The effects of low- and high-intensity exercise on Holstein dairy cattle muscle composition and thermoregulatory capabilities

by

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Abstract

Heat stress, the inability of the body to maintain thermal homeostasis, is one of the most significant health challenges that faces the dairy industry. The economic impact of heat stress has cost the dairy industry an estimated $1-$2 billion per year. Mitigation of heat stress on dairy herds is essential to the success of the industry and welfare of the cattle. One proposed strategy for mitigation of heat stress is exercise-induced heat acclimation. In humans, rats, and horses, exercise has been documented as promoting changes in skeletal muscle fiber types and vasculature that are more conducive to maintaining homeostatic temperatures.

To test the utility of exercise as a method to mitigate heat stress, three 8-week exercise trials involving a total of 71 Holstein dairy heifers were completed over a two-year period; the first trial (trial 1) occurred during the early summer of 2016, the second (trial 2) during the late summer of 2016, and the third (trial 3) during the early summer of 2017. Trial 1 and 3 heifers were kept in drylot pens before trial commencement, while trial 2 heifers were kept on multi-acre pasture. Heifers were split into groups within trials 1 and 2 to perform one of three activities: sedentary, low-intensity exercise, or high-intensity exercise. Heifers in trial 3 in 2017 performed only sedentary or low-intensity exercise treatments. Skeletal muscle composition of biopsies from the semitendinosus (Chapter 2; trial 1 and 2) and biceps femoris (Chapter 3; trial 3) muscles were analyzed within and across treatments. Chapter 2 data were combined for analysis utilizing a randomized complete block design to account for differences between trial. Percentage of fiber types, cross-sectional surface area, and oxidative capacity were measured for Chapters 2 and 3, with capillary density being an additional measurement for Chapter 3. In addition, for Chapter 3, core body temperature (via rectal thermometer) and mean skin temperature (via infrared

thermometer) were obtained weekly, and ear temperatures from CowManager Sensors were obtained hourly.

Time by treatment interactions were analyzed via a two-way repeated measures ANOVA for body weight, fiber type percentages, cross-sectional surface area, oxidative capacity, and temperature data. For Chapter 2, there were no time by treatment interactions of fiber type percentages, indicating that time and type of exercise did not affect fiber type percentages; however, in Chapter 3 there was a time by treatment interaction for type I fiber percentages, with sedentary treatment decreasing type I fibers over time and low-intensity treatment increasing type I fiber percentages. Cross-sectional surface area and oxidative capacity of fiber types were unaffected in Chapters 2 and 3 and capillary density was unaffected by exercise treatment in Chapter 3.

In Chapter 3, temperature data were evaluated for mean skin temperature to core body temperature ratio, temperature trends during a period of ambient cooling, and temperature trends during a period of ambient heating. There was a time by treatment interaction for the temperature data during the period of cooling; heifers that underwent low-intensity treatment had lower temperatures during the cooling period than those completing sedentary treatment. Additionally, the sensitivity of the CowManager ear tags were compared with rectal temperatures (core body temperatures) and infrared temperatures of the left ear (ear temperature); this occurred to determine if the ear tags were sensitive enough to detect minute differences between treatments after a period of exercise. As there were differences between treatments indicated by rectal temperatures, but not by the ear tags, and, as temperature trends wavered depending on the obtainment method, we concluded that the ear tags are not sensitive enough to detect differences in body temperature after periods of exercise.

Both high and low-intensity exercise did not seem to consistently effect skeletal muscle among Chapters 2 and 3; however, Chapter 3 data analyses did show promise with low-intensity exercise potentially improving cooling capabilities during a period of ambient cooling. Additionally, CowManager ear tags may not be beneficial in determining the differences in body temperature after a period exercise. Further investigation into the effects of exercise on fatty liver disease and urine-nitrogen content may be warranted, as well as investigation into utilizing longer trial periods for dairy cattle exercise.

Keywords: Dairy, Exercise, Muscle Fibers, Thermoregulation

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Dedication

This is dedicated to my current and future nieces and nephews.

Chapter 1 - Review of Literature

Importance of Dairy Products and the Dairy Industry

The dairy industry is of worldwide significance as an agricultural giant, producing 800 billion kilograms of milk in 2014. In the same year, the global dairy industry market was valued at $336 billion (EIC, 2015). Although milk consumption has not been rising in countries that are major producers of milk, such as the European Union and the USA, the FAO (2017) has reported that developing countries are importing milk at an increased rate. At the same time, the FAO (2017) has stated that developing countries are beginning to increase their own annual milk production to meet the demand for increased consumption among the local population. A 22% increase in milk production is anticipated through 2027 and it is projected that developed and developing countries will produce 9 and 33 percent more milk, respectively (FAO, 2018).

Dairy products are commodities in developing countries due in part to their cost-effective nutritional value. Among these products, cow's milk is demonstrated to be consumed most often (Nuñez, 2016). Cow's milk has been recommended for children, the elderly, and women to ensure proper growth, strong bones, and to reduce the risk of osteoporosis, respectively (Holt et al., 2011; Wolfe, 2015). Cow's milk has also been documented as one of the most concentrated and digestible forms of protein, vitamins, and minerals available, containing approximately 3.2% protein, 2.6% casein, 0.6 % whey protein, 4.6 % sugar, and 3.9% fat (Belitz et al., 2008).

Depending on the age of the consumer, Smith et al. (1999) stated cow's milk meets 20-28% of the daily protein requirement. Casein and whey protein (80% of milk protein and 20% of milk protein, respectively) have been considered a source of complete human protein, meaning, when evaluated, they contained all nine essential amino acids for humans and were easily digestible (Hoffman & Falvo, 2004). Casein and whey proteins have been calculated to have a

1.00 Protein Digestibility Corrected Amino Acid Score, meaning that their protein composition is 100% available for protein synthesis in the human body (Hoffman & Falvo, 2004). Vegetable proteins have typically been documented as containing inferior proteins, meaning they lack one or more of the essential amino acids for humans (Hoffman & Falvo, 2004). An exception to the previous statement would be soybean protein. Just like milk protein, Michelfelder (2009) indicated soybeans provide all nine essential amino acids to humans upon digestion and intestinal absorption. Soy protein has been calculated to have a 1.00 Protein Digestibility Corrected Amino Acid score (Hoffman and Falvo, 2004).

Despite the similarities in protein quality and content, there is a key benefit to utilizing animal milk products over vegetable-based products like soymilk; cow's milk has been reported as being more efficient in delivering calcium to human consumers. Depending on the age of the consumer, others have demonstrated cow's milk meets 52-65% of the daily dietary reference intake (DRI) of calcium (Feskanich et al., 2003; Chevalley et al., 2008; Skinner et al., 2011). Other calcium-rich foods, such as seeds and beans, contain less than 35% DRI of calcium, depending on the age of the consumer. Whole milk has been shown to contain approximately 305 mg of calcium, while unfortified soy milk has been shown to contain approximately 10 mg of calcium (Heaney et al., 2000). Calcium-fortified soy milk is assumed to contain the same amount of calcium that is in cow's milk (~300 mg); however, Heaney et al. (2000) reported that the absorbability of calcium from calcium-fortified soy milk (0.237 fractional absorbability) was not comparable to calcium sources from cow's milk (0.306 fractional absorbability).

Despite documentation of nutritional value, there are inconsistent reports about the benefits and detriments of consuming milk products. Some researchers have linked milk consumption to increased obesity rates (Berkley et al., 2005), cancer (Ornish et al., 2001; Larsson et al., 2004;

Ursin et al., 1990; Qin et al., 2009; Mettlin & Piver, 1990), mortality rates (Michaelsson et al., 2014;), iron deficiency in children (Ziegler, 2011), acne (Spencer et al., 2009; LaRosa et al., 2016: Adebamowo et al., 2006; Adebamowo et al., 2008;), and bone degeneration (Michaelsson et al., 2014). Although this is not a conclusive list, most studies promoted high daily milk consumption in their methods (>24 ounces of milk/day). Greater than 24 ounces of milk per day is more than the daily recommended amount of milk. Additionally, confounding variables, such as memory-based surveys and subjective self-assessments, were present in these studies and most of these studies have been countered by additional studies.

Others have reported that milk consumption has no effect on or reduces the risk of cardiovascular disease (Alexander et al., 2016; Qin et al., 2015; Chowdhury et al., 2014; Hu et al., 2014; Rice, 2014; Muldowney & Kiely, 2011; Drouin-Chartier et al., 2016), mortality (O'Sullivan et al., 2013), bone fractures (Bian et al., 2018; Sahni et al., 2014; Høidrup et al., 1999; Bischoff-Ferrari et al., 2011; Kanis et al., 2005), type 2 diabetes (Chen et al., 2014; Hirahatake et al., 2014; Tian et al., 2017; Gijsbers et al., 2016; Hruby et al., 2017; Santaren et al., 2014; Aune et al., 2013), hypertension (Soedamah-Muthu et al., 2012; Machin et al., 2014; Appel et al, 1997; Karanja et al., 1999; Beltran-Barrientos et al., 2018), obesity (Beck et al., 2017; Lin et al., 2012; Carruth & Skinner, 2001; Moore et al., 2006), and cancer (Davoodi et al., 2013). The variability has led meta-analyses to conclude the following: that, despite the weakness of some articles, the benefits of milk consumption outweighed the detriments, that harmful effects tended to only occur with excessive consumption of milk products, and that they felt the need for further investigation on the long-term and short-term health effects from consumption of different dairy products (Bian et al., 2018; Qin et al., 2009; Davoodi et al., 2013; Louie et al., 2011; Thorning et al., 2016; Guo et al., 2017; Lu et al., 2016; Alvarez-Leon et al., 2006).

In conclusion, the majority of existing studies detail a beneficial relationship between milk consumption and various human health concerns; however, many nutritionists concluded there is need for more research in this area. Despite the inconsistency on the effects of milk consumption, there is substantial scientific evidence milk products are a source of complete, digestible protein and absorbable calcium in the diet. Because of its contributions to health and the global economy, the dairy industry is important for the success of society. Despite its importance, the dairy industry is at risk. Heat stress, one of the most significant health ailments affecting dairy cattle, is increasing in incidence and severity due to climate change.

Climate Change: Definition, Trends, and Predictions

Climate is defined as the statistics of weather over a period of 30 years (IPCC, 2013); thus, climate change is a variation in the Earth's weather patterns over time (NASA, 2014). Climate change is present when weather data averages are statistically significant between years (IPCC, 2013). Climate change can manifest as fluctuations in temperatures or wind speed or in the amount of snow, rainfall, or wildfire incidences (Karl & Trenberth, 2003).

Global temperature is often evaluated when analyzing climate change. The average atmospheric temperature has fluctuated since the 1850's (figure 1.1); however, a trend of record-level, increasing temperatures began in the 1980's (Morice et al., 2012). According to data provided by NASA's Goddard Institute for Space Studies (2018) and data analyses provided by Hansen et al. (2010), the earth's average atmospheric temperature has increased by 1°C over the past 38 years and is projected to increase 5.6°C over the next century. The Intergovernmental Panel on Climate Change (IPCC) attributed this increase in climatic temperatures to the amount of greenhouse gas emissions (IPCC, 2019). The temperature increase, both the past increase and the

predicted future increase, have been designated as “Global Warming” or the “Greenhouse Effect” by scientists, politicians, and the public.

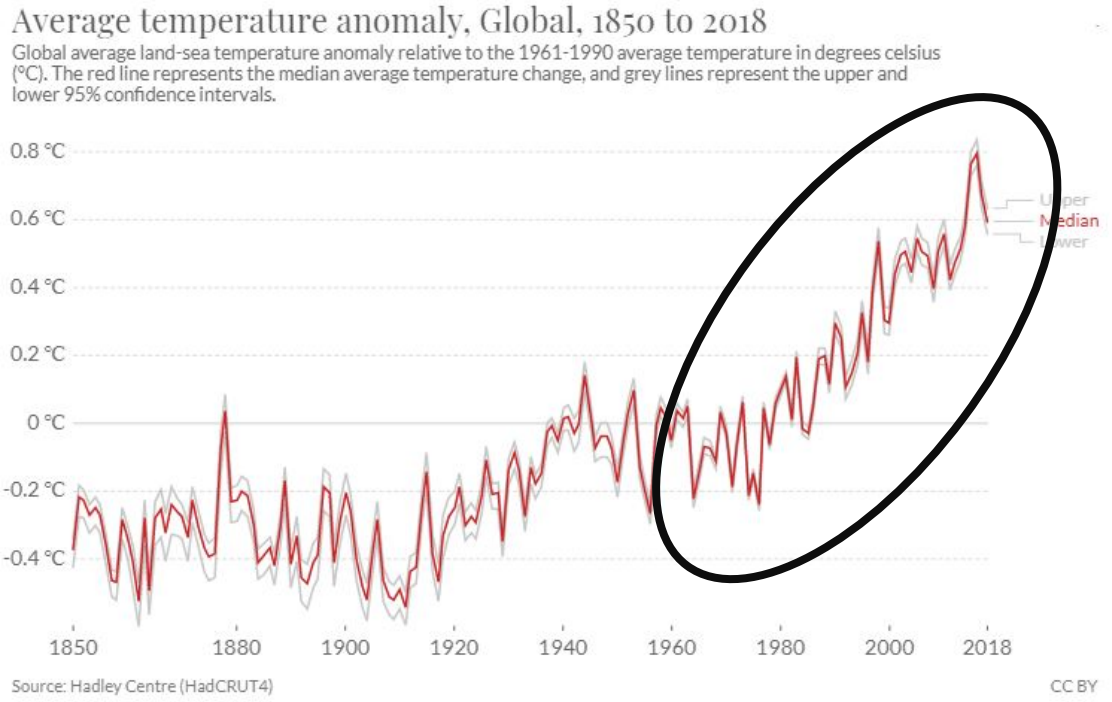


Figure 1.1 Global Average temperature Anomaly from 1961-1990.
Recorded global temperatures have fluctuated from the mid-1800’s up to 2017; however, an overall increase in temperature can be seen beginning in the 1960’s through 2017 (circled). Average global temperature increased by approximately 0.9℃ over the 57-year period (Hadley Centre, 2019).

