109863510, [6]110644506.

SNP Array

A total of 24 DNA samples, as outlined in Table 4.1, were genotyped using a BovineSNP50 v3.0 Infinium BeadChip and visualized using an Illumina iScan System. Three of these samples had a technical replicate, while the remaining samples were run only once. Quality control filtering of samples based on a 99.7% call rate threshold excluded one of the 24 samples. Additionally, 51959 SNP markers (97.63%) passed the GenTrain Score threshold (0.7), with a mean GenTrain Score of 0.847 ± 0.052. Out of the 51959 SNPs, 42207 SNPs (81.23%) had a minor allele frequency above 5%.

The 42207 SNPs that passed quality control were used to create a hierarchical clustering dendrogram and perform a principal component analysis (PCA) (Figure 4.1). There is obvious clustering of Akaushi cattle relative to the Senepol, Galloway, and Cross cattle which are more closely related to one another.

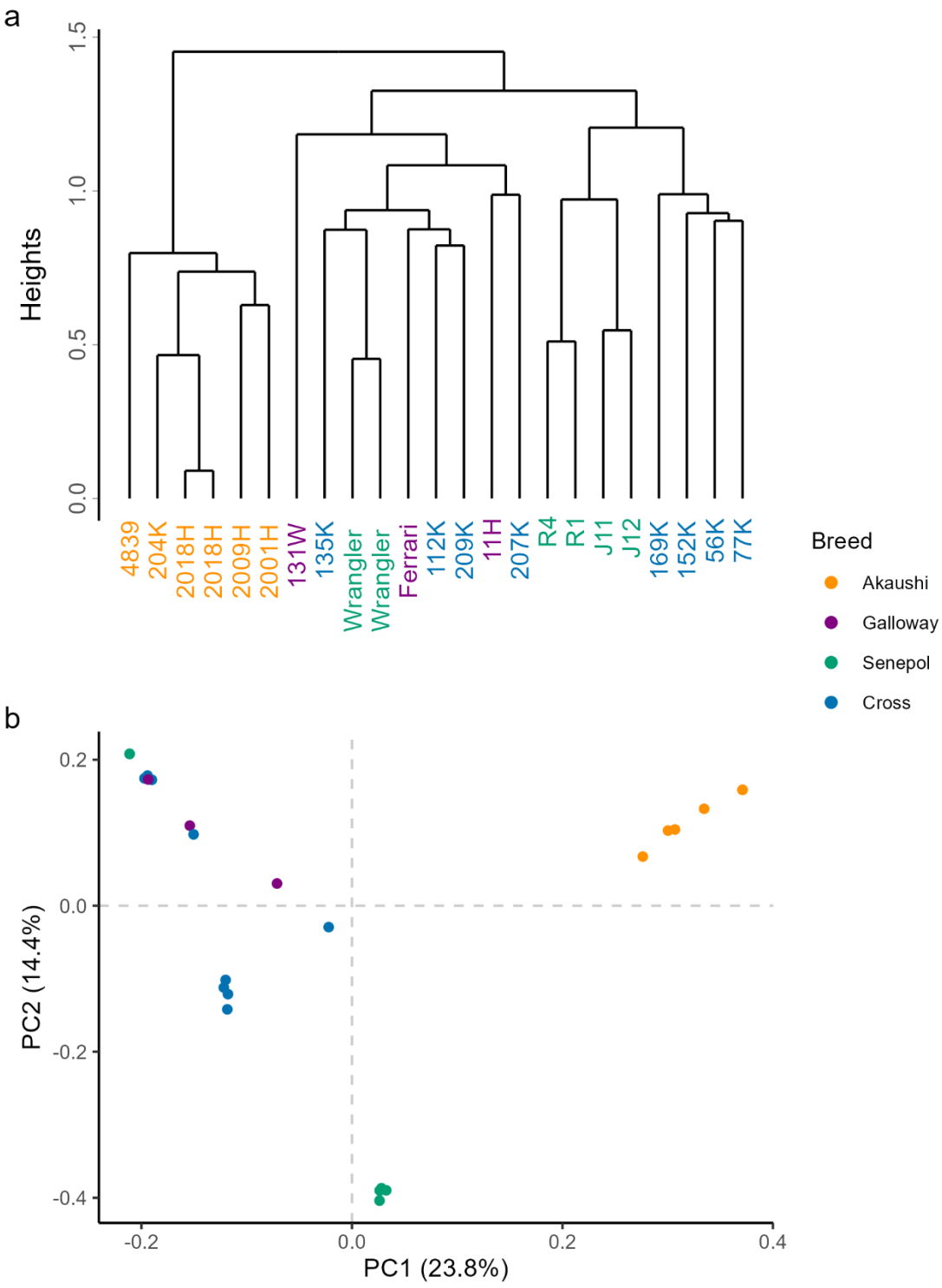

Figure 4.1: Population structure of cattle using genotyping data displayed in both hierarchical clustering dendrogram (a) and principal component analysis (PCA) (b).

Genome-Wide Association Study (GWAS)

As phenotypic data on marbling for our cattle was outside the scope of the current project, mock data was generated to test the pipeline as outlined in the methods. Values were based on a presumed average beef marbling standard (BMS) score of the cattle breeds using the Japanese BMS scale. In the mock dataset, Akaushi (Wagyu) cattle were made to have the highest BMS of 10.32 ± 0.45, whereas the others were much lower. Galloway were made to have a BMS of 5.37 ± 0.21, Senepol were made to have a BMS of 3.88 ± 0.32, and Senepol/Galloway crosses were made to have a BMS of 4.90 ± 0.44. For the individual BMS scores of each cattle used for the mock data, see Table A.3 in the Appendix.

These BMS values were then compared to the genotypes of the 42207 SNPs and were given a probability value of association. Correction for multiple testing identified a p-value threshold of 1.736111×10^{-4}. A total of 12 SNPs across the bovine genome passed this threshold as visualized in the Manhattan plot displayed in Figure 4.2. Details on the 12 SNPs are outlined in Table 4.5. The lowest p-value among the 12 SNPs was $p = 1.79 \times 10^{-7}$ for the SNP named Hapmap47186-BTA-114970.