Proposed Causes of Global Warming

Cooke et al. (2016) reported 97% of the scientific community from published climate research indicated global warming is occurring and is attributable to humankind (Cook et al., 2016). When evaluating the cause of global warming, the main concern was the release and subsequent atmospheric occupation of greenhouse gases (Thompson, 2010). The Earth warms

continually from solar radiation but, when the sun's radiation onto the Earth is decreased, such as when one half of the lateral hemisphere is facing away from the sun, the planet begins to cool, releasing heat into the atmosphere for dissipation.

Greenhouse gases (i.e. methane, carbon dioxide, nitrous oxide, etc.) are released into and subsequently occupy the troposphere (the lowest region of the atmosphere) and stratosphere (the highest region of the atmosphere). Greenhouse gases absorb heat in the form of infrared radiation (IR) and the earth gives off heat in the form of IR. The greenhouse gases absorb the IR energy that comes into contact with their weakly bonded molecules and trap the heat within the Earth's atmosphere. Solar radiation (a majority of which is in the visible light spectrum of electromagnetic radiation) can still mostly pass from the sun and through the atmosphere to the planet surface, but the infrared radiation dissipated by the earth is trapped within the atmosphere.

In 2016, there were 6,511 million metric tons of carbon dioxide released annually into the atmosphere compared to 2,000 million metric tons of carbon dioxide in 1990. The United States (~15.3% of annual emissions) and China (~29.2% of annual emissions) were the top contributors to greenhouse gas emissions in 2016 (Le Quere et al., 2017; Peters et al., 2017; Jackson et al., 2017). Annual average global concentrations of carbon dioxide have increased from less than 320 parts per million (ppm) in the 1960's to over 400 ppm as of 2016 (Dlugokencky, 2018). This increase in greenhouse gases in the Earth's atmosphere were attributed to transportation (28.5% of total greenhouse gas emissions), electricity production (28.4%), industry (22%), commercial and residential release (11%), agriculture (9%), and land use and forestry (11%; EPA, 2019).

Effects of Global Warming on Seawater Levels, Freshwater Resources, and Rainfall

Long-term and unprecedented global warming has potentially catastrophic consequences. The Earth's polar ice caps have been melting, with the runoff contributing to an increased sea

level; the sea level rose 7.6 ± 3.9 millimeters between 1992 and 2017 (Shepherd et at., 2018; Willis et al., 2018; Zabel et al., 2014). Fretwell et al. (2013) predicted "the ice sheets of Antarctica hold enough water to raise global sea levels by 58 meters". In addition to collected run-off from the melting ice, the sea level is also presumed to have risen due to the expansion of seawater molecules from increased atmospheric temperatures (IPCC, 2013).

The increased sea level encroaches upon land, presumably reducing available space for agriculture, wildlife habitats, and urbanization. In addition, it creates a constant loop in which the amount of atmospheric heat that is absorbed into the ocean is released back into the atmosphere over time (Dahlman & Lindsey, 2020; Cheng et al., 2020). According to the IPCC 5th Assessment Report (2014), only 10% of the energy trapped by greenhouse gases is dedicated towards melting sea ice, ice caps, glaciers, and warming the land mass; the remaining 90% is absorbed into the oceans, with the oceans commonly being referred to as Earth's "heat sink". A "heat sink" is defined as "a substance or device that absorbs or dissipates especially unwanted heat" (Merriam Webster, 2020). The oceans are assumed to absorb the unused environmental heat from the atmosphere due to water's high heat capacity: 3.900 joule/gram°C for sea water compared to 0.96 joule/gram°C for light concrete (Waples & Waples, 2004). The oceans are vast, containing approximately 1,335,000,000 km^3 of water and thus provide ample supply of water molecules for heat absorption.

Heat absorbed into the ocean moves from water source to water source, process to process, until the heat-producing energy is ultimately released back into the environment and re-trapped by greenhouse gases. The heat transfer cycle from the sun to the Earth's surface and then between the ocean and atmosphere and the atmosphere and ocean continues on, assumingly trapping increasingly greater quantities of heat.

In contrast to sea water, drinkable water has been documented as evaporating at greater rates (evaporation rates vary depending on the location and amount of water present) due to an increase in atmospheric moisture holding capacity (Bates et al., 2008); for every 1°C increase in atmospheric temperature, there is a 7% atmospheric moisture capacity increase (Trenberth, 2008). During drought conditions generated by increased water evaporation, the frequency of rainfall was decreased and the intensity of rainfall was increased; this caused heavy rainfall (>7.6 mm per hour) versus light rainfall (<2.5mm per hour; Monjo, 2016; Easterling et al., 2017). When evaporated water is precipitated after drought conditions, the receiving soil is often dry and compacted, reducing the capability of the soil to absorb the heavy rainfall. The combination of drought-induced heavy rainfall and compacted soil has been attributed to causing floods (Trenberth, 2011). Over the next century, drought and flooding is projected to increase in prevalence as the atmospheric temperature increases (Easterling et al., 2017).

To summarize: climatologists have identified that a significant increase in the Earth's average global temperature has occurred over the past century and have projected that it will continue increasing at a greater and faster rate over the next century. The increasing temperature within the Earth's atmosphere is increasing the evaporation of fresh water, increasing the incidence of drought and flooding, causing a rise in the sea level, and is causing a feedforward circle that re-introduces increasingly greater quantities of heat from the ocean and back into the atmosphere.

Global Warming Impact on the Dairy Industry: Heat Stress

Global warming is predicted to increase the incidence and severity of heat stress on dairy cattle, with a presumed decrease in profit for the dairy industry and a negative impact upon animal welfare (Silanikov et al., 2015; Martinsohn et al., 2012; Nardone et al., 2010; Von Keyserlingk et al., 2013). In 2003, the dairy industry experienced a net loss of $1 billion due to heat stress alone

(St-Pierre et al., 2003). Using present-day milk pricing, losses to the dairy sector due to heat stress are expected to exceed $2 billion annually by 2099 (Mauger et al., 2015). Both the monetary losses recorded in 2003 and the predicted monetary losses over the next century are attributed to decreased milk production during times of elevated ambient temperatures. Most calculated or projected losses do not include costs associated with heat abatement and minimization techniques for dairy cattle, individual animal deaths, or culling due to heat stress.

In addition to economic concerns, animal welfare is a significant concern within the dairy industry. Increased ambient temperatures cause discomfort as well as promote mastitis and decreased fertility. Cattle mortality rates increase during hot environmental conditions (Elvinger et al., 1991; Bishop-Williams, 2015; Vitali et al., 2015). For every one unit increase in the heat stress index, mortality rate increased approximately 1.03 times (Bishop-Williams et al., 2015). A prolonged period of hot weather, increased the heat stress index of dairy cattle by 8.6 units and resulted in a mortality rate increase of 1.27 times (Bishop-Williams, 2015).

Global warming results in greater average ambient temperatures. High ambient temperatures within livestock dense regions, are presumed to result in increased incidences of heat stress and heat stress-induced fatalities among dairy herds. Heat stress within dairy cattle results in decreased profit for the dairy industry and decreased health and welfare for dairy cattle.

Heat Transfer and the Effects of Heat on Dairy Cattle

To understand how high environmental temperatures physiologically affect dairy herds, the methods of heat transfer must be thoroughly understood. Heat is the transfer of energy between objects. Based upon the consequences of the first two laws of thermodynamics, heat moves along a warm-to-cold concentration gradient and can neither be created nor destroyed (Carnot, 1824; Clausius, 1850). Temperature is the amount of molecular movement within a system and

determines the amount of heat released or absorbed within a system. High temperatures increase molecular movement while low temperatures slow movement. Matter can be heated by one of two ways: energy movement from a warmer object or through energy form conversion. Energy exists in one of nine forms: chemical, thermal, nuclear, electrical, mechanical, gravitational, radiant, sound, and elastic. Any of these forms of energy can be converted to heat, which can then be transferred from a warmer to cooler object. There are four methods of heat transfer: conduction, convection, evaporation, and radiation.

Conduction is a heat transfer mechanism that occurs within a solid or between an immobile object of a greater temperature and an immobile object of lesser temperature. Heat, by way of its concentration gradient, will transfer from the warmer area or object to the cooler area or object. For example, a hot dairy cow lying on sand in her stall will conductively transfer heat from her epidermis to the sand molecules; this removes the heat from the cow, subsequently cooling her body, as heat is absorbed into the sand by way of the temperature concentration gradient. When the air is dry and a dairy cow's skin is wetted, conduction transferred approximately 20% of the total heat generated towards the environment (Mondaca et al., 2013). When using a conductive heat exchanger under cattle bedding, 20-90% of heat transferred from the exchanger to the cow was attributed to conduction (Rojano et al., 2011). Therefore, depending on the environmental temperature, a reasonable amount of heat can be conductively transferred between a dairy cow and its environment.

Convection utilizes moving currents, such as flowing water or wind, to transfer heat away from a warmer surface and towards a cooler surface. For example, a dairy cow standing in front of a fan. Heat will move from the cow and into the cooler molecules of air flowing over the cow's body. Heat will be carried away via the wind, providing a constant source of cooler molecules for

the cow's epidermal heat to transfer into upon contact. Fournel et al. (2017) noted that the addition of fans to circulate air along dairy cattle's bodies increased the amount of heat lost to the environment and subsequently decreased cattle rectal temperature (−0.5°C).

A warm surface (or warm ambient environment) that has a layer of liquid on it will conductively and convectively transfer heat to liquid molecules, resulting in evaporation. Misters are often used to cool dairy cows by coating their hair and epidermis with a layer of water. The dairy cow is warmer than the water, thus heat conductively and convectively transfers from the dairy cow's skin into the water on the skin surface. The heat causes an increase in the kinetic energy of the water and ultimately results in a phase shift from liquid to vaporization of the water. The water vapors float away, taking kinetic energy with them and removing kinetic energy from the water. The lower kinetic energy of the water allows more heat to be transferred from the skin surface to the water, cooling the skin surface. Fournel et al. (2017) noted that a combination of misters and fans reduced cattle's heat load by -0.8°C through increased evaporative cooling.

All objects are presumed to radiate heat. Radiation is a heat transfer mechanism that utilizes electromagnetic (EM) waves. Electromagnetic waves take on many forms, including gamma rays, infrared, microwaves, radio waves, light, and x rays. Most of the radiation emitted by mammalian bodies is in the form of infrared radiation (Gulyaev & Godik, 1984). Most of the radiation emitted by the sun is in the form of x-rays, ultraviolet rays, visible light, and infrared rays, all of which can be absorbed by cooler objects. An example of radiation is two cows of different temperatures standing 6 inches apart on a cool day. One cow is warmer than the other and is radiating EM waves through the environment. The second, cooler cow, absorbs heat via the EM radiation from the warmer cow and its body temperature increases. Another example of radiation would be a cow standing in the sun on a hot day. If the cow is of a cooler temperature than the EM waves being

supplied by the sun, the cow will absorb the heat from the sun's EM and its body temperature will increase.

Physiological Response of Heat Transfer in Mammalian Bodies

The cow's thermoregulatory response to body temperature relies on a stream of processes through its nervous system. Body temperature (T_B) is detected via visceral and peripheral sensory nerves within the dermis, liver, skeletal muscle, and hypothalamus. Thermoreceptive sensory nerves contain portions of three different types of thermoreceptors: hot receptors, cold receptors, and some that can detect both hot and cold. Thermoreceptors contain transient receptor potential (TRP) protein channels. When TRP channels encounter molecules of hot or cold temperatures, these channels may be opened. Some channels respond to very high temperatures (relative to the T_B), some respond to cool temperature, cold temperatures, or warm temperatures. When TRP channels are opened by interacting with molecules of a specific temperature, there is an influx of sodium that rushes into the sensory nerve cell and an afferent action potential is generated. This action potential propagates from the distal end of the sensory nerve and travels to the hypothalamus for signal interpretation. The greater the number of "hot" or "warm" molecules, the more openings of the heat-sensitive TRP channels, thus increasing the frequency of heat-designating afferent action potentials to the hypothalamus for interpretation. The frequency of specific channel activation dictates what temperature the hypothalamus interprets (Zheng, 2013; Wang & Siemens, 2015; Zhang, 2018).

If the T_B is outside of the appropriate temperature range, the hypothalamus will trigger an autonomic efferent action potential along the body's motor neurons (Song et al., 2016). This efferent action potential travels from the central nervous system to effector cells, cells of the body that generate a response to changes in the ambient environment (Wetsel, 2011). Efferent signals

cause the effector cells to respond appropriately (Kamm & Siemens, 2016). For instance, an effector can cause a response by increasing thermogenic-induced shivering if the T_B is interpreted as too cold. Inversely, if the T_B is considered to be too hot, effector neurons send responses for increased vascular dilation to increase blood flow to the dermis for heat dissipation.

Effector activation is decreased if there is a decrease in the amount of efferent action potentials coming from the hypothalamus. Once the body reaches a T_B that the hypothalamus interprets as acceptable, the hypothalamus will send efferent action potentials to effectors at a decreased rate, causing minimization of responses such as shivering or vascular dilation. A mature, lactating dairy cow's acceptable ambient temperature range is between 5°C and 25°C (Berman et al., 1985). When the hypothalamus interprets ambient temperatures greater than 25°C, effectors are likely activated and heat abatement techniques are initiated. Heat abatement techniques are managed to eliminate or mitigate the effects of *heat stress* in the dairy cow.

Heat Stress Defined

In mammals, Lenis et al. (2016) noted heat abatement is made possible via sweating, increased blood flow to the dermis, increased respiratory rate, and behavioral adaptations. Sweating releases moisture onto the skin's surface for evaporative cooling. Increased blood flow to the dermis directs blood flow away from the body's core and towards the skin surface. Here, surface capillaries are closer to the ambient environment and allow for conductive, evaporative, radiative, and convective heat transfer into the environment. An elevated respiratory rate increases blood flow through the lungs. The lungs are mucous membranes coated in moisture. This moisture can evaporate if heated to the point of vaporization. Evaporation of this moisture decreases the heat load in the lungs and core. In addition to these physiological adaptations to overheating,

several species have developed behavioral adaptations. These adaptations will be addressed for dairy cattle as a component of this thesis.

Heat abatement is slim to none when temperature, humidity, and solar radiation are great enough to prevent heat loss from the dairy cow. In other words, when the concentration gradient of combined heat, moisture, and EM waves are greater than the heat dissipating, heat may instead be absorbed. Heat builds up within the cow, increasing body temperature over time. This increase in heat load causes additive thermal stress.

Stress is defined as any effect that results from a disruption of homeostasis within an organism. Heat stress results from high ambient temperatures. Just as each species has a homeostatic 'set-point' for normal core body temperature, each species also has its own threshold for heat stress. In dairy cattle, ambient temperatures over 25°C trigger an action potential from the body's thermoreceptors to the hypothalamus; this causes efferent action potentials to flow from the hypothalamus to body tissues to activate one or more heat abatement mechanisms. If the cow's body cannot release heat and the body temperature continues to rise, heat stress may exacerbate to the point of death.

As homeotherms increase in size, their body volume increases when compared to their body surface area; thus, as an animal increases in size, its surface area to volume ratio decreases. A larger animal, such as a dairy cow, will have greater quantities of heat-generating tissues, such as adipose and muscle, and longer distances for heat to flow from the viscera and out towards the body's surface for dissipation (Polsky & Von Keyserlingk, 2017). Thus, larger animals produce greater quantities of heat and have more tissue mass to circulate heat; this makes them more susceptible to heat stress than a smaller ruminant, such as a goat.

Dairy cattle are sensitive to high temperatures, in part, because of their large size, but their predisposed sensitivity to thermal stress also results from lactation processes and ruminal digestion. Fermentation by microbes within the rumen produces 30-70 kilocalories per 100 kilocalories of carbohydrate digested versus the monogastric digestive tract which produces 5-7 kilocalories per 100 kilocalories of carbohydrate digested (Armstrong & Blaxter, 1956). The average dairy cow's total diet (ranges from 10-30 kg of dry matter per day) is comprised of 60-70% carbohydrates, thus a dairy cow's rumen can produce greater quantities of heat when compared to a monogastric animal (Herdt, 2014; National Research Council, 2001).

In addition to heat generated by the rumen, Holter (1976) demonstrated lactation also increased heat load within the dairy cow when one day post-lactation, the average body temperature of fasting cows was 39.8℃, while 31 days post-lactation the average temperature of fasting cows was 37℃. Lactating dairy cows produced up to 48.5% more heat than non-lactating dairy cows (Purwanto et al., 1990). Breeds, such as Holstein-Friesians, which are considered high milk-yielding (≥31.6 kg/day) are more susceptible to heat stress than lower milk-yielding breeds (Prathap et al., 2017; Igono et al., 1985; Purwanto et al., 1990).

When Holstein-Friesian (Holstein) cattle were first transported from their native Netherlands to North America in 1852, their mature body weight was less than 540 kg and they produced less than 3,000 kg of milk annually (Houghton, 1897). Northern Netherland's environment is considered oceanic, with cool temperatures year-round; however, the North American environment is intensely regional in terms of climate. The North American environment remains highly versatile and is only increasing in versatility as global warming is exacerbated. Currently, American dairy cattle weigh, on average, 680 kg and produce approximately 10,433 kgs of milk annually. Larger body sizes generate more heat and are more susceptible to heat stress,

including detrimental drops in milk production. As the weight of the dairy cow approaches 500 kgs, the efficiency curve of milk production to post-calving body weight decreases in slope, indicating that as a dairy cow's weight surpasses 500 kg, milk production does not increase linearly. Once the cow surpasses 580 kgs, there is a negative slope for milk efficiency to post-calving body weight (Rehak et al., 2012). Holsteins above 500 kgs may produce more milk annually; however, they have significantly less feed efficiency with greater body weights, thus causing organizations, such as the USDA, to use selection indices that place a negative accentuation on greater Holstein body sizes (Miglior et al., 2005).

In addition to their large body size, Holstein-Friesian dairy cattle have been shown to have less active sweat glands than other breeds of dairy cattle, such as Jerseys (Gebremedhin et al., 2008). As a result, Holsteins are less readily able to lose heat through evaporative cooling. In addition, sweat glands that *were* active were often functionally inhibited by properties of the hair or by the color of the cow (Gebremedhin et al., 2008). Increased black:white hair ratios perpetuates heat stress because there is greater absorption of heat by darker colored cows (Hillman et al., 2001). Despite their predisposition to heat stress, the Holstein breed is one of the most utilized breeds of dairy cattle across the U.S. According to the Council of Dairy Cattle Breeding (2019) in 2018, 86% of dairy cows worldwide were of the Holstein breed.

To summarize, the larger the animal, the more heat they generate and the more heat-trapping tissue mass they have. When considering heat stress, an agricultural animal of concern is the large-bodied, high milk-producing Holstein dairy cow. Holsteins are the most common breed of dairy cow across the globe; however, they are very susceptible to heat stress due to their large size (average of 680 kg), their heat-generating ruminal digestion (produces 30-70 kilocalories of heat for every 100 kilocalories of carbohydrate digested), and high milk production (lactation

increases a dairy cow's heat load by 48.5%). The incidence and severity of heat stress in dairy cattle are both predicted to increase as global ambient temperatures continue to increase (Klinedinst et al., 1993).

Heat Index of Dairy Cattle: TNZ and the THI

Basal metabolic rate is the rate of total energy expenditure for mammals at rest. The range of acceptable ambient temperatures at which basal metabolic rate does not increase or decrease is referred to as the thermoneutral zone (TNZ; Berman et al., 1985; Yousef, 1985b). Berman et al. (1985) and Yousef (1985b) define the TNZ as having two distinct boundaries: the lower critical temperature (LCT) and the upper critical temperature (UCT). Below the LCT is an ambient temperature at which a mammal must increase thermogenic mechanisms, such as shivering, to increase the amount of heat within the body to maintain its body temperature. Above the UCT is an ambient temperature at "which metabolic heat production increases as the animal augments heat loss activities" (Collier et al., 2011; Yousef, 1985b).

A mature, lactating dairy cow's TNZ is between the range of 5℃ and 25℃, with 5℃ as the LCT and 25℃ as the UCT (Berman et al., 1985). In addition to ambient temperature, breed, age, stage of lactation, sex, stage of adaptation, and the thickness and length of hair on the cow's body also affect the TNZ (Collier et al., 2011; Kingma, 2012; Noordhuizen, 2015). When ambient temperatures cause the dairy cow to fluctuate outside of the TNZ, discomfort, stress, or death can occur, depending on the extremity of the fluctuation. When above the UCT, **hyper**thermia, or heat stress, ensues and when below the LCT, **hypo**thermia ensues.

Hypothermia is a condition in which the dairy cow loses more body heat than it is producing. If significant, vital organs, such as the heart, liver, and kidneys, begin to shut down. Hyperthermia is a condition in which the dairy cow has gained more heat than lost and body

temperature has exceeded what the hypothalamus deems as acceptable. Heat stress occurs from hyperthermia. High ambient temperature, high ambient humidity, direct solar radiation, or a combination of all three, contribute to a decreased heat transfer from the dairy cow to the environment (Jackson & Rosenberg, 2010).

Ambient temperature contributes to the concept of the TNZ; however, ambient humidity does not. Ambient humidity impacts the ability of the dairy cow to abate heat as increased atmospheric humidity decreases the ability of a cow to dissipate heat through evaporative cooling. The temperature-humidity index (THI) encapsulates the effects of both ambient temperature and ambient humidity (Berry et al., 1964).

The THI is an index that combines the effects of ambient temperature and ambient humidity; further analysis revealed at what temperature hyperthermic stages occur. Although initially utilized for humans, Berry et al. (1964) adapted the concept of the THI to be used for predictive measurements of hyperthermia in dairy cattle. In 2012, Collier et al. proposed an index threshold of 68 ≤ (Figure 2) be used as the predictive measurement for hyperthermic stress in dairy cattle; an index level of 68 is used as it considers rectal temperature versus respiration rate, rectal temperature versus milk yield, and the temperature humidity index.

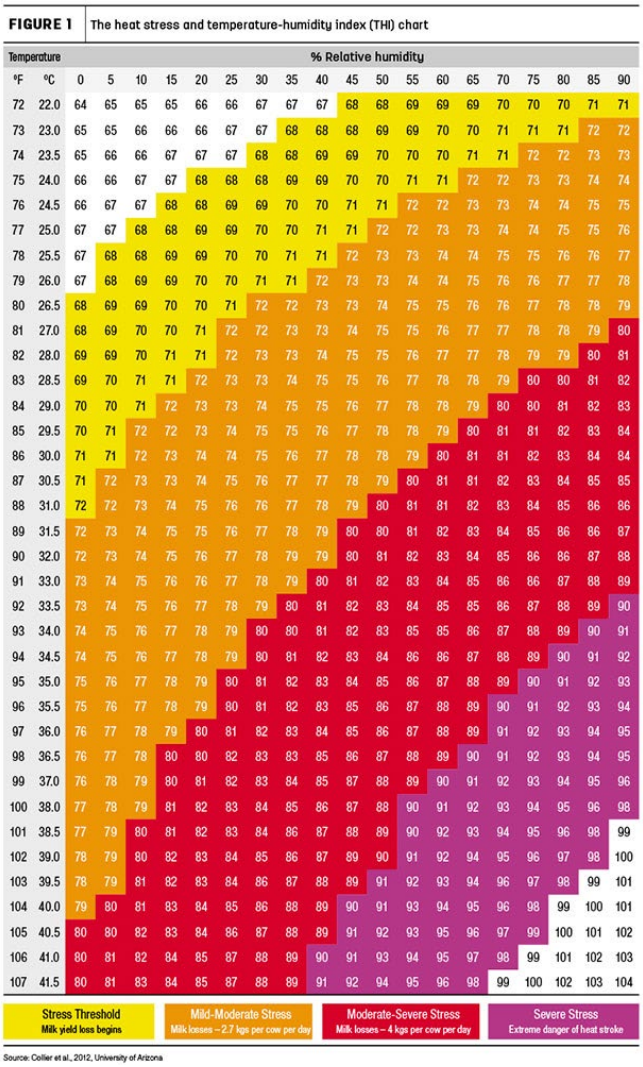


FIGURE 1 The heat stress and temperature-humidity index (THI) chart

Temperature		% Relative humidity																		
°F	°C	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
72	22.0	64	65	65	65	66	66	67	67	67	68	68	69	69	69	70	70	70	71	71
73	23.0	65	65	66	66	66	67	67	68	68	68	69	69	70	70	71	71	71	72	72
74	23.5	65	66	66	67	67	67	68	68	69	69	70	70	70	71	71	72	72	73	73
75	24.0	66	66	67	67	68	68	68	69	69	70	70	71	71	72	72	73	73	74	74
76	24.5	66	67	67	68	68	69	69	70	70	71	71	72	72	73	73	74	74	75	75
77	25.0	67	67	68	68	69	69	70	70	71	71	72	72	73	73	74	74	75	75	76
78	25.5	67	68	68	69	69	70	70	71	71	72	73	73	74	74	75	75	76	76	77
79	26.0	67	68	69	69	70	70	71	71	72	73	73	74	74	75	76	76	77	77	78
80	26.5	68	69	69	70	70	71	72	72	73	73	74	75	75	76	76	77	78	78	79
81	27.0	68	69	70	70	71	72	72	73	73	74	75	75	76	77	77	78	78	79	80
82	28.0	69	69	70	71	71	72	73	73	74	75	75	76	77	77	78	79	79	80	81
83	28.5	69	70	71	71	72	73	73	74	75	75	76	77	78	78	79	80	80	81	82
84	29.0	70	70	71	72	73	73	74	75	75	76	77	78	78	79	80	80	81	82	83
85	29.5	70	71	72	72	73	74	75	75	76	77	78	78	79	80	81	81	82	83	84
86	30.0	71	71	72	73	74	74	75	76	77	78	78	79	80	81	81	82	83	84	84
87	30.5	71	72	73	73	74	75	76	77	77	78	79	80	81	81	82	83	84	85	85
88	31.0	72	72	73	74	75	76	76	77	78	79	80	81	81	82	83	84	85	86	86
89	31.5	72	73	74	75	75	76	77	78	79	80	80	81	82	83	84	85	86	86	87
90	32.0	72	73	74	75	76	77	78	79	79	80	81	82	83	84	85	86	86	87	88
91	33.0	73	74	75	76	76	77	78	79	80	81	82	83	84	85	86	86	87	88	89
92	33.5	73	74	75	76	77	78	79	80	81	82	83	84	85	85	86	87	88	89	90
93	34.0	74	75	76	77	78	79	80	80	81	82	83	85	85	86	87	88	89	90	91
94	34.5	74	75	76	77	78	79	80	81	82	83	84	86	86	87	88	89	90	91	92
95	35.0	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93
96	35.5	75	76	77	78	79	80	81	82	83	85	86	87	88	89	90	91	92	93	94
97	36.0	76	77	78	79	80	81	82	83	84	85	86	87	88	89	91	92	93	94	95
98	36.5	76	77	78	80	80	82	83	83	85	86	87	88	89	90	91	92	93	94	95
99	37.0	76	78	79	80	81	82	83	84	85	87	88	89	90	91	92	93	94	95	96
100	38.0	77	78	79	81	82	83	84	85	86	87	88	90	91	92	93	94	95	96	98
101	38.5	77	79	80	81	82	83	84	86	87	88	89	90	92	93	94	95	96	98	99
102	39.0	78	79	80	82	83	84	85	86	87	89	90	91	92	94	95	96	97	98	100
103	39.5	78	79	81	82	83	84	86	87	88	89	91	92	93	94	96	97	98	99	101
104	40.0	79	80	81	83	84	85	86	88	89	90	91	93	94	95	96	98	99	100	101
105	40.5	80	80	82	83	84	86	87	88	89	91	92	93	95	96	97	99	100	101	102
106	41.0	80	81	82	84	85	87	88	89	90	91	93	94	95	97	98	99	101	102	103
107	41.5	80	81	83	84	85	87	88	89	91	92	94	95	96	98	99	100	102	103	104

Stress Threshold — Milk yield loss begins

Mild-Moderate Stress — Milk losses – 2.7 kgs per cow per day

Moderate-Severe Stress — Milk losses – 4 kgs per cow per day

Severe Stress — Extreme danger of heat stroke

Source: Collier et al., 2012, University of Arizona

Figure 1.2 Temperature-Humidity Index of Dairy Cattle

Ambient humidity is on the X axis, while temperatures in Fahrenheit and Celsius are displayed parallel to one another on the Y axis. The point at which a dairy cow begins to become heat stressed is shown in yellow and begins at 68. The stress threshold increases as the values increase, moving from the orange region, to red, to purple as severity increases. (Collier et al., 2012).