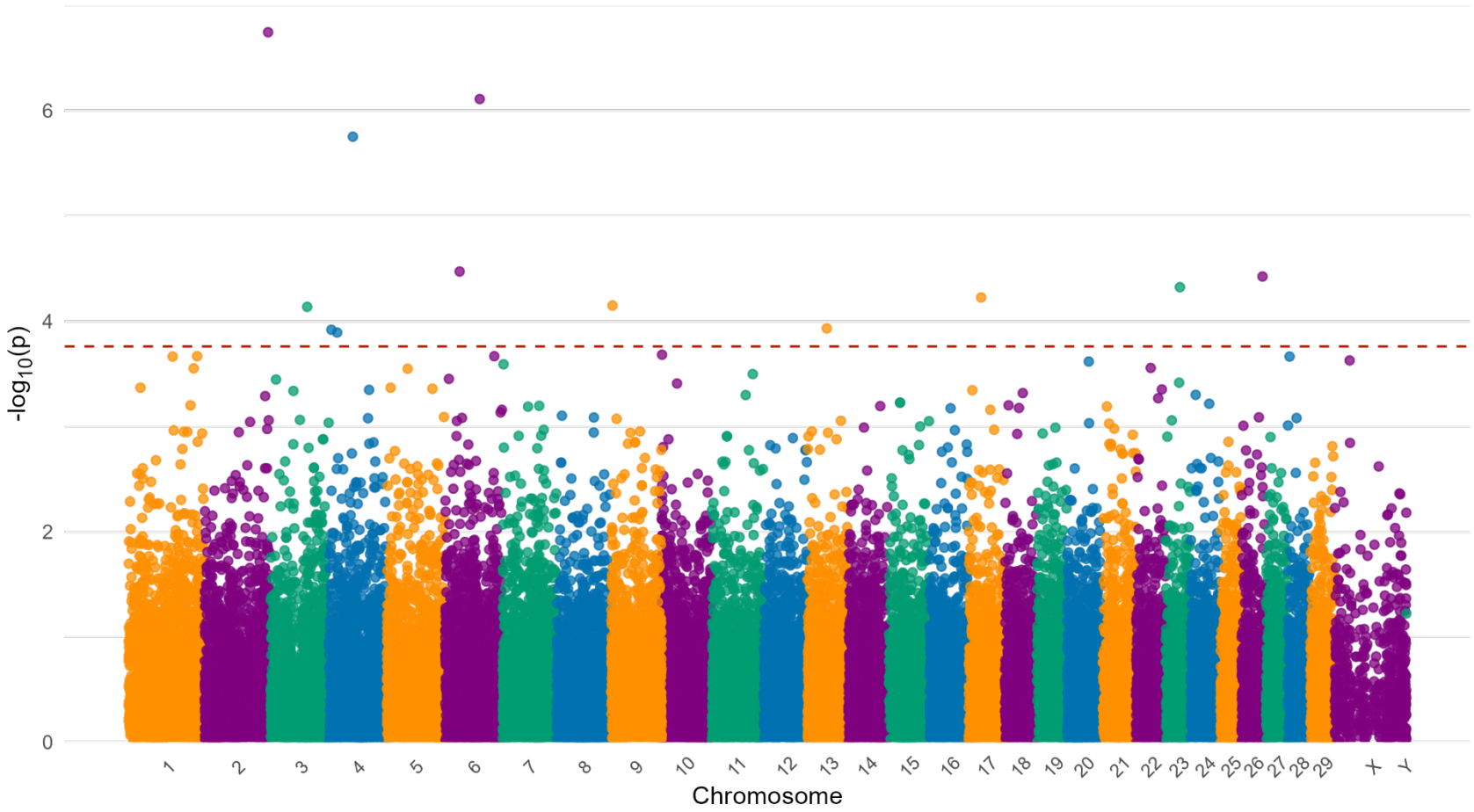

Figure 4.2: Manhattan plot of SNPs associated with mock BMS values for Akaushi, Galloway, Senepol, and Cross cattle. The dashed red line indicates the p-value threshold (1.736111×10^{-4}) and SNPs above that are considered statistically significant.

Table 4.5: Statistically significant SNP markers that might be associated with fat marbling traits.

SNP Name	SNP ID	Chr*	Position*	p-value
Hapmap47186-BTA-114970	rs41618854	2	131194707	1.79×10^{-7}
Hapmap53126-rs29022865	rs29022865	3	76153357	7.30×10^{-5}
ARS-BFGL-NGS-96185	rs109583632	4	5422821	1.20×10^{-4}
ARS-BFGL-NGS-52712	rs109837538	4	16372283	1.28×10^{-4}
BTB-00180208	rs43387851	4	49723245	1.76×10^{-6}
Hapmap58595-rs29013454	rs29013454	6	28873303	3.37×10^{-5}
Hapmap23983-BTC-070420	rs109898977	6	70431058	7.73×10^{-7}
BTB-00378544	rs43582937	9	2952558	7.09×10^{-5}
UA-IFASA-3757	rs29017988	13	40234544	1.17×10^{-4}
ARS-BFGL-NGS-100397	rs110629564	17	25776770	5.94×10^{-5}
Hapmap39414-BTA-56194	rs41667517	23	30599886	4.74×10^{-5}
ARS-BFGL-NGS-117756	rs109199542	26	43936742	3.76×10^{-5}

Chr: chromosome.
*ARS-UCD1.3 Bovine Genome Assembly genomic coordinates.

A total of 21 genes where the genomic coordinates lie within a maximum genomic distance of 300kb were identified surrounding the 12 SNPs as outlined in Table 4.6. The genes identified encompass a large variety of functions. Gene functions were obtained using human homologs, as they represent similar roles in bovine models and are much more characterized. No SNPs were located within exon regions of the genes identified, so no pathogenicity analyses of amino acid substitutions were completed.

Table 4.6: Genes of proximity to the SNPs identified in the GWAS.

SNP ID	Allele	Closest Genes (Strand)[1]	Relative Position for SNP	Gene Function[2]
rs41618854	A/G	*ALPL* (-) *RAP1GAP* (+)	Intron Variant Downstream (~30.2kb)	Bone Formation Cell Growth and Differentiation
rs29022865	T/C	ENSBTAG00000063253 (-)* ENSBTAG00000065262 (+)*	Intron Variant Downstream (~14.5kb)	- -
rs109583632	A/G	*DDC* (+)	Intron Variant	Neurotransmitter Metabolism
rs109837538	C/T	ENSBTAG00000025604 (+)** *ICA1* (-)	Upstream (~3.6kb) Upstream (~16.7kb)	- Associated with Diabetes Mellitus
rs43387851	T/G	*NME8* (+) *SFRP4* (-)	3' UTR Variant Downstream (~8.6kb)	Neural Development Osteoblast Proliferation
rs29013454	C/T	*UNC5C* (+) *PDHA2* (-)	Upstream (~63.1kb) Upstream (~231.9kb)	Neural Development Pyruvate Metabolism
rs109898977	T/G	*KDR* (-) *KIT* (+)	Downstream (~136.5kb) Downstream (~177.0kb)	VEGF Signal Transduction Stem Cell Maintenance
rs43582937	A/C	ENSBTAG00000055087 (-)**	Downstream (~97.3kb)	-
rs29017988	T/C	*RALGAPA2* (-) *KIZ* (+)	Upstream (~46.1kb) Upstream (~255.7kb)	Cancer Development Centromere Localization (Mitosis)
rs110629564	C/T	ENSBTAG00000064864 (+)*	Downstream (~38.8kb)	-
rs41667517	G/T	*ZNF165* (-) *OR1F12* (-)	Upstream (~0.67kb) Downstream (~5.4kb)	Transcription Factor Olfactory Receptor
rs109199542	C/T	*OAT* (-) *CHST15* (-)	Downstream (~108.3kb) Upstream (~188.3kb)	Neurotransmitter Metabolism Proteoglycan Formation in ECM

[1]Gene orientation: (+) is forward and (-) is reverse.

[2]Gene functions from studies involving human subjects.

*Long Non-Coding RNA, **Hypothetical Protein.

UTR: untranslated region, VEGF: vascular endothelial growth factor, ECM: extracellular matrix.

Discussion

The aim of our genotyping efforts was to identify specific genetic variations associated with marbling characteristics in Akaushi cattle. These genetic markers could potentially be used to predict relative fat marbling characteristics of cattle bred with Akaushi. Additionally, a genome-wide association study was conducted using mock marbling data to build a model that outlines genetic differences between Akaushi and our other cattle breeds used in this study. No obvious genetic variations were determined to be associated with Akaushi marbling characteristics.

Seven SNPs previously associated with marbling characteristics in Wagyu cattle breeds with high intramuscular fat content were genotyped as outlined in Table 4.4 (Abe et al., 2009; Taniguchi et al., 2004, Hudson et al., 2021; Ishii et al., 2013; Uemoto et al., 2011). No obvious unique genotype patterns were recognized among Akaushi cattle in comparison to both Senepol and Galloway breeds. As no markers were unique to Akaushi cattle at this phase, it is unlikely that these variations are associated with the higher intramuscular fat generally observed in Akaushi cattle. Marbling is undoubtedly a complex trait, and many genetic variations may play a role in increasing intramuscular fat (Hudson et al., 2021). Additionally, genetic variations associated with superior intramuscular fat are not consistent among different breeds of cattle (Barendse et al. 2006; Barendse et al. 2009; Barendse et al. 2010; Barendse 2011; Bolormaa et al. 2011; Hudson et al., 2021). This indicates that it is likely necessary to look beyond the seven previously identified SNPs and perform a more comprehensive SNP analysis of the bovine genome and compare that analysis to marbling traits.

To evaluate an array of genetic variations associated with marbling, the BovineSNP50 v3.0 Infinium BeadChip was used to identify genotypes of many genetic loci which were subsequently analyzed in a genome-wide association study (GWAS). The genotyping information allowed for clustering of sample population structure visualized in Figure 4.1, indicating clear distinctions between Akaushi and our other cattle breeds. Additionally, the clustering of Galloway, Senepol, and Cross cattle breeds is consistent with the assumption that they would have similar genetic profiles due to the Cross group being offspring of the two breeds.

GWAS was conducted to establish a model for SNPs associated with marbling in Akaushi using a mock dataset that estimates the likely BMS scores of each breed in the study. The GWAS yielded a total of 12 SNPs which are associated with the assigned BMS values (Figure 4.2 and Table 4.5). Eighteen genes with well characterized functions were within or near the genomic coordinates of the SNPs that were identified. These genes have been associated with many different functions (Table 4.6).

A possibly important marker is rs29013454 ($p = 3.37 \times 10^{-5}$), which is approximately 231.9kb upstream of the gene *PDHA2*. *PDHA2* encodes a subunit of the pyruvate dehydrogenase complex (PDC) which converts pyruvate to acetyl-CoA, an intermediate that plays a pivotal role in protein, carbohydrate, and lipid metabolism (Pinheiro et al., 2012; Shi and Tu, 2015). In lipid metabolism, acetyl-CoA is used in the citric acid cycle forming citrate, which is then transported out of the mitochondrial matrix, and subsequently used in *de novo* fatty acid synthesis (Tan & Jiang, 2024). Acetyl-CoA is also involved in the *de novo* biosynthesis of palmitic acid under the catalytic effects of *FASN* (Ordovás et al., 2008; Roy et al., 2006). Altered abundance of PDC components may modify acetyl-CoA production and subsequent fatty acid synthesis. However, due to rs29013454's distance from *PDHA2*, it is relatively improbable that this variation directly influences regulation of *PDHA2*. On the other hand, since regulatory elements hundreds of kilobases away from the genes they modulate have been previously reported, it is possible that regulatory elements of *PDHA2* exist in the region of rs29013454 (Roessler et al., 1997; Dileone et al., 1998; Kleinjan et al., 2001).