If environmental temperatures are above the UCT (25°C) and the THI is above a threshold of 68, dairy cows tend to exhibit behavioral adaptations and neurological, cardiovascular, hormonal, nutritional, reproductive, and lactational fluctuations to help them regulate their body temperature.

Behavior during Heat Stress Conditions

A dairy cow responds to heat stress by altering its behavior. In order to dissipate heat, the cow increases salivation and panting, and seeks shade. In order to reduce heat production from ruminal digestion, the cow decreases dry matter intake. Heat stress also causes cows to group

themselves around resources, such as water or shade (Polsky & Von Keyserlingk, 2017; Mader at el., 2002;); grouping, due to radiation between cattle, is suspected to increase heat loads (Wieman et al., 1992; Wellman, 1973). If heat stress persists in cattle, death is the probable outcome (Vitali et al., 2015).

Cows pant to compensate for their high ratio of inactive active sweat glands. Panting is defined as a rapid increase in respiratory rate in an effort to increase conductive, convective, and evaporative heat loss through the respiratory tract. Cows that are panting present with their mouths open and with tongues extended to increase air flow throughout the respiratory tract (Avendano-Reyes, 2012). The respiratory tract is comprised of the nose, mouth, pharynx, larynx, trachea, bronchi, and bronchioles. The bronchioles and bronchi form interconnecting branches within the moist fibrous tissues of the lungs. Due to the multitude of branches that air intake passes through, there is an increased surface area for convective and conductive cooling along the respiratory tract tissues; convective heat transfer because air is passing along the blood-dense respiratory tissues and conductive because the air, relatively cooler than the body's temperature, is inhaled and cools the blood flowing through respiratory tissues. The respiratory tract, including the bronchial tubes and bronchioles, also contains moisture along its entire surface and is in the form of mucus or saliva. The moisture allows for evaporative cooling from the respiratory tract, thus increasing heat transfer from the body and to the environment.

Increased salivation and drooling typically occur in conjunction with panting. Ptyalism, the condition of excessive saliva production or reduced saliva clearance, can be attributed to the increased anxiety that is caused by heat stress as well as by the hypothalamic stimulus to increase saliva spread for increased evaporative cooling from the respiratory tract (Hainsworth, 1967). The

cow's mouth is open to increase air flow through the respiratory tract (panting) and allows the increased saliva spread to escape the orifice, resulting in drooling.

Dry matter intake decreases during heat stress to limit the amount of heat produced by rumination and digestion. The rumen produces 7 to 8% of the daily heat production of a dairy cow fed a maintenance ration (Nutrition Reviews, 1968). As stated previously, fermentation by microbes within the rumen produces 30-70 kilocalories per 100 kilocalories of carbohydrate digested versus the monogastric digestive tract which produces 5-7 kilocalories per 100 kilocalories of carbohydrate digested (Armstrong & Blaxter, 1956). The average dairy cow's total diet (ranges from 10-30 kg of dry matter per day) is comprised of 60-70% carbohydrates, thus a dairy cow's rumen can produce a lot of heat when compared to a monogastric animal (Merck, 2019; NRC, 2001). Decreasing dry matter intake limits the amount of food being fermented in the dairy cow's rumen and thus limits the amount of heat being produced. West (2003) reported a decrease in dry matter intake (DMI) of 0.85 kg for every 1°C increase in ambient temperature that was above the thermoneutral zone of the cow. However, this decrease in DMI is detrimental to the dairy cow. The net result is decreased lactation (Kadzere et al., 2002; Tao et al., 2011), body condition score (Garnsworthy, 1988; Avendano-Reyes et al., 2009), immune function (do Amaral et al., 2010, 2011), reproductive performance (Hansen, 2009), and welfare (Kadzere et al., 2002; Polsky & Von Keyserlingk, 2017)

The THI and core body temperature are correlated with how much time a cow will stand; a THI of 68 or above was correlated with standing behavior changes in cattle. When core body temperature was above 38.8°C, cows were 50% more likely to stand than lie down (Allen et al., 2015). Standing time increased in heat stressed cattle by 1.9 hours/day (2.6 hours/day for the coolest environmental conditions measured and 4.5 for the hottest environmental conditions

measured) (Cook et al., 2007). It is assumed that standing time increases during incidents of heat stress due to increased radiating surface area, increased evaporative cooling from active sweat glands, and increased surface area exposed to air and wind for convective cooling (Silanikove, 2000; Berman, 2003; Maia et al., 2005). However, increased standing time results in decreased blood flow to the udder (subsequently decreasing lactation) (Rulquin and Caudal, 1992), increased likelihood of laminitis (Cook et al., 2004), and increased maintenance requirements when DMI is already low (West, 2003).

Cows seek shade in an attempt to decrease the amount of solar radiation onto their bodies (Quartermain et al., 1960). Respiration rates and core body temperatures decreased when cattle were in shaded conditions during hot ambient temperatures (>26.7°C) (Rhoad, 1940; Gaalaas, 1945; Seath & Miller, 1948), therefore it is assumed that cattle seek out shade to decrease the heat load within their body. Schutz et al. (2011) demonstrated that dairy cattle seemed to prefer shade over sprinklers, despite the increased heat-abatement that sprinklers provide and, according to a study by Shutz et al., (2010), are willing to compete against one another for access to shade. During hot days, Hagenmaier et al., (2016) found that DMI increased with shade provision and Fisher et al., (2008) found that milk production increased by 3 % for shaded cows versus not shaded cows; increases in DMI and lactation capabilities indicate reduced effects of heat stress on cattle in shaded environments. Cows seek shade because it minimizes radiation absorption from the sun and decreases the effects of heat stress.

Agitation and aggression are common with any form of stress on the body (Kruk et al., 2004). Although not extensively researched in dairy cattle, stressors cause a response in humans and mice that stimulates aggression, frustration, and agitation towards fellow species-members and other species (Kruk et al., 2004). Veenema and Neumann (2007) wrote “Aggression is

functional when it involves survival of the individual or species", indicating that during times of stress, when homeostasis is non-existent, animals may become more aggressive to ensure survival. This includes aggression over sources of shade and water as animals compete to access the resources that are imperative to their survival. Polsky & Von Keyserlingk (2017) reported a need for further research on the relationship between heat stress, aggression, agitation, and frustration in dairy cows.

To summarize, heat stress causes a variety of behavioral changes in dairy cattle. Decreased visitation to provided food and decreased food intake, increased agitation or aggression towards other cows, increased open-mouth panting and drooling, shade seeking, and cattle grouping, are all behavioral changes documented in heat stressed cattle. These behavioral changes are often associated with underlying physiological changes that occur due to heat stress.

Physiological and Anatomical Changes during Heat Stress Conditions

The Neurohormonal System during Heat Stress Conditions

The brainstem and the hypothalamus exert control over the autonomic nervous system, which is comprised of two distinct components: the parasympathetic and the sympathetic pathways. Both neural systems initiate and modulate involuntary responses towards the presence or absence of stimuli in order to maintain physiologic homeostasis.

The parasympathetic nervous system is often referred to as the "rest and digest" pathway. Parasympathetic tone is activated at rest, in the absence of stress, to increase activity of the digestive, urinary, renal, and glandular systems. At the same time, there is a concurrent decrease in heart and respiratory rate.

The sympathetic nervous system regulates opposing actions and is often referred to as the "fight or flight" pathway. This system is stimulated when external stressors tax the body system.

The sympathetic nervous system engages in order to provide a means by which an individual can escape from that which is inducing stress in order to restore homeostasis.

Both the parasympathetic and sympathetic nervous systems work in tandem. Consider, for instance, what transpires when sympathetic tone allows for escape from a stressor. As soon as a stressor is removed or inhibited, parasympathetic tone increases and reversion to a homeostatic state is achieved; physiological parameters will stabilize to the cow's baseline levels. The prioritized tone (sympathetic versus parasympathetic) is dependent upon the frequency of afferent nerves firing from a stressful stimulus and towards the hypothalamus for stimuli interpretation and nervous tone propagation.

Heat stress activates the sympathetic nervous system. The nervous and endocrine systems work together to amplify the stress response. In the case of heat stress, the body works as a collective unit to increase heat abatement, decrease heat generation processes, and remove itself from a hot environment. To facilitate these responses, the sympathetic nervous system relies primarily upon two hormones, epinephrine (adrenaline) and norepinephrine (noradrenaline), in addition to glucocorticoids. Adrenergic, pre-synaptic neurons release acetylcholine in response to afferent action potentials alerting the hypothalamus to increased ambient temperatures; the acetylcholine binds to nicotinic acetylcholine receptors on adjacent synapses and causes release of norepinephrine. Norepinephrine then binds to adrenergic receptors of effector neurons; this instigates *some* of the cow's physiological heat abatement techniques, more specifically those initiated by beta-1 and alpha receptors. This sequence of events occurs at varying intensities when the dairy cow is in an ambient environment with a temperature above its thermoneutral zone of 25°C ± 1°C (Roenfeldt, 1998; Bligh, 1973). Norepinephrine release propagates the following regulatory actions to minimize the amount of energy dedicated to functions that are deemed "non-

essential" in the moment, such as digestion and urination. Energy is instead harnessed and put to use for those actions that enable "escape" from the stressor: skeletal muscle contraction, vision, and energy flow. During heat stress, epinephrine is progressively secreted (Alvarez & Johnson, 1973; Johnson & Vanjonack, 1975) from the cow's adrenal medulla and produces similar effects to norepinephrine: salivary gland inhibition (Kadzere et al., 2002), increased heart and respiratory rate (Kadzere et al., 2002; Vermunt & Tranter, 2011; Yousef, 1985b), increased glycolysis (Baumgard & Rhoads, 2013; Min et al., 2017), decreased digestive activity (Nardone et al., 2010; Soriani, 2013), and increased blood pressure (Yousef, 1985b). Epinephrine functions with norepinephrine to reduce processes that generate heat and instead support processes that abate heat from the cow's body.

Epinephrine and norepinephrine are the primary hormones whose concentrates fluctuate during times of heat stress; however, there are others (table 1.1). Thyroxine, cortisol, growth hormone, antidiuretic hormone (ADH), insulin, insulin-like growth factor 1 (IGF-1), adiponectin, leptin, follicle stimulating hormone (FSH), luteinizing hormone (LH), progesterone, estradiol, prostaglandin, androstenedione, prolactin, and the renin-angiotensin-aldosterone system (RAAS) all experience changes in their basal levels in the face of heat stress. Collectively, these hormones work towards decreasing reproductive efficiency, decreasing lactation, increasing utilization of heat abatement techniques, and reducing heat-generating processes or "unessential" processes, such as urine formulation and excretion.

Table 1.1 Neurohormonal Fluctuations for Cattle in High Ambient Temperatures				
Hormone	**Role**	**Reported Change**	**Effect**	**Literature**
Thyroxine	Cellular metabolism	↓	Hypothyroidism; infertility; fatigue	*Johnson et al., 1988* *Magdub et al., 1982* *Collier et al., 1982*
Cortisol	Metabolism regulation; Reduce inflammation; Regulates blood sugar levels through gluconeogenesis;	↑	Suppressed immune system; increased blood pressure; serotonin decrease	*Kim et al., 2017* *Das et al., 2016* *Titto et al., 2017* *Abilay et al., 1975* *Silanikove, 2000*
Growth Hormone	Stimulates release of IGF-1; bone growth; lipolysis; increases metabolic rate	↓	Decreased milk production; initiate negative energy balance	*West, 2003* *Igono et al., 1988* *McGuire et al., 1991*
Antidiuretic Hormone	Fluid regulation throughout the body	↑	Sweating inhibition long-term; decreased urinary output	*El-Nouty et al., 1980* *Shafie & Badreldin, 1962*
Insulin	Metabolism of fats and glucose	↑ ; ↓	Increased: increased gluconeogenesis; reduced insulin sensitivity Decreased: Weight loss, negative energy balance, reduced milk yield	*Itoh et al., 1998* *Wheelock et al., 2010* *Baumgard & Rhoads, 2007;* *Guo et al., 2017*
Insulin-Like Growth Factor-1	Nutrient metabolism	NC ; ↓	Decreased: reduced milk production, decreased reproductive performance	*McGuire et al., 1991* *Hirayama et al., 2004* *Chaiyabutr et al., 2008* *Collier et al., 2008;* *Rhoads et al., 2010* *Wheelock et al., 2010*
Leptin	Decreases lipogenesis; increases lipolysis	↑	Negative energy balance; increased insulin resistance; cardiovascular problems	*Bernabucci et al., 2006*
Follicle Stimulating Hormone	Growth of ovarian follicles	↑	Decreased fertility; decrease reproductive efficiency	*Wolfenson, 1995* *Roth et al., 2000*
Luteinizing Hormone	Ovulation and development of corpus luteum	↓	Reproductive inefficiency; decreased fertility	*Gilad et al., 1993* *Madan & Johnson, 1973* *Wise et al., 1987* *Lee, 1993*
Progesterone	Establishes and maintains pregnancy	↑ ; ↓ ; NC	Decreased: decreased embryo viability; embryo loss	*Trout at el., 1998* *Abilay et al., 1975* *Vaught et al., 1977;* *Younas et al., 1993* *Howell et al., 1994* *Ronchi et al., 2001* *Rosenburg et al., 1977* *Jonsson et al., 1997* *Bridges et al., 2005;* *Roth at el., 2000* *Guzeloglu et al., 2001*
Prolactin	Milk production; potential fluid regulator during heat stress	↑	Potential increase of fluid in blood plasma	*Davis et al., 2016* *Alamer, 2011*
Estradiol	Maintenance of oocytes	↓	Decreased reproductive efficiency; decreased milk production;	*Bridges et al., 2005*

In summary, the nervous and endocrine systems work in conjunction to minimize heat-generating processes or processes that are not necessary for escape from the stressor. The sympathetic nervous system is activated during periods of heat stress, subsequently generating a cascade of hormones to generate effector responses, such as sweat gland stimulation. In addition to helpful hormonal fluctuations, hormonal changes occur that can negatively impact the health of the cow and her progeny. Reproductive efficiency, embryo health, and lactation production all decrease in response to hormonal circulatory changes.

The Cardiovascular System during Heat Stress Conditions

Heat stress influences adjustments in the cardiovascular system of the dairy cow. Several changes occur to mediate increasing body temperatures, including: diversion of blood flow from renal and splanchnic vascular beds and towards the peripherals, heart, and lungs, increased respiratory rate, increased heart rate, and fluctuations in blood chemistry (Rowell, 1986; Wolfenson et al., 1981; Wojtas et al., 2014; Iguchi et al., 2012; Kopecek et al., 2001; Sidhu et al., 2011; Johnson & Proppe, 1996).