The most significantly associated SNP, rs41618854 ($p = 1.79 \times 10^{-7}$), resides within an intronic region of *ALPL*, a gene that is associated with bone formation (Numa-Kinjoh et al., 2015), and is highly expressed in bovine embryonic stem cell lines (Wang et al., 2005). This SNP is also approximately 30.2kb downstream of *RAP1GAP*, which modulates the activity of RAP1, a key protein in cell growth and differentiation pathways while also being associated with many types of cancer (Kim et al., 2012). The second most significantly associated SNP, rs109898977 ($p = 7.73 \times 10^{-7}$), is downstream of both *KDR* (~136.5kb) and *KIT* (~177.0kb); which are involved in endothelial cell growth and

stem cell proliferation, respectively (Vandenbark et al., 1992; Ziegler et al., 1999). However, the distance of the SNP to these two genes indicates a lower probability of the SNP being involved with the regulation of the gene. The SNP rs43387851 ($p = 1.76 \times 10^{-6}$) lies within the 3' UTR of *NME8* and is approximately 8.6kb downstream of *SFRP4*. *NME8* is associated with neural development, while *SFRP4* is involved in osteoblast proliferation (Liu et al., 2014; Nakanishi et al., 2008). Two notable SNPs include rs29022865 ($p = 7.30 \times 10^{-5}$) and rs109583632 ($p = 1.20 \times 10^{-4}$), which are each intronic variants of ENSBTAG00000063253 and *DDC*, respectively. ENSBTAG00000063253 is a long non-coding RNA transcript, whereas *DDC* is a protein that primarily catalyzes dopamine and serotonin biosynthesis (Bertoldi, 2014). The SNP rs41667517 ($p = 4.74 \times 10^{-5}$) is positioned 666bp upstream of the gene *ZNF165*, a member of the zinc-finger family of proteins. This family of transcription factors in eukaryotic organisms are known to play key roles in regulating expression of genes important for cell growth, proliferation, differentiation, and apoptosis (Dong et al., 2004).

Lastly, it is important to note the variants rs41618854, rs29022865, and rs109583632, which are located within intronic regions of genes (*ALPL* and *DDC*), or non-coding RNA transcripts that could modulate alternative splicing or regulatory functions of the RNA transcripts. Additionally, rs43387851 which resides in the 3' UTR of *NME8* could alter the mRNA stability or disrupt post-translational regulation.

One limitation of this work is the variability observed within the genetic variants previously associated between different cattle breeds with superior marbling characteristics (Hudson et al., 2021). Marbling is clearly a complex trait that can involve the functions of many genes, however, variations in genes influencing marbling traits between cattle breeds may imply convergent evolution through high selective pressures for marbling traits across different geographical regions. Therefore, the SNPs that have previously been associated with marbling may not be applicable to Akaushi cattle due to this inconsistency. Another limitation is a lack of true BMS values for our animals and use of a mock dataset. While meat quality assessments were initially planned, they were not conducted at this point as they fell beyond the scope of this stage of the project. True BMS scores might be similar to input values of the mock dataset, however, some

unintentional biases may be introduced due to the spread of mock BMS scores. Mock data could be indicative of general differences between our cattle breeds which is consistent with population structure outlined in Figure 4.1. This would indicate that the allelic differences between the SNPs are simply the holistic differences between breeds rather than highlighting genetic differences associated with Akaushi marbling characteristics. Many of the genes found in the GWAS function in neurological pathways, in addition to proliferation and maintenance of different cell types, including osteoblasts, stem cells, and endothelial cells. This may suggest distinct neurological and developmental characteristics between Akaushi cattle and other cattle breeds.

This GWAS pipeline shows promise for determining SNPs related to marbling of Akaushi cattle. However, as a mock dataset was used, it is important to perform this study with measured meat quality metrics in the future. Additionally, it would be valuable to include a higher density SNP array that covers more loci across the bovine genome to increase coverage. Adding more cattle breeds known for their superior intramuscular fat in addition to Akaushi could illuminate the steps of lipid metabolism unique within these breeds. Understanding which steps in the lipid biosynthesis and trafficking pathways are affected by variations associated with each breed could yield breeding optimization strategies that ultimately lead to increased marbling characteristics in composite cattle breeds. If convergent evolution is a factor in marbling, this would explain the inconsistencies in genetic variations across cattle breeds (Barendse et al. 2006; Barendse et al. 2009; Barendse et al. 2010; Barendse 2011; Bolormaa et al. 2011). Interestingly, the different Wagyu breeds: Japanese Black, Japanese Brown (Akaushi), Japanese Shorthorn, and Japanese Polled, are primarily produced in different regions of Japan (Motoyama et al., 2016). Since marbling is a complex trait, selection for marbling in these different regions may have arisen through different stages within lipid metabolism pathways. Krusinski et al., however, has recently reported that diet plays a more significant role in the nutritional profile of beef than breed in Red Angus x Akaushi crosses (2022). This may be because the marbling characteristics of Akaushi were not passed to the crosses used in Krusinski et al. as it is a complex trait (2022). This finding suggests that both diet and genetics will need to be controlled in the optimization of

marbling characteristics of the proposed Climate Master Composite breed. Clearly, more research is required on the genetic profile of Akaushi cattle in relation to marbling.

Other research avenues could explore the neural development and function of Akaushi cattle. Considering that four of the genes identified in the GWAS influence neural development or neurotransmitter metabolism, this could indicate a possible variation in neurological functions. These neurological functions could affect the temperament of Akaushi. Temperaments of Akaushi have not been adequately reported in previous literature, however, Japanese Black cattle have been shown to have a calm demeanor (Takeda et al., 2017). Japanese Black cattle are also highly susceptible to environmental stressors which has been known to affect their temperament, which is likely similar for Akaushi (Nabenishi & Yamazaki, 2017; Nakajima et al., 2021). In addition to temperament, variations in neurotransmitter metabolism may increase the susceptibility to neurodegeneration through unregulated production of toxic neurotransmitters, such as dopamine and serotonin production which are both influenced by *DDC* function (Bertoldi, 2014; Harris et al., 2014). This information could potentially provide a better understanding of the suitability of Akaushi for use in future cattle breeding work. Selection for marbling traits in cattle to improve meat quality, however, may enable adverse traits to arise within the novel composite breed that would not be desirable.