Blood flow to the skin increases during heat stress. In human forearms, heat stress increased blood flow to the cutaneous tissues by 7-8 liters/minute; research has not been performed on dairy cattle to measure exact volume of blood flow to the peripherals (Detry et al., 1972; Rowell, 1974). Sidhu et al. (2011), confirmed that, in humans, blood is increasingly diverted from renal and splanchnic vascular beds and directed towards the skin and heart during periods of heat stress; the same is presumed to occur in cattle. The increase in cardiac blood flow must, anatomically, also increase blood flow through the lungs; cardiac output of the heart equals 100% of pulmonary cardiac output (Levitzky, 2013).

Blood flow during heat stress is controlled by two main nerve types: autonomic adrenergic vasoconstrictor nerves and autonomic active vasodilator nerves (Johnson & Proppe, 1996). These

nerves are activated by the thermoregulatory response from the pre-optic area of the hypothalamus and they trigger the effect of increased blood flow to the skin and lungs for heat dissipation. Blood distribution is controlled by the pre-optic area of the hypothalamus for artery and vein plexuses that run deeply and superficially within the cutaneous layers (Braverman, 1989; Conrad, 1971). Within the artery and vein plexuses are three types of minute blood vessels: true capillaries, nutritional vessels, and arteriovenous anastomoses (AVAs) (Johnson & Proppe, 1996; Braverman, 1989; Daniel & Prichard, 1956; Gemmell & Hales, 1977).

True capillaries provide exchange of nutrients to and from the cutaneous tissues. They branch off the arteriolar shunt, provide nutrients to the surrounding tissues, and return back to the vascular shunt. Nutritional vessels are found superficially within the skin, are smaller than a true capillary, and have thick walls, providing more resistance to blood flowing between vessels. Nutritional vessels dilate during times of stress to increase oxygen, energy, and heat delivery to the skin and muscles. Arteriovenous anastomoses are connections between different blood vessels, such as an artery to an artery, vein to vein, or artery to vein. The walls of AVAs and the precapillary sphincters that precede them are dilated when signaled by efferent action potentials flowing along active vasodilator nerves, thus heat stress increases blood flow through them. Arteriovenous anastomoses cannot carry nutrients to tissues, as they have no transport method; however, AVAs can conductively transfer heat to surrounding tissues.

True capillaries and nutritional vessels can be found all throughout the body, but AVAs are found only in select areas; the areas and the quantity of AVAs differ between species (Braverman, 1989; Daniel & Prichard, 1956; Johnson & Proppe, 1996). Cattle have higher quantities of AVAs in their scrotum, cheeks, ears, forehead, teats, mucous membranes, and limbs (Amakiri, 1976; Goodall, 1955; Nisbet, 1956). In sheep, AVAs lost less heat to surrounding tissues

per volume of blood when compared to heat lost from true capillaries or arterioles; however, during times of heat stress, total heat loss was equal between AVAs and true capillaries, indicating that AVAs have more blood flowing through them at a given time than do true capillaries (Johnson et al., 1986). There are large quantities of interpulmonary AVAs in mammals, allowing for increased respiratory heat loss during times of heat stress; this is specifically important when referencing cattle, due to the prioritized evaporative heat dissipation from their lungs (Lovering et al., 2014).

Heart rate increases during heat stress to meet the demand of blood flow diversion to the skin and lungs for increased heat dissipation (Johnson & Proppe, 1996). Approximately 40% of the heart rate increase is accredited to local activation, meaning that blood temperature in conjunction with heart-local thermoreceptors in the sinoatrial node directly impacts heart rate; 60% of the increase is accredited to hypothalamic stimuli (Crandall & Wilson, 2015; Wilson & Crandall, 2011). A normal heart rate for dairy cattle is between 48-84 beats per minute, but during heat stress it is common for a cow's heart rate to exceed 90 beats per minute (Merck Veterinary Manual, 2019). In humans, heart rate has been reported to increase by 30 beats per minute for every 1°C increase in internal body temperature; at this time, the ratio of increasing internal heat increment to heart beats per minute has not been measured in cattle (el-Sherif et al., 1970; Faithful et al., 1984; Koroxenidis et al., 1961; Wyss et al., 1974).

Heart rate is directly proportional to respiratory rate (RR). As heart rate increases, delivery of blood through the pulmonary artery and towards the lungs increases, driving the need for increased blood oxygenation; RR increases to deliver the required oxygen into the blood. Respiratory rate also increases to facilitate evaporative cooling from the lungs. Respiratory rate is modulated by efferent nerves from the medulla oblongata and pons centers of the brain stem. The resting respiration rate in cattle is 26-50 breaths/minute (Merck Veterinary Manual, 2019). In

cattle, there is an increase in RR of 2.8-3.3 breaths/minute for every 1℃ increase in THI's greater than 78 (Gaughan et al., 2000). This leads to RRs of 40-60 breaths per minute during temperatures of 24℃-28℃ (Hahn et al., 1997). Core body temperature is also correlated with RRs. Gaughan et al. (2000) found that, with a core body temperature of 40.1℃, cattle had a RR of 133 breaths/minute and, at 40.7℃, had a RR of 200 breaths/minute; a 0.6℃ increase in core body temperature instigated a 67 breaths/minute increase in RR; however, Spiers et al. (1994), and Gaughan et al. (1999), all concluded that while RR rose exponentially during initial signs of heat stress (fast and shallow breathing), there is a RR threshold. Meeting the threshold causes mammals to revert towards deeper and slower breaths after prolonged heat stress rather than the initial quick pants. Gaughan et al. (2000), concluded that this breathing reversion was due to a metabolic shift that attempts to mediate the increasing heart rate by inducing a deeper, open mouth breath versus rapid panting. The predicted impact of ambient temperature on respiratory rate depends on age, sex, lactation status, genotype, phenotype, feeding time, and body condition score (Gaughan et al., 2000).

Blood composition changes due to increased respirations per minute, as well as due to hormone restriction/release during heat stress. As ambient temperature, THI, and body temperature increase, there is a decrease in circulating carbon dioxide (CO_2), glucose, and electrolytes, as well as fluctuations in amino acids, immune cells, and circulating hormones (Kim et al., 2018; Guo et al., 2017). There are increases in circulating heat shock proteins (HSPs), non-esterified fatty acids (NEFAs), bicarbonate ions, and hydrogen ions (Kim et al., 2018; Guo et al., 2018; Ribeiro et al., 2018).

Decreases in circulating CO_2 can be attributed to increased respiratory rate (McDowell, 1972). Increasing respiratory rates increase the speed at which carbon dioxide leaves the blood

(McDowell, 1972). Through alveolar exchange between the blood, pulmonary tissues, and air, breathing releases carbon dioxide from the respiratory tract and into the environment. Loss of CO2 resulted in respiratory alkalosis in cattle (Hales, 1967); to combat this, bicarbonate ions and hydrogen ions are released into the blood via the kidneys and intracellular mechanisms. If respiratory alkalosis is not mediated due to low levels of bicarbonate or hydrogen ions, the decreased partial pressure of CO2 reduces blood perfusion to the brain and can lead to altered neurologic function, tremors, and tetany (Mitchell et al., 1972).

Electrolytes decrease in the blood due to increased sweating. Despite the sparsity of active sweat glands, dairy cattle still sweat and lose electrolytes. Sweating rate varies between cattle breeds (Gebremedhin et al., 2008). For example, Holstein cattle sweat, on average, 213 g/m^2-h, while Jersey cattle sweat 642 g/m^2-h (Gebremedhin et al., 2008). Cattle have mostly apocrine sweat glands (Findlay & yang, 1950; Dowling, 1955). Apocrine sweat glands are controlled by adrenergic nerves and are activated during sympathetic nervous responses (Hodge & Brodell, 2018). Apocrine glands contract during stress, pushing oily sweat from the glomerulus of the gland, into the apocrine excretory duct, and ultimately into the piliary canal of a corresponding hair follicle; the sweat rises in the piliary canal and spills onto the surface of the skin from the hair follicle (Hu et al., 2018). Apocrine sweat is composed of proteins, lipids, steroids, and electrolytes (Hu et al., 2018; Semkova et al., 2015). In contrast to the sodium ion release during human sweating, electrolytes in cattle sweat are primarily potassium ions; there are at least 4-5 times more potassium ions than sodium ions in cattle sweat (Johnson, 1970). When potassium was decreased in dairy cattle, as it would be during sweating, there was a linear decrease in milk yield and DMI, as well as an increased incidence of hypokalemia (West et al., 1992; Merck Veterinary Manual, 2019). Hypokalemia can cause muscle weakness, muscle fasciculations, lethargy, and death; the

effect of hypokalemia is compounded by a decreased DMI, thus decreased potassium intake, during times of heat stress (Merck Veterinary Manual, 2019).

Activation of heat shock genes causes increased production and dissemination of HSPs towards misfolded proteins within the cytoplasm. Heat shock proteins intake the non-functional (misfolded or denatured) proteins (NFPs) and bind them to a hydrophobic strip within the HSP cavity. Adenosine triphosphate (ATP) then binds to the ATP binding site in the HSP molecule thus causing a chaperonin "lid" on the top of the HSP to close and trap the NFP. With the NFP trapped, hydrophobic molecules rearrange the NFP back to its correct formation. Adenosine triphosphate binds to the ATP binding site and allows the chaperonin "lid" to open, releasing the correctly folded protein into the surrounding cytosol. This process helps to mitigate the damage of elevated temperatures on the tissues of the body. Heat shock proteins (HSPs), specifically HSP70 and HSP90, increase in blood circulation as a result of heat stress, with HSP70 taking the predominant role in reducing the effects of high temperature on protein degradation within livestock (Archana et al., 2017; Deb et al., 2014).

Literature varies regarding non-esterified fatty acids (NEFAs) and their levels within the blood during heat stress conditions; some literature suggests that NEFAs increase within the blood, while others suggest that levels are stagnant or decreasing (Wheelock et al., 2010). The majority of literature suggests an increase in circulating NEFAs due to the negative energy balance induced by heat stress (O'Brien et al., 2010; Bernabucci et al., 2010; Lamp et al., 2015; Shehab-El-Deen et al., 2010; Grummer, 1993). The negative energy balance results in catabolism of triglycerides into glycerol and NEFAs and thus ensues circulation of fatty acids within the blood. NEFAs, in addition to the increase triggered by heat stress, are already circulating in high amounts due to the negative balance created by milk production, thus heat stress worsens an already dangerous

metabolic process. Due to their inflammatory properties, circulating NEFAs (greater than 0.2 mM) can cause Fatty Liver Disease, ketosis, and decreased insulin secretion as well as increased incidence of milk fever, placental retention, abomasum displacement, metritis, and mastitis (Drackley, 1999). While circulating NEFAs are acceptable in small amounts, anything greater than 0.2mM can be toxic to the cow and cause detrimental implications.

In conclusion, respiratory and heart rate as well as blood flow, pressure, and composition all change in response to heat stress. Respiratory rate, as well as heart rate, increase to meet the demand for increased blood flow and blood pressure throughout the body. Blood flow decreases from the splanchnic bed and towards the heart, lungs, and peripheral tissues for heat abatement. Blood pressure increases to deliver blood to the peripherals quicker and with enough force to cause portals, such as arteriovenous anastomoses, to dilate, allowing for further increasing of blood flow to the dermal layers. Blood composition fluctuates to meet the demands of the body during periods of heat stress: electrolytes decrease in the blood due to sweating and decreased DMI. NEFA's increase in circulation due to the negative energy balance driven by increased energy usage and decreased DMI. Heat shock proteins increase in the blood as they are the main constituents repairing heat-damaged or misfolded proteins. Decreased amounts of CO2 circulation occur due to the rapid breathing response towards heat stress and can cause acidosis of the blood; this results in respiratory alkalosis in cattle. Overall, the cardiovascular system is greatly affected by heat stress, with modifications occurring that can prove fatal if not remedied.

The Immune System during Heat Stress Conditions

Heat stress impacts both the humoral and the adaptive immune responses by altering function of toll-like receptors, cytokines, and inflammatory processes. Heat stress causes immunodeficiency in both cows and their calves (Lacetera et al., 2005; Lacetera et al; 2006; Do Amaral et al., 2009; Elvinger et al., 1991; Kamwanja et al., 1994; Do Amaral et al., 2010; Do

Amaral et al., 2011; Tao et al., 2012a; Laporta et al., 2017; Nardone et al., 1997; Tao et al., 2019). The driving forces behind decreased immune function during heat stress conditions are: increased inflammatory processes due to the stress reaction, apoptosis of intestinal barriers, decreased DMI, increased exposure to and better growth conditions for bacteria, and behavioral changes.

Toll-like receptors (TLRs) are proteins embedded into the surface of the innate immune system's sentinel cells. Toll-like receptors recognize microbial structures and initiate activation of the sentinel cells within the body, subsequently increasing cytokine production and inflammation. Heat stress has been linked with activation of TLRs in cattle and swine, specifically TLR2 and TLR4 (Ju et al., 2014; Bharati et al., 2017). TLR2 and TLR 4 are both are associated with recognition of lipopolysaccharides (LPS) and endogenous materials in the blood and are positively correlated with increasing humoral cytokine production.

Lipopolysaccharide, a pro-inflammatory endotoxin, increases in circulation during heat stress due to apoptosis of intestinal cells (also known as "leaky gut" syndrome) (Hall et al., 1999; Wernstedt Asterholm et al., 2014; Bradford et al., 2015). By increasing upregulation of TLRs through receptor activation, increases of LPS in the blood stimulate pro-inflammatory cytokine production of TNFα, interleukin-1α, interleukin-6, and IFN-γ (Carroll et al., 2009; DuBose et al., 2002).