In conclusion, this study showed that Akaushi cattle were genetically distinct through population structure analysis, however, it did not yield any explicit genetic variations that clearly influence marbling characteristics in Akaushi cattle through targeted sequencing and GWAS. The closest association in variations to lipid metabolism is rs29013454, an upstream variant to *PDHA2*, in which regulation of the gene could affect acetyl-CoA biosynthesis. Other SNPs near genes involved in neurological, growth, and cell maintenance functions were associated with Akaushi cattle. While the effects of the SNPs on these genes could be null, it provides a direction for future research involving Akaushi cattle. These factors could contribute to a better understanding of genetic differences between Akaushi and other cattle breeds, especially with respect to

marbling, which may be used to improve carcass characteristics for the cattle industry in the future.

Chapter Contributions

NSERC funding acquisition was completed by Dr. John Church and Dr. Paul J. Adams. Samples were collected and sent to the Applied Genomics Centre by Dr. John Church and Wyatt Cook. DNA extraction and quality assessment was completed by Robert J. Wester. Amplification, fragmentation, hybridization, staining, extension and imaging steps of the BovineSNP50 v3.0 BeadChip workflow was completed by Robert J. Wester. Data handling and PLINK exportation from GenomeStudio 2.0 was completed by Robert J. Wester. SNP data quality control was completed by Robert J. Wester. Population structure analysis was completed by Robert J. Wester. GWAS bioinformatics pipeline and downstream analysis was completed by Robert J. Wester

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CHAPTER 5: General Discussion

The primary purpose of the Climate Master Cattle project is to develop a novel *Bos taurus* breed that could withstand the challenges of climate change, and also have improved meat quality. While this work did not ultimately accomplish the final goal, it includes the initial first steps of the project and justifies its feasibility and a need for continuation of the overall project.

In Chapter 2, the SNP markers rs517047387 and rs383143898 were confirmed to be directly correlated with the SLICK and polled phenotypes within our cattle populations, respectively. These phenotypes appear to follow an autosomal dominant inheritance pattern based on our results, which is similar to previous literature findings (Littlejohn et al., 2014; Medugorac et al., 2012). These markers can be subsequently used to reliably predict whether these phenotypes of interest are present in *Bos taurus* cattle. Thus, providing the cattle industry with a cost-effective and accurate genetic tool for tracking these phenotypes.

In Chapter 3, the heat tolerant capabilities of our Climate Master Hybrid with the SLICK genotype were assessed by measuring respiration rate, internal ruminal temperature, relative activity levels, and HSP70 gene expression and blood plasma concentrations. Climate Master Hybrids with the SLICK genotype had significantly reduced respiration rates and increased activity levels compared to our animals without the SLICK genotype during periods of elevated temperature and humidity as indicated by the THI. Similar respiration rate findings have previously been identified in SLICK cattle (Carmickle et al., 2022; Dikmen et al., 2014; Littlejohn et al., 2014). In addition, increased activity levels in cattle with the SLICK genotype to our knowledge have not been previously reported, yet a connection between activity levels and heat stress has been established (Heinicke et al., 2019). Both respiration rate and activity levels have been characterized as strong indicators of animals impacted by heat stress (Heinicke et al., 2019; Lees et al., 2019). No differences were identified between cattle groups for both HSP70 gene expression and blood plasma concentrations, which may indicate that all groups in this population could be initiating heat stress responses at similar environmental conditions. However, cattle with the SLICK genotype show the adaptive

capability to reduce the magnitude of heat stress indictors, as shown by their respiration rates and activity levels observed in this study. Therefore, animals with the SLICK genotype have a superior ability to reduce the effects of heat stress through responses and innate abilities affecting heat absorption and dissipation. These results also show that respiration rate and activity levels, collected using novel collection techniques utilizing both drones and boluses that can assess heat tolerant capabilities of the SLICK genotype. To our knowledge, this study is among the first to use the Moonsyst bolus to assess activity levels in cattle as a response to heat stress. Since heat stress causes many adverse effects in cattle, and animals with the SLICK genotype show reduced impacts from these effects, the SLICK genotype appears to be a promising solution for cattle sustainability in response to rising global temperatures (*Climate Change*, n.d.; IPCC, 2022). Furthermore, this study demonstrates that the genetic influence from a cold adapted temperate breed like Galloway may not completely inhibit the heat tolerance capabilities inherent to the SLICK genotype. While the SLICK genotype is a promising solution to cattle heat stress, our Climate Master Hybrid may additionally possess cold tolerant capabilities which is important for temperate environments in Canada.

Another interesting finding in Chapter 3 was that Akaushi cattle had significantly lower rumen temperatures compared with the other cattle breeds in our study population. While all cattle group medians remained within optimal temperature ranges for fermentation (Beatty et al., 2008), heat stress indictors have been associated with changes in the cattle gut microbiome (Czech et al., 2022; Kim et al., 2020). Furthermore, variations in gut microbial diversity and composition have been associated with marbling characteristics (Sato et al., 2024). Therefore, the lower ruminal temperature observed in this population of Akaushi cattle may have implications on its marbling characteristics.

Chapter 4 aimed to reveal genetic markers associated to marbling traits in Akaushi cattle. This involved genotyping along with a genome-wide association study (GWAS). No alleles were found to be unique to Akaushi cattle compared to non-Akaushi cattle of the seven SNPs genotyped. Population structure analysis of the SNP array results indicated that Akaushi cattle were genetically distinct from the rest of the cattle breeds. The GWAS was conducted using a mock dataset generated to predict marbling

characteristics for each individual as an assessment of the marbling characteristics was outside the scope of the current stage of this project. The GWAS identified 12 SNPs significantly associated with the predicted marbling attributes. One candidate SNP identified, rs29013454, could be associated with marbling traits in Akaushi, which may modulate the activity of *PDHA2*. *PDHA2* is involved in the oxidative decarboxylation of pyruvate, forming acetyl-CoA, an important intermediate in lipid, carbohydrate, and protein metabolism (Pinheiro et al., 2012; Shi and Tu, 2015). Completing another GWAS with measured marbling characteristics of Akaushi in the future is paramount to determining markers associated with marbling.

Continuation of the project does require additional research to adequately validate the potential benefits of the Climate Master Hybrid to producers. Since the Climate Master Hybrid breed has genetic influence from Galloway, a winter hardy breed, an assessment of its cold tolerant capabilities is still required. The cold tolerance capabilities will show whether this novel breed can handle extreme cold as well as hot temperatures, as indicated in Chapter 3, that could arise from climate change. One example indicator of cold stress is a reduction in feed efficiency, which leads to higher production costs to reach final carcass weights similar to animals not affected by cold stress (Terry et al., 2021; Toghiani et al., 2020). After cold tolerance assessments, the meat qualities of the Climate Master Hybrid should be assessed and compared to Akaushi cattle. This assessment could be paired with a GWAS to determine genetic markers associated with the marbling characteristics of Akaushi, providing an array of genetic predictors of marbling traits. Finally, crossbreeding the Climate Master Hybrid with Akaushi would be the final step in the breeding plan. Marbling characteristics of the proposed Climate Master Composite breed should then be determined to assess how well the marbling characteristics from Akaushi were implemented in the composite breed. It would be important to characterize both the heat and cold tolerant traits of the proposed Climate Master Composite to confirm these capabilities traits were transferred as expected. If the Climate Master Composite has superior marbling, heat tolerance, and cold tolerance as predicted, it would present novel and potentially profitable and sustainable genetic attributes for cattle producers, increasing the chances of consideration and ultimately adoption of these new traits in the future.

The SLICK phenotype clearly provides superior heat tolerance capabilities that have been observed in our Climate Master Hybrid as well as in other research projects (Carmickle et al., 2022; Dikmen et al., 2014; Littlejohn et al., 2014). Introduction of the SLICK genotype could be beneficial to many cattle breeds throughout the beef industry, however many breeds have been bred for specific traits for multiple generations (Johnson, 2023). As such, the breed's characteristics may have been curated over many years (Johnson, 2023). In this case, crossbreeding with Senepol to introduce the SLICK genotype may produce adverse effects to the overall characteristics inherent in the original breed, such as changes in growth rates. However, the CRISPR/Cas9 system, a gene editing tool, could introduce the SLICK genotype into breeds of cattle without crossbreeding, which could dramatically accelerate genetic improvements in cattle (Carmargo et al., 2023). A possible caveat with gene editing is that the SLICK mutation on its own may not produce an animal with the SLICK phenotype and may only be expressed when a set of genetic dependencies are met. If the expression of the SLICK phenotype is not dependent on other genetic influences, it could be introduced into various *Bos taurus* breeds used in the beef industry, providing the benefits of the SLICK phenotype while maintaining the breed's previously curated characteristics.

According to previous findings, genetic markers associated with marbling in beef cattle are inconsistent across cattle breeds (Hudson et al., 2021). Lipid metabolism involves many genes that could affect total intramuscular fat content, providing many possible mutation sites for increased marbling characteristics (Tan & Jiang, 2024). The inconsistencies in genetic markers associated with marbling characteristics across various breeds may suggest the marbling trait is an adaptation that has arisen through convergent evolution. To assess this, a large GWAS could be conducted with many different breeds known for their marbling characteristics, along with an Angus control group. Each of the breeds would undergo high density genotyping and marbling and meat quality characteristic assessments. Genetic markers identified in this proposed GWAS that are unique to each of the breeds could determine physiological differences between the differentially adapted marbling characteristics, constructing a large collection of markers each responsible for marbling. Downstream implementation of these genetic variations could provide insights to the development of marbling, and potentially be used to

optimize the marbling characteristics in beef cattle through genetically guiding breeding programs.

The Climate Master Cattle project is a huge undertaking with many major potential benefits to the beef industry, and possibly the environment. Heat and cold tolerance will both be extremely important in the future as weather patterns due to climate change become more volatile. The overall accomplishments of this work include the development of a genotyping tool that accurately predicts the SLICK and polled phenotypes of cattle and the heat tolerant capabilities exhibited by the SLICK genotype within a hybrid animal that may potentially have cold tolerant capabilities. Additionally, genetic differences between Akaushi and non-Akaushi cattle were determined, but their functions with respect to marbling characteristics were not confirmed. Overall, the SLICK genotype is important to developing heat tolerance in cattle in temperate environments, and its autosomal dominant inheritance pattern makes it easily introduced into new cattle breeds, including those that are cold tolerant.

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Appendix

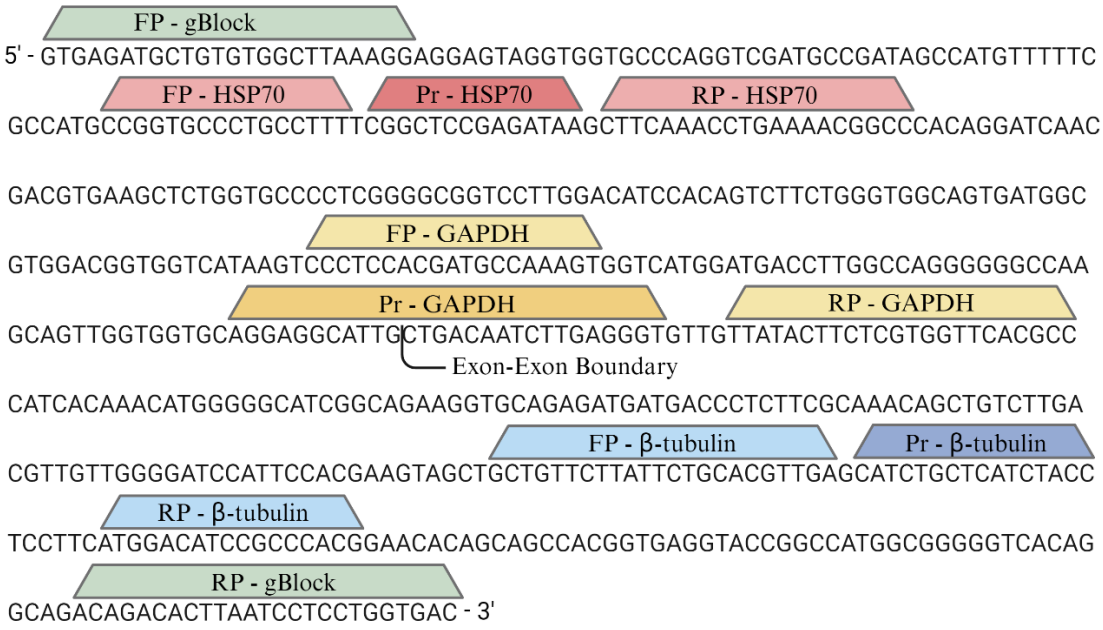

Figure A.1: Annotated nucleotide sequence of gBlock for HSP70 qPCR optimization. Each assay has a binding site for the forward primer (FP), TaqMan Probe (Pr), and reverse primer (RP). To generate more of the gBlock, a forward and reverse primer flank the assay binding sites for PCR amplification. The probe of the GAPDH assay is across an exon-exon boundary, which specifically targets cDNA. Created with BioRender.com.