TNFα induces cell proliferation, survival, differentiation, and apoptotic factors (Parameswaran & Patial, 2010) and causes apoptosis of bovine hepatocytes and mammary cells during instances of heat stress (Lmao et al., 2006). In some publications, TNFα decreased in circulation (Do Amaral, 2010; Do Amaral, 2011); it is unknown why this occurred but may be attributed to stage of lactation. Interleukin-1α increases during heat stress and is responsible for raising body temperature through inflammation and fever (Carroll et al., 2009); this initiates a

positive feedback loop of increasing body temperature. Interluekin-6 is a pleiotropic cytokine and increases during heat stress, subsequently inducing a cascade of negative health effects (Tanaka et al., 2014): fibrosis (Duncan & Berman, 1991), joint swelling and edema (Nakahara et al., 2003), osteoporosis (Kotake et al., 1996), autoimmunity (Kimura and Kishimoto, 2010), chronic inflammation (Kimura and Kishimoto, 2010; Kishimoto, 1989), thrombocytosis (Ishibashi et al., 1989), anemia (Nemeth et al., 2004), and amyloid A amyloidosis (Gillmore et al., 2001). Interferon-γ (IFNγ) increases during heat stress and is attributed as a primary activator of macrophages and is associated with increased incidence of autoimmune diseases (Baccala et al., 2005).

When immune cells function appropriately during times of infection by foreign substances, they often function successfully. However, long-term exposure to high heat loads and the subsequent long-term production and presence of cytokines can cause detrimental health effects on cattle.

Although not a comprehensive list, the following are the major diseases of concern in regards to heat stress: mastitis (Das et al., 2016), fatty liver disease (Skibiel et al., 2018), laminitis (Shearer, 2005; Cook et al., 2007; Cook et al., 2004b), displaced abomasum (Vermunt & Tranter, 2011), acidosis (Das et al., 2016), and ketosis (Gantner et al., 2016).

Mastitis occurs when mammary tissues become inflamed. Behavioral changes in heat stressed cattle can contribute to foreign body infection through the mammary canals, leading to activation of inflammatory properties. For instance, if a cow attempts to cool herself by lying on a feces-soaked concrete ground, she may introduce fecal bacteria into her teat canals (Vermunt & Tranter, 2011). Warm environments also increase environmental pathogen load, increasing the likelihood of contaminating udders during warm ambient temperatures (Godden et al., 2003). As

systemic inflammatory cytokines are increased in circulation during heat stress, mammary tissue can also become inflamed despite avoidance with infective pathogens (Igono et al., 1988; Giesecke, 1985; Wohlgemuth et al., 2016); incidence of mastitis increases during heat stress conditions (Pragna et al., 2017; Hammami et al., 2015; Jingar et al., 2014). Mastitis results in decreased milk production in cattle (Seegers et al., 2003; Hortet & Seegers, 1998; Hoblet et al., 1991; Houben et al., 1993; Firat, 1996; Deluyker et al., 1991; Lescourret & Coulon, 1994; Luquet et al., 1992; Myllys & Rautala, 1995; Ostergaard & Grohn, 1999; Rajala-Schultz & Grohn, 1999; Wilson & Sears, 1992; Wolf & Jahnke, 1990) and changes in the milk composition of cattle, goats, and sheep (Seegers et al., 2003; Leitner et al., 2004; Bruckmaier et al., 2005; Paixao et al., 2017; Leitner et al., 2004).

Laminitis is inflammation of the laminar tissues of cattle hooves. As cytokines increase in circulation during heat stress, inflammation is more prevalent in tissues such as the laminae of the hoof (Shearer, 2005; Shearer, 2011; Gressley, 2014; Katz and Bailey, 2012; Bergsten, 2003). Due to inflammation induced by LPS circulation and cytokine production, vascular changes, cellular apoptosis, oxidative damage, and basement membrane degradation all occur (Katz and Bailey, 2012; Herdt, 1991). In addition, behavioral changes during heat stress, such as increased standing time and thus uneven pressure on the hoof areas (Van Der Tol et al., 2002; Cook et al., 2007; Allen et al., 2015; Alam et al., 2011), contributes to the incidence of laminitis in heat stressed dairy cattle (Bergsten, 2003; Leonard et al., 1994). Nutrition also plays a large role in the incidence of laminitis in heat stressed dairy cattle; heat stress-induced acidosis of the rumen increases the likelihood of white line disorder and laminitis in dairy cattle (Nocek, 1997; Shearer, 2005; Gressley, 2014; Moser & Divers, 1987; Abdela, 2016). Results of laminitis include incurred medical costs (Ronk, 2016; Ozsvari, 2017), reduced fertility (Ronk, 2016; Weaver, 1985; Lucey et al., 1986a; Collick

et al., 1989; Sprecher et al., 1997; Barkema et al., 1994), cattle culling (Ronk, 2016; Booth et al., 2004; Amory et al., 2008; Randall et al., 2016), and decreased milk production (Ronk, 2016; Archer et al, 2010; Bicalho et al., 2008; Moser & Divers, 1987; Booth et al., 2004; Amory et al., 2008; Randall et al., 2016).

High temperatures have been associated with increased incidence of acidosis in dairy cattle (Mishra et al., 1970; Bandaranayaka & Holmes, 1976; Niles et al., 1998). Heat stress causes acidosis of the rumen by decreasing saliva ingestion, decreasing dry matter intake, and predisposing cattle to a preference for high-energy concentrates over fiber and forage (Shearer, 2005). Saliva contains bicarbonate. During temperate conditions, saliva and its bicarbonate components are ingested, subsequently flowing into the rumen and balancing the low pH of fermented feedstuffs. During heat stress, drooling increases, increasing the amount of saliva that escapes the mouth and decreasing the amount of saliva and bicarbonate that is ingested into the rumen. In dairy cattle, an estimated 108-308 liters of saliva flows between the mouth and rumen per day (Erdman, 1988). Salivary flow provides 1134-3234 grams of sodium bicarbonate per day for the dairy cattle rumen (Shearer, 2005). During heat stress the salivary flow is expected to decrease due to drooling and dehydration (Shearer, 2005; Das et al., 2016) subsequently decreasing total buffering capacity and the pH of the rumen; this combination instigates rumen acidosis (ruminal pH<6 [Merck Veterinary Manual, 2019]) (Dale et al., 1954). Decreased dry matter intake and a preference for high-energy concentrates exacerbates the decreased ruminal pH and further advances the severity of acidosis in suffering cattle (Shearer, 2005). Results of acidosis include depressed feed intake, decreased milk production, liver abscesses, fluctuations in milk composition, laminitis, rumenitis, erosion and ulceration of the luminal epithelium, sepsis, and, in

severe cases, death (Merck Veterinary Manual, 2019; Krause & Otzel, 2006; Plaizier et al., 2008; Radostits et al., 2007; Abdela, 2016).

It is hypothesized that ketosis (acetonaemia) occurs during heat stress due to the decreased dry matter intake. Ketosis is the process by which fat is mobilized during times of blood glucose deficits. Since feed intake decreases, glucose in the blood decreases and causes increased lipomobilization. A standard test for ketosis is the levels of blood β-hydroxybutyric acid (BHBA); ketosis is observed with associated BHBA values ≥14.4 mg/dl. Lipomobilization and high ketone values within the circulatory system are associated with reduced milk production (Duffield, 1997), weight loss, and displaced abomasum (Oeztel, 2007). These results are speculated to occur due to the increased ketone bodies within the blood, but the exact pathogenesis is unknown (Merck Veterinary Manual, 2019). This author speculates that the decreased glucose observed during heat stress, rather than merely the presence of ketone bodies, accounts for the adaptive changes seen in ketotic cattle.

Displaced abomasums are also common among heat stressed cattle (Garcia, 2006; Vermunt & Tranter, 2011). A displaced abomasum is when the abomasum, a digestive compartment in a ruminant, fills with gas and, instead of lying on the bottom of the abdominal cavity, rises to the top, thus becoming “displaced” from its normal position. Ketosis incidence is often tied with incidence of displaced abomasums; both occur from lack of nutrients/digestion and, during heat stress, lack of feed intake. Lack of feed intake decreases contractions of the abomasum and causes air to remain stationary in the compartment. The results of a displaced abomasum are decreases in milk yield (Deluyker et al., 1991; Detilleaux et al., 1997; Constable, 2019), abomasal ulcers (Palmer & Whitlock, 1984; Cable et al., 1998; Constable, 2019), and peritonitis (Constable, 2019; Wittek, 2019).

While there are many detrimental effects on the health and immune system of the heat stressed cow, there are also indirect health effects on calves in utero and on nursing calves. Heat stress during late gestation causes uterine ischemia resulting in an under-developed placenta (Tao et al., 2011; Alexander & Williams, 1971); this reduces the quantity of oxygen and nutrients that are supplied to the calf in utero. A direct effect of an under-developed and dysfunctional placenta is growth retardation and immune dysfunction of the calf after parturition (Oakes et al., 1976; Collier et al., 1982; Tao et al., 2012a; do Amaral et al., 2011; Monteiro et al., 2014). In utero, calves have decreased levels of circulating insulin, glucose, and IGF-1 (Tao et al., 2014; Guo et al., 2016), while also having increased concentrations of catecholamine, epinephrine, and norepinephrine (Limesand et al., 2005; Limesand et al., 2006; Thorn et al., 2009; Tao et al., 2013). Maternal immunoglobulin levels in colostrum are altered, providing decreased passive immunity for calves after birth (Monteiro et al., 2014; Nardone at al. 1997; Stott et al., 1976; Donovan et al., 1986); calves will have impaired immune function for the remainder of their lives due to the lack of immune transfer from their mother (Tao et al., 2012). Overall, calf growth is impaired both in utero and postnatally and immune function is compromised for the remainder of the calf's life.

Health status of heat stressed cattle and their calves is altered during and after periods of heat stress. Due to heat stress, prevalence of disease is increased, milk production and reproductive performance decreased, and calf immunity compromised.

The Digestive System during Heat Stress Conditions

Heat stress has a variety of effects on the digestive system of dairy cattle. There is an increased cost of maintenance for body functions (Collier et al., 2006) such as increased respiration and cooling efforts, driving the need for greater-than-average nutrition; however, despite the increased need for more intake, dry matter intake (DMI) actually decreases during heat stress (Johnson et al., 1963). The decrease in DMI results in decreased body condition score (Cummings

and Foster, 2003; Roche et al., 2008), muscle mass (Bauman & Currie, 1980), fat mass (Bauman & Currie, 1980), ruminal pH (Beckett et al., 2016), nutrient uptake by the portal vein (McGuire et al., 1989), milk production (Hristov et al., 2000; Martin & Sauvant, 2002; West, 2003; Rhoads et al., 2009; McDowell, 1972; Moallem et al., 2010; Aganga et al., 1990; Soriani et al., 2013; Fuquay, 1981; Beede and Collier, 1986), placental nutrient delivery (Regnault et al., 2003; Morrison, 2008), and nutrient passage rate (Warren et al. 1974; Faichney & Barry 1986; Silanikove 1992; Bernabucci et al. 2009). Reductions in DMI also result in increased circulation and hepatic uptake of non-esterified fatty acids (NEFAs) (Bauman & Currie, 1980; Bell, 1995; Bauman, 2000; Bauchart, 1993), fatty liver disease (FLD) (Drackley, 1999; Schäff et al., 2012; Kuhla et al., 2009), incidence of ruminal acidosis (Dale et al., 1954; Shearer, 2005; Kadzere et al., 2002; Sanchez et al. 1994; Conte et al., 2018), and ketosis (Duffield, 2000; Gillund et al., 2001).

To reduce body heat, body function maintenance increases (Collier et al., 2006). Maintenance increases to increase sweating, heart rate, and respiratory rate. However, despite the increase in nutrient requirements for maintenance, DMI decreases during periods of heat stress. Dry mater intake decreases due to heat stress (Spiers et al., 2004; Moallem et al., 2010), with West (2003) finding a decrease in DMI of 0.85 kg per degree (°C) increase. A reduction in ruminal heat production, and thus overall body heat production, is the physiological goal for decreasing DMI; however, decreases in DMI create additional health problems. Though DMI depression occurs during heat stress, there is an increased digestibility of nutrients within the rumen (Warren et al., 1974; Mulligan et al., 2001) and an increase in ghrelin, the compound that causes appetite stimulation and hunger (Pearce et al., 2014; Polsky & Von Keyserlingk, 2017); however, as blood is re-directed to peripheral locations, portal vein nutrient uptake decreases, ultimately causing deficient uptake of any nutrients the cow does happen to ingest (McGuire et al., 1989). Body

condition score decreases due to the muscle and fat mass loss (Bauman & Currie, 1980) that occurs during DMI reduction. Intake reduction impairs successful mammary development and the amount of nutrients delivered to mammary tissues for milk synthesis, decreasing the amount of milk produced (Lough et al., 1990; Tao et al., 2011; Collier et al., 1982b; West, 2003; Rhoads et al., 2009; McDowell, 1972; Moallem et al., 2010; Aganga et al., 1990; Soriani et al., 2013; Fuquay, 1981; Beede and Collier, 1986). In addition to the reduction of nutrients to the mammary gland, direction of nutrients decreases to the placenta and uterus as well (Dreiling et al., 1991; Reynolds et al., 2006; Bell & Ehrhardt, 2002; Limesand et al., 2004; Yates et al., 2011). The lack of nutrients flowing to these reproductive tissues decreases fetus growth (Collier et al., 1982b; Wolfenson et al., 1988; Avendano-Reyes et al., 2006; Adin et al., 2009; do Amaral et al., 2009; do Amaral et al., 2011; Tao et al., 2011; Tao et al., 2012b; Bell et al., 1989), fetus and calf health (Merlot et al., 2008; Reynolds et al., 2010; Machado-Neto et al., 1987; Tao et al., 2012a; Nardone et al., 1997; Hough et al., 1990; Yasuda et al., 2006), calf weight (Tudor, 1972), placental weight (Collier et al., 1982b; Bell et al., 1989), and abortion (Wu et al., 2006).

Ruminal pH also decreases during heat stress as a direct effect of decreased DMI. During prehensile and mastication processes, saliva is generated in the mouth and subsequently swallowed. Cattle saliva has a high buffering capacity and balances the acids produced by the ruminal bacteria. In dairy cattle, an estimated 108-308 liters of saliva flows between the mouth and rumen per day (Erdman, 1988). Salivary flow provides 1134-3234 grams of sodium bicarbonate per day for the dairy cattle rumen (Shearer, 2005). During heat stress, salivary flow is expected to decrease due to drooling and dehydration (Shearer, 2005; Das et al., 2016) subsequently decreasing total buffering capacity and the pH of the rumen; the combination of buffer deficiency and low pH instigates rumen acidosis (ruminal pH<6 [Merck Veterinary Manual,

2019]) (Dale et al., 1954). Decreased dry matter intake (less fill within the rumen) and a preference for high-energy concentrates exacerbates the decreased ruminal pH and further advances the severity of acidosis in heat stressed cattle (Shearer, 2005).

Ketosis is a result of an imbalance in energy demand, excessive adipose tissue mobilization, and increased ketone body production in the hepatic tissue. Heat stress and the resultant decrease in DMI can lead to ketosis if the energy requirement to intake ratio substantially increases (Rajala-Schultz et al., 1999b; Dohoo & Martin, 1984; Ganter et al., 2016). Incidence of ketosis is linked to incidence of fatty liver disease, which is further linked to heat stress due to increased circulation and hepatic uptake of NEFAs (Bauman & Currie, 1980; Bell, 1995; Bauman, 2000; Bauchart, 1993; Kuhla et al., 2009; Drackley et al., 2005; Schäff et al., 2012). Mobilization of NEFAs occur during heat stress and DMI deficiency due to breakdown of NEFAs from adipose tissue; lipid breakdown is initiated during a negative energy balance to provide energy substrates for tissue use. The NEFAs increase through circulation, eventually coming to the liver for re-direction or lipolysis for gluconeogenesis; however, during heat stress, blood flow through the portal vein of the liver decreases (McGuire et al., 1989), causing fatty acids to stagnate in the liver and result in fatty liver disease (Herdt, 1988).

Overall, heat stress results in a decrease in DMI that negatively impacts cow health and longevity, calf and fetus health, and milk production. The decrease in DMI is often considered one of the main and most dangerous implications of heat stress due to the severity and incidence of health problems that occur in both cow and calf. While treatments have been investigated on their ability to increase DMI during heat stress, at the time of this thesis, the treatments have not proven both significant and efficient (Fuquay, 1981; Collier et al., 1982a; Beede & Collier, 1986; Huber

et al., 1994; Sanchez et al., 1994; West, 1994; West, 1998; Grummer, 1990; Morrison, 1983, Conte et al., 2018; Robinson, 2013; Collier & Beede, 1985).

Lactation during Heat Stress Conditions

Heat stress induces changes in the lactation of dairy cattle, as well as in the composition of milk obtained during heat stress conditions (Reyad et al., 2016; Pragna et al., 2017; Polsky & Von Keyserlingk, 2017). Lactating dairy cattle are more affected by heat stress than their non-lactating counterparts; this is due to an increased metabolic rate associated with milk production, as well as the high amount of heat generated by the udder (heat loss equivalent to 388 $J/s \cdot m^2$) (Purwanto et al., 1990; Gebremedhin & Wu, 2016). In addition, higher milk-producing dairy cattle, such as the Holstein breed, have a greater predisposition towards heat stress than lower milk-producing cattle (Spiers et al., 2004). During heat stress, dairy cattle DMI decreases, resulting in less digested nutrients for milk synthesis within the udder; this decreases the amount of milk produced (West, 2003; Rhoads et al., 2009; McDowell, 1972; Moallem et al., 2010; Aganga et al., 1990; Soriani et al., 2013; Fuquay, 1981; Beede and Collier, 1986). Collier et al., (2008) also found a connection between heat stress and the upregulation of protein chaperone genes, as well as interference with cell transport in the mammary epithelial cells, resulting in disruption of lactation processes. During late gestation, heat stress impairs mammary development, further decreasing the amount of milk produced (Tao et al., 2011; Collier et al., 1982b). Heat stress during the dry period has been linked to decreased milk production in the subsequent lactation (Tao & Dahl, 2013; Wolfenson et al., 1988; do Amaral et al., 2009). Heat stress-induced increases in udder temperature have also resulted in increased incidences of mastitis, further reducing profitability due to cattle health concerns and degraded milk quality (Igono et al., 1988).

Ingraham (1979) reported that, for every increase in THI, milk production decrease by 0.32 kg. West (2003), found that milk yield can decrease by 600 to 900 kg per dairy cow per lactation, without the effects of mastitis. With current prices (April, 2019) at $0.31 per kg of fluid milk, the cost of heat stress on milk yield can, at a 600 kg loss per lactation, amount to $186 per cow per lactation (AMS-USDA, 2019). Cows lactate up to once per year, with the average dairy farm housing 120 cows: this amounts to over $20,000 lost on average per farm per year (ERS-USDA, 2006). Other reports have demonstrated the cost of milk yield loss due to heat stress to total $39,000 on average per year per farm (Key et al., 2014).

Milk composition also changes in response to heat stress. Reyad et al., (2016) found that total solids, solids-not-fat, fat, protein, lactose, and ash decreased during incidences of heat stress (Reyad et al., 2016). Bandaranayaka & Holmes, (1976) and Richardson et al., (1961) found that, during heat stress, short-chain fatty acids (SCFA) and medium-chain fatty acids (MCFA) were decreased while long-chain fatty acids (LCFA) increased in milk. Immunogloblins G and A also decrease in milk content during heat stress (Nardone et al., 1997), decreasing the effects of transferrable immunity to calves. Protein decreases can be attributed to decreased DMI and thus decreased nutrient supply for milk synthesis (Cowley et al., 2015). Smith et al., (1983) hypothesized that fat synthesis or a predisposition towards LCFA circulation within the udder was changed due to lipomobilization induced by heat stress; at this point in time, the exact mechanism of fat distribution fluctuations within the udder is unknown (Hammami et al., 2015). Heat stress decreases the quality of milk, negatively impacting calf health and nutrition, as well as decreasing the amount of milk viable for consumers.

To summarize, heat stress negatively impacts cattle lactation by decreasing cattle milk quality and the amount of milk produced. Mammary cell proliferation is decreased, subsequently

impairing mammary development during late gestation; this decreases the amount of milk produced. Nutrient delivery to the mammary chain is also decreased during heat stress, as DMI decreases and any circulating glucose is delivered to more imperative physiological processes that lactation. The decrease in milk production negatively impacts dairy farms by decreasing profits and calf health through a reduction in quantity and quality of milk produced.

The Reproductive System during Heat Stress Conditions

Effects of heat stress on the developing reproductive system and fetus are detrimental. Heat stress decreases fertility through reductions in DMI, LH, progesterone, and estradiol (Johnson et al., 1963; Wise et al., 1988; Rosenburg et al., 1977; Stott & Williams, 1962; Christison & Johnson, 1972; Folman et al., 1983; Gwazdauskas et al., 1981). Dominance degree for the dominant follicle (Badinga et al., 1993; Honig et al., 2016; Wolfenson et al., 1995), oocyte and embryo quality (Alfujairi et al., 1993; Gordon et al., 1987; Hansen, 1997; Monty & Racowsky, 1987; Rutledge et al., 1999; Hansen et al., 2000; Roth, 2008; Al-Karanani et al., 2002), ovarian activity (Alves et al., 2014; Wilson et al., 1998), conception rates (Lavon & Ezra, 2011; Wolfenson & Roth, 2019; Schuller et al., 2014; Hahn et al., 2003; Morton et al., 2007; Garcia-Ispierto et al., 2007; Nabenishi et al., 2011; Dash et al., 2016), pregnancy rates (Putney et al., 1989; Dash et al., 2016; Khan et al., 2013; El-Tarabany & El-Bayoumi, 2015; Oseni et al., 2005; Amundson et al., 2006), uterine and umbilical blood flood (Reynolds et al., 1985; Bell et al., 1987; Thureen et al., 1992; Regnault et al., 2007; Limesand et al., 2018; Schroder & Power, 1997; Reynolds et al., 2006; Yates et al., 2011; Tao & Dahl, 2013; Dreiling et al., 1991), oxygen delivery to the fetus (Schroder & Power, 1997; Regnault et al., 2003), and fetal growth (Wolfenson et al., 1988a; Tao et al., 2012; Collier et al., 1982b; Skibiel et al., 2018; Bell et al., 1989; Wu et al., 2006; Tudor, 1972; Tao & Dahl, 2013) are all decreased as a result of heat stress.

A reduction in dry matter intake, and decreases in luteinizing hormone, progesterone, and estradiol secretion all contribute to the decrease in reproductive efficiency of cattle during heat stress inducing conditions. Feed intake impacts a wide variety of physiological functions in dairy cattle, including reproduction, and is decreased during heat stress (Johnson et al., 1963). The decrease in dry matter intake affects all reproductive stages in dairy cattle (Krishnan et al., 2016). Jonsson et al. (1997) reported that a negative energy balance prolongs the postpartum period, as well as inhibits fertility in dairy cows. The increases in insulin and glucose from a negative energy balance are correlated with delayed ovulation (Nmez et al., 2005) which is subsequently correlated with a longer calving interval and reductions in birth weight (Savasani et al., 2015; Johnson et al., 1963). Decreased feed intake affects the amount of digested nutrients available for the uterus and umbilical cord; this is further exacerbated by blood flow being re-routed from the uterus to peripheral tissues during heat stress conditions (Reynolds et al., 1985; Bell et al., 1987; Thureen et al., 1992; Regnault et al., 2007; Limesand et al., 2018; Schroder & Power, 1997; Reynolds et al., 2006; Yates et al., 2011; Tao & Dahl, 2013; Dreiling et al., 1991). In addition to the decrease in nutrients delivered to the uterus and umbilical cord, oxygen delivery to the fetus is also impaired (Schroder & Power, 1997; Regnault et al., 2003). With both nutrient and oxygen restriction occurring at a result of heat stress and re-routed blood flow, the growth rate of the fetus and subsequent calf birth weight are decreased (Wolfenson et al., 1988a; Tao et al., 2012; Collier et al., 1982b; Skibiel et al., 2018; Bell et al., 1989; Wu et al., 2006; Tudor, 1972; Tao & Dahl, 2013).

Luteinizing hormone (LH) functions to trigger ovulation and corpus lutea development; LH is required for conception and increases estradiol secretion from the maturing follicle. Luteinizing hormone decreases during heat stress, delaying or prohibiting ovulation of the egg from the ovary (Wise et al., 1988; Wolfenson et al., 1993). Luteinizing hormone release during

estrous was reduced after 5 days in a hot environment from 5.6 pulses per 8 hours in cooled cattle versus 3.1 pulses per 8 hours in heat stress cattle (Wise et al., 1988). Progesterone, the hormone responsible for pregnancy maintenance, is also decreased during heat stress (Wise et al., 1988; Rosenburg et al., 1977; Stott & Williams, 1962), increasing the likelihood of embryo and fetus abortion. Estradiol prepares the uterus for pregnancy after ovulation and assists in maintaining pregnancy. Estradiol is secreted by the corpus lutea, which is developed by LH surges throughout estrus; therefore any fluctuations in LH are going to have an indirect effect of estradiol secretion. Estradiol, in conjunction with the decrease in LH, decreases during heat stress (Wolfenson et al., 1997; Wolfenson et al., 1995; Wilson et al., 1998). Wilson et al. (1998) reported that at day 21 of estrus, the serum estradiol of heat stressed cattle was lower (approximately 3.5 pg/ml; $P<0.001$) than cattle treated with a thermoneutral environment. Due to the decreases in pregnancy hormones, ovarian activity is decreased and follicle degree of dominance is altered (Alves et al., 2014; Wilson et al., 1998; Badinga et al., 1993; Honig et al., 2016; Wolfenson et al., 1995). The dominance and size of subsequent dominant follicles is presumably altered due to LH and estradiol fluctuations (Wilson et al., 1998; Wolfenson et al., 1995); size of dominant follicles decreases in heat stressed cattle and ultimately reflects whether or not the follicle contains a mature egg for conception or not. Number of viable follicles for dominance selection is also decreased during heat stress (Wilson et al., 1998; Wolfenson et al., 1995), ultimately impacting availability of follicles for dominance and successive maturity.

Not only are follicles impacted, but oocyte and subsequent fertilized embryo quality are also negatively affected (Alfujairi et al., 1993; Gordon et al., 1987; Hansen, 1997; Monty & Racowsky, 1987; Rutledge et al., 1999; Hansen et al., 2000; Roth, 2008; Al-Karanani et al., 2002). Oocyte development does not commonly progress to the blastocyst stage in heat stressed cattle

(Al-Katanani et al., 2002) due to reduced follicular development and lower steroid concentrations in fluid within the follicle (de S-Torress Junior et al., 2008; Gendelman et al., 2010; Al-Katanani et al., 2002). In vitro experimentation documented a decrease in oocyte cleavage rate (and thus blastocyst formation) of 30 to 65% (Schrock et al., 2007; Edwards et al., 2005; Lawrence et al., 2004). When blastocysts *are* formed during heat stress conditions, they are often of lower quality (Chambers & Tomlinson, 2009) and this subpar quality is typically maintained through all phases of conception. As such, the embryo is often compromised as well, but is only significantly compromised until day 3 of pregnancy; most of the deleterious effects on the embryo occurs during day 1 of pregnancy with subsequent resistance to maternal heat stress occurring after day 3 (Ealy et al., 1993).

Due to hormone and follicle fluctuation, as well as degraded oocyte quality, conception rates are lower in heat stressed cattle. Conception rate (CR) refers to the number of cows that conceive from insemination service. Conception rate significantly decreases during heat stress conditions (Lavon & Ezra, 2011; Wolfenson & Roth, 2019; Schuller et al., 2014; Hahn et al., 2003; Morton et al., 2007; Garcia-Ispierto et al., 2007; Nabenishi et al., 2011; Dash et al., 2016). In Schuller et al. (2014), the CR during heat stress conditions decreased from 31% to 12% from 1 hour of exposure to an average THI of 73. Nabenishi et al., (2001) described a CR decrease from 38.2% during summer months (June to September) to 29.5% during cooler months (October to June).

Heat stress has a large detrimental effect on the reproductive system of dairy cattle. Not only is fertility decreased, but any embryo that does progress, is at an increased risk for abortion when THI is high. Manifestation of hormonal changes is the driving force behind the decrease in fertility, as pregnancy hormones such as LH, estradiol, and progesterone all decrease during

incidences of heat stress. Hormonal fluctuations affect the physiology downstream from initial hormone surges and impacts the follicular development on the ovary which directly impacts oocyte cleavage and embryo formation. If pregnancy during heat stress is successful, the embryo is at an increased loss risk due to the quality of blastocyst formed during conception, maternal stress hormones, and uterine temperatures. Because of heat stress, conception rate is significantly lower in heat stressed cattle. A low conception rate among cattle herds is associated with fiscal losses of $622.40 per dairy cow for dairy farmers (Kim & Jeong, 2018). Thus, in addition to animal welfare concerns surrounding heat stress and reproduction, farmers' financial losses provide yet another reason why novel and efficient heat stress mitigation strategies are required.