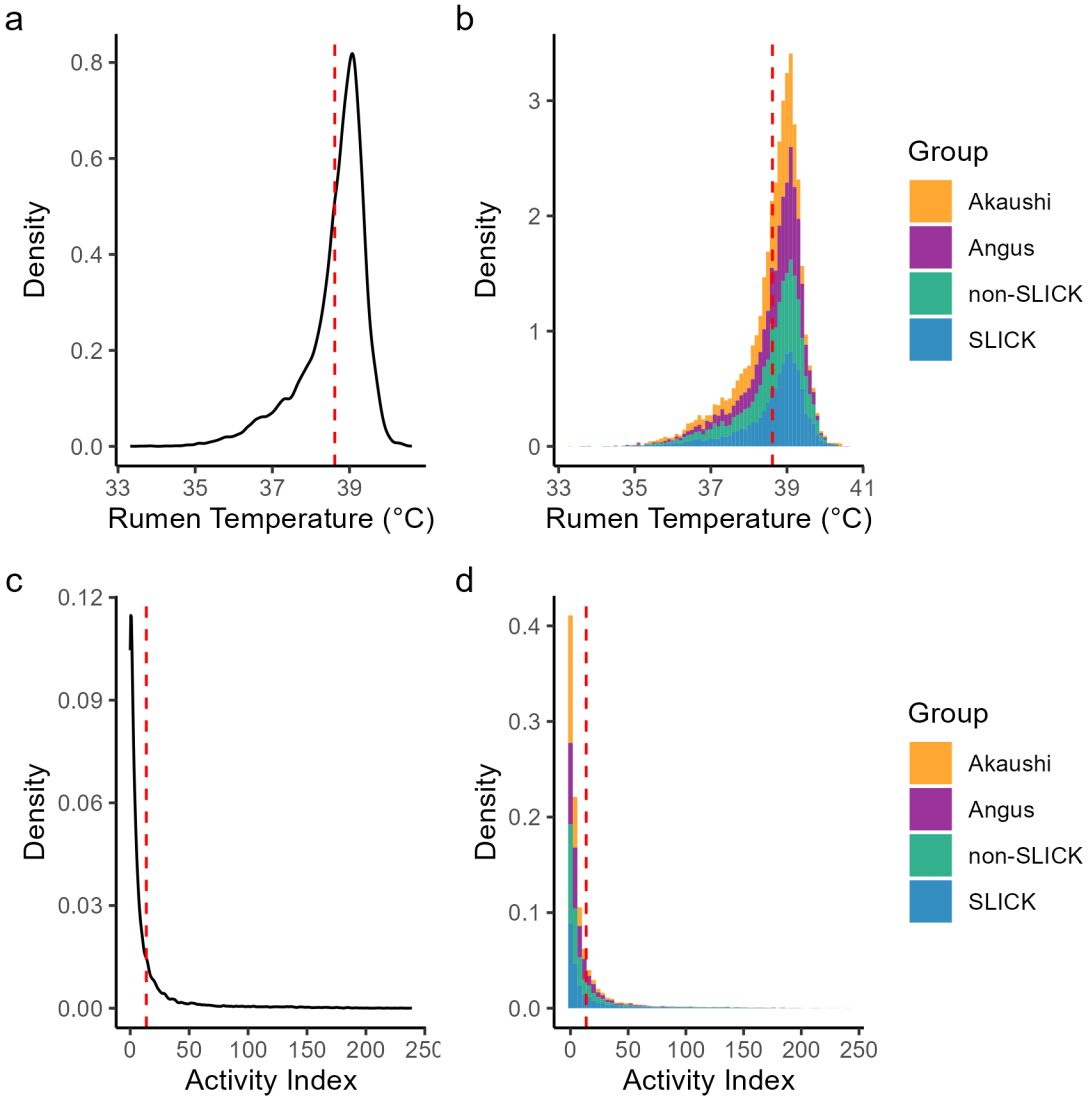

Figure A.2: Distribution plots of bolus data. Internal rumen temperature (TR) of combined data density plot (a), and histogram by group (b). Activity index (AI) density plot of combined data (c) and histogram by group (d). Red dashed line represents the mean of data.

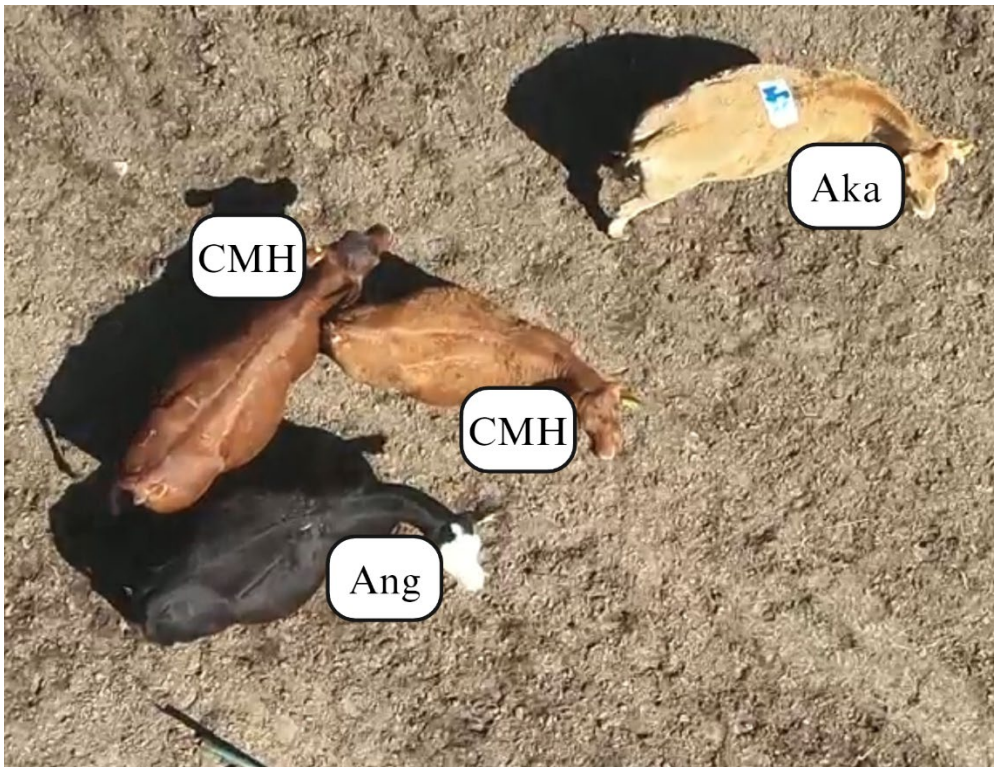

Figure A.3: Example coat colours of the cattle used for the respiration rate data collection. Akaushi (Aka), Angus (Ang) and the Climate Master Hybrid (CMH) are pictured here. Drone footage captured by the DJI Mavic Enterprise Advanced Quadcopter by Matthew Francis. Created with BioRender.com

Table A.1: Bolus Kruskal-Wallis test results for all THI ranges comparing cattle groups for both T_R and A_I. All p-values were adjusted for FDR using the Benjamini-Hochberg procedure ($\alpha = 0.05$).

THI Range	T_R p-value	A_I p-value
THI < 60	5.58×10^{-47}	3.15×10^{-24}
60 ≤ THI < 65	1.52×10^{-32}	7.61×10^{-34}
65 ≤ THI < 70	4.89×10^{-22}	1.34×10^{-24}
70 ≤ THI < 75	4.52×10^{-5}	5.85×10^{-32}
75 ≤ THI	0.0405	2.76×10^{-12}

T_R: Rumen Temperature, A_I: Activity Index

Table A.2: Bolus pairwise Wilcoxon tests for each cattle group for each THI range for both T_R and A_I. All p-values were adjusted for FDR using the Benjamini-Hochberg procedure ($\alpha = 0.05$).

THI Range	Group 1	Group 2	T_R p-value	A_I p-value
THI < 60	Akaushi	Angus	1.40×10^{-28}	9.46×10^{-19}
	Akaushi	non-SLICK	7.50×10^{-27}	4.62×10^{-4}
	Akaushi	SLICK	5.99×10^{-43}	6.09×10^{-18}
	Angus	non-SLICK	0.162	8.16×10^{-8}
	Angus	SLICK	5.96×10^{-3}	0.957
	non-SLICK	SLICK	0.351	4.67×10^{-7}
60 ≤ THI < 65	Akaushi	Angus	3.38×10^{-18}	4.10×10^{-29}
	Akaushi	non-SLICK	3.69×10^{-10}	1.17×10^{-12}
	Akaushi	SLICK	1.51×10^{-33}	1.02×10^{-27}
	Angus	non-SLICK	0.327	3.71×10^{-4}
	Angus	SLICK	2.27×10^{-4}	0.483
	non-SLICK	SLICK	5.33×10^{-5}	1.43×10^{-4}
65 ≤ THI < 70	Akaushi	Angus	4.53×10^{-17}	3.53×10^{-16}
	Akaushi	non-SLICK	1.03×10^{-13}	3.77×10^{-7}
	Akaushi	SLICK	6.71×10^{-20}	3.89×10^{-22}
	Angus	non-SLICK	0.926	8.63×10^{-3}
	Angus	SLICK	0.701	0.0106
	non-SLICK	SLICK	0.701	3.02×10^{-6}
70 ≤ THI < 75	Akaushi	Angus	1.02×10^{-5}	1.85×10^{-14}
	Akaushi	non-SLICK	9.37×10^{-3}	1.56×10^{-7}
	Akaushi	SLICK	1.18×10^{-3}	1.69×10^{-28}
	Angus	non-SLICK	0.133	0.0769
	Angus	SLICK	0.104	9.48×10^{-8}
	non-SLICK	SLICK	0.907	4.94×10^{-10}
75 ≤ THI	Akaushi	Angus	0.0345	4.10×10^{-4}
	Akaushi	non-SLICK	0.385	2.19×10^{-3}
	Akaushi	SLICK	0.108	1.75×10^{-10}
	Angus	non-SLICK	0.209	0.712
	Angus	SLICK	0.385	3.51×10^{-6}
	non-SLICK	SLICK	0.485	7.81×10^{-4}

T_R: Rumen Temperature, A_I: Activity Index

Table A.3: Mock marbling data for GWAS.

Well	Sample	Sex	Breed	BMS
A01	J11	M	Senepol	3.6
B01	J12	M	Senepol	3.5
C01	131W	F	Galloway	5.6
D01	R4	F	Senepol	3.7
E01	R1	F	Senepol	4.1
F01	11H	F	Galloway	5.2
G01	2018H	F	Akaushi	10.7
H01	56K	F	Cross	4.9
A02	77K	M	Cross	5.5
B02	112K	M	Cross	4.7
C02	135K	M	Cross	4.8
D02	152K	M	Cross	4.9
E02	169K	M	Cross	5.7
F02	207K	F	Cross	4.3
G02	209K	M	Cross	4.6
H02	Wrangler	M	Senepol	4.2
A03	Ferrari	M	Galloway	5.3
B03	2018H	F	Akaushi	10.7
C03	112K	M	Cross	4.7
D03	Wrangler	M	Senepol	4.2
E03	2009H	F	Akaushi	9.7
F03	2001H	F	Akaushi	10.6
G03	204K	M	Akaushi	9.8
H03	4839	M	Akaushi	10.4

BMS: Beef Marbling Standard score