Strategies for Abatement of Heat Stress

Dairy farmers can reduce the severity and incidence of heat stress through a number of methods. They can provide free-standing shade in the form of trees or other structures (Bond et al., 1967; Buffington et al., 1983), water misters and sprinklers (Harner et al., 2003), free access to drinking water (Stermer et al., 1986), fans, and air conditioning (Hansen et al., 2001; Jordan, 2003; Berman et al., 1985; Takamitsu et al., 1987; Frazzi et al., 2000; Folman et al., 1979; Calegari et al., 2014; Bucklin et al., 2009). Genetic selection, timed artificial insemination (AI), and nutritional strategies, may also provide some relief for dairy cattle imposed with heat stress (Carabano et al., 2019; Gray et al., 2011; Dikmen et al., 2014; Ravagnolo & Misztal, 2000; Berman, 2011; Hoffman, 2010), but would take time, money, production and health decreases, and an increased understanding of cattle genomics.

Water misters, sprinklers, and free access to drinking water can decrease heat stress effects by reducing respiration rate and the body's surface and internal temperatures (Harner et al., 2003); the water helps to cool or maintain temperature in cattle through evaporative and convective

cooling properties. Drinking water not only cools the internal temperature through convection and conduction of water through the digestive tract, but also provides more water for excretion of sodium and potassium for evaporative cooling via sweating. Drinking water that is 28 ℃, 22℃, 16℃, or 10℃ can lower body temperature in dairy cattle by .47℃, .59℃, .57℃, and .75℃ respectively (Stermer et al., 1986). Despite the positive effects of utilizing water for cooling, as global temperatures increase, the supply of drinking water is predicted to decrease and the cost of using misters or sprinklers will rise; therefore, free access to water may no longer be possible or cost-effective in the short-to-long term future (Trenberth, 2011; Tanaka et al. 2005). In addition, Nardone et al. (2010) estimates that temperature increases will cause an increase in animal water consumption by a factor of two to three, further reducing the amount of water available for agricultural use.

Shade is also extremely effective in reducing the radiant heat load of dairy cattle. Structures or trees can provide shade and reduce the radiant heat load by 30% in dairy cattle (Bond et al., 1967; Buffington et al., 1983); however, land availability diminishes as world population and sea levels rise, eliminating select areas for agricultural use (Thornton, 2010). Areas most likely to be significantly affected by climate change alone include Africa, South America, India, and Europe by a maximum land loss of 18%, 21%, 17%, and 4%, respectively (Zhang & Cai, 2011). India and China are the second and third highest milk producing regions, with Europe and Asia being the highest producers of milk around the world (FAO, 2019). The decrease in accessible agricultural land impacts the ability of dairy farmers to plant trees and construct shade-providing buildings for dairy herds.

Fans and air conditioning can decrease the heat load of dairy cattle, with Hahn et al. (1969) showing an average decrease in rectal temperature of 0.4℃ and an increase in milk yield of

0.5kg/cow per day with use of an air conditioning system. Use of a simple box fan can decrease a dairy cow's body temperature by .1-.6℃ (Berman et al., 1985; Takamitsu et al., 1987; Frazzi et al., 2000; Folman et al., 1979; Calegari et al., 2014), while a more advanced ceiling fan/mister combo can cool cattle by 4.2℃ (Bucklin et al., 2009). However, fans and air conditioning rely on electricity to function and from 2006 to 2013, the cost of electricity increased by 15% in the U.S. and by 17% in the European Union (U.S. Energy Information Administration, 2019; Eursostat, 2018). Electricity cost is projected to continue increasing over the next decade, reducing the cost efficiency of fans and air conditioning (Savenia Labs, 2018; Marcy & Metelitsa, 2014; Fournel et al., 2017).

Basal heart rate, respiratory rate, and temperature (Carabano et al., 2019), as well as hair color, shed rate, and the body mass to surface area ratio (Gray et al., 2011) are all genetic indicators of a cow's disposition to heat stress. Most research involving genetic selection for positive heat stress indices have taken extended periods of time, have not been fully understood (ie. lacking genomics studies on the genetic markers of heat stress), or have not shown significant improvements to warrant the potential decrease in milk production of mixed breeds. Typically, high producing cattle breeds are from more temperate climates, while their low-producing counterparts are from warm climates; production and climate are inversely proportional in the case of dairy cattle (Berman, 2011; Hoffman, 2010). This relationship provides a significant challenge for cross breeding cattle with heat stress resilience to cattle with high annual milk yield. Carabano et al. (2019) recommends focusing on selecting for heat tolerance in high producing breeds rather than selecting for higher milk production in high-temperature-resistant breeds. Ravagnolo & Misztal (2000) believe that since the genetic correlation between production and heat stress is so small (-0.3), that productive cows can still be selected among heat-resistant breeds. Ravagnolo's

suggestion can be seen in Holstein cattle transposed with the SLICK haplotype from Senepol cattle; the heat tolerance was improved in the SLICK Holsteins (Dikmen et al., 2014). However, selecting for any trait can take generations of data collection and trial and error, lengthening the amount of time required for improving heat resistance among dairy cattle (Garcia-Ruiz et al., 2016). Since global temperatures are predicted to increase quickly over the next decade, genetic selection may not be the most time advantageous towards improving heat stress resistance in dairy cattle, but would likely be valuable for reducing incidence of heat stress in future generations.

Timed artificial insemination (TAI) occurring with the season rather than artificially inseminating upon estrus detection, is one proposed method of reducing the effects of heat stress on the reproductive system of dairy cattle. The main TAI protocol (Ovsynch) involves injecting GnRH (or GnRH agonist) and $PGF_{2\alpha}$ (Pursely et al., 1995; Burke et al., 1996; Pursley et al., 1997) and, when performed around the summer months, can improve annual pregnancy rate by up to 40% depending on calving interval and days postpartum (Hansen & Arechiga, 1999; Are´chiga et al., 1998a; de la Sota et al., 1998). There are many different TAI protocols with various timing periods, hormone use, and efficiencies; however, most are iterations of the Ovsynch protocol. Most protocols involve a lot of handling and labor and thus a lot of time dedicated towards correctly timing the hormone doses and insemination periods with cattle ovulation (Colazo & Mapletoft, 2014). Ahmadi & Ghaisari (2007) reported that utilizing Ovsynch during the summer months was not significantly different than using CIDR implantation and an estradiol benzoate injection. In addition, TAI is subject to error due to asynchrony between ovulation, hormone injection, and insemination, resulting in a low synchronization rate of 68% (Colazo et al., 2009). Iterations of Ovsynch have also become increasingly complex protocols that eliminate their usage among typical dairy farmers (Macmillan, 2010).

Nutritional modifications are another way that animal scientists and farmers have tried to mitigate the effects of heat stress on dairy cattle. Timing meals (Robinson, 2013; Collier & Beede, 1985), increasing dietary energy through fat and fiber supplementation (Collier & Beede, 1985; Drackley et al., 2003; Wamtjes et al., 2008; Gallardo et al., 2001; Moallem et al., 2010; Serbester et al., 2005; Halachmi et al., 2004; Kanjanapruthipong et al., 2010; Gonzalez-Rivas et al., 2018), microbial additives (Schingoethe et al., 2004; Bruno et al., 2009; Zhu et al., 2016; Dias et al., 2018; Salvati at el., 2015; Boyd et al., 2011), mineral supplementation (Al-saiady et al., 2004; Soltan, 2010; Seijan et al., 2012; Calamari et al., 2011; Oltramari et al., 2014; Sanz Fernandez et al., 2014; Weng et al., 2018), vitamin supplementation (De et al., 2014; Padilla et al., 2006; Di et al., 1997; Maciejewski Lenoir et al., 2006; Cheng et al., 2006; Zimbelman et al., 2010; Zimbelman et al., 2013; Rungruang et al., 2014; Wrinkle et al., 2012), and plant extract supplementation (Pan et al., 2014; Pompeu et al. 2011; Boyd et al., 2011; Benchaar & Calsamiglia, 2008; Zhang et al., 2014) have all been researched for their effects on heat stress.

Fuquay (1981) wrote that "dietary emphasis should be to increase intake or alter levels of proteins, amino acids, or other nutrients to improve the conversion of feed units into production units." One method of increasing intake was suggested by both Robinson (2013) and Collier & Beede (1985). Both articles recommended feeding cattle during the coolest part of the day while appetite is stimulated as well as feeding multiple, small meals to keep feedstuff from warming in the sun. Ingestion of warm feed contributes to the overall heat load by increasing ruminal temperature. These suggestions are likely the easiest and most effective strategy for reducing heat load in dairy cattle; however, it is likely that cattle, despite being fed during cooler periods of the day, will still be suffering from increased body heat load obtained during the hot periods, thus a decreased DMI will persist (West et al., 2003). To encourage feeding during heat stress conditions,

shade and water should be provided over feed bunks and alleys (West, 1997; Collier & Beede, 1985).

Digested fat produces less heat than the ruminal fermentation of carbohydrates or protein (Wang et al., 2010) so, dairy nutritionists have suggested increasing fat density of rations while decreasing carbohydrate-rich rations (Drackley et al., 2003; Wamtjes et al., 2008; Gallardo et al., 2001; Moallem et al., 2010; Serbester et al., 2005; Wang et al., 2010). Most of these studies, while detailing an increase in milk yield during heat stress conditions, did not elaborate on the effects of increased fat density on the temperature of the dairy cattle studied and whether or not DMI actually increased. Moallem et al. (2010) did indicate a significant decrease in metabolic heat production of -1.3 MCal/day when dairy cattle were fed 300 g/day of calcium salts of fatty acids and Wang et al. (2010) reported a significant decrease in rectal temperature at 14:00 from feeding a 1.5% saturated fatty acid, but no change in DMI; as temperature fluctuates daily, the significant difference only taking effect at 14:00 does not seem promising for the treatment promoting heat loss in heat stressed dairy cattle. Furthermore, increasing the composition of fats within the diet often decreases intake by compromising palatability (Grummer, 1990; Drackley et al., 1992). Increasing dietary fat may also have a negative impact on the digestion and utilization of other nutrients such as calcium and cellulose (Tillman & Brethour, 1958; Davison & Woods, 1960; Glasser et al., 2008; Boerman et al., 2015). Despite the potential of reducing ruminal heat generation by decreasing carbohydrate fermentation, increasing the dietary fat to carbohydrate ratio has either resulted in decreased dry matter intake, no change in any indicator besides milk yield, and/or potential nutrient deficiencies.

By decreasing the dietary NDF from roughage in feedstuff, energy density of rations increase, as does palatability and digestibility; this is hypothesized to increase DMI and ameliorate

some of the health effects witnessed with the DMI decrease during heat stress (Min et al., 2019). Feed intake and milk yield were significantly higher when dairy cattle were fed soy hulls or cassava chips versus roughage NDF (neutral detergent fiber), but cattle body temperatures were not measured during these treatments (Halachmi et al., 2004; Kanjanapruthipong et al., 2010). Some fibers are slower to ferment than others, such as crushed corn (Gonzalez-Rivas, 2016). Gonzalez-Rivas et al. (2018) fed a total mixed ration in addition to ground corn and found that the rectal temperature of treated cows decreased by 0.2°C. However, an appropriate balance of fiber sources must be delicately maintained to avoid ruminal acidosis (West, 1999; Conte et al., 2018). Ruminal acidosis is caused by excess feeding of fermentable carbohydrates, resulting in a pH drop in the rumen (Merck, 2015). Ruminal acidosis can cause decreased feed intake (when feed intake is already low due to heat stress), decreased milk production, diarrhea, and/or death

Microbial additives, specifically yeast cultures, have become an interesting topic of research among dairy scientists. *Saccharomyces cerevisiae* seems to be the most common additive explored for mitigating the effects of heat stress. Schingoethe et al. (2004) explored using the yeast culture to improve lactation efforts during heat stress, but did not find significance. Bruno et al. (2009) found that 30 g/day of the yeast culture improved performance, as well as milk yield and composition. When provided with 240 g/day of *S. cerevisiae* dairy cattle had greater feed efficiency (Zhu et al., 2016) while Dias et al. (2018) found that, at select times during the day (namely at hour 15:00), 15 g/day of *S. cervisiae* caused rectal and skin temperature to significantly decrease by 0.29°C and 0.35°C, respectively. Dias et al. (2018) also found that respiration rate significantly decreased by 5.3 breaths per minute when cattle were given the 15g/day dose. Salvati et al. (2015) used live yeast and found significant benefits for milk production, as well as a lower respiratory rate (56 breaths per minute for control cattle to 48 breaths per minute for cattle

receiving 10 g/day of live yeast) during the treatment period. A combination of *Lactobacillus acidophilus* and *Propionibacterium freudenreichii* improved milk yield and composition, as well as the digestibility of CP (crude protein) and NDF subsequent to microbial supplementation (Boyd et al., 2011). While there are significant effects from supplementing microbial additives, the use of these additives mostly effects milk production with some effect of respiratory rate and a potential effect on overall body temperature. Further evaluation of microbial cultures on body temperatures, behavior, and animal welfare should be evaluated to determine if these cultures are truly making cattle more resistant to heat stress.

Several minerals have been explored for their effects on improving cattle heat tolerance, specifically selenium (Se), chromium (Cr) and zinc (Zn). The effects of selenium supplementation to the diet improved milk composition, immune function, and overall cattle health when supplied (Seijan et al., 2012; Calamari et al., 2011; Oltramari et al., 2014). In addition, inorganic selenium (sodium selenite) supplementation of 0.278 mg/kg of DM decreased respiratory rate of cattle while organic selenium (selenium yeast) supplementation of 0.278 mg/kg of DM decreased cattle surface temperatures (Oltramari et al., 2014). It is unknown why this difference occurred between organic versus inorganic minerals, but could have something to do with the fact that organic selenium contained *Saccharomyces cerevisiae*, whose heat stress mitigation benefits were discussed previously. Chromium has also been explored and has improved milk yield, DMI, and conception rates when fed 4 g/day of Cr yeast (Al-saiady et al., 2004) or 6 mg/head/day of a standard Cr supplement (Soltan, 2010). Zinc supplementation of 35 mg/kg Zn hydroxychloride and 40 mg/kg of Zn methionine was speculated to improve mammary epithelium stability, but the results were not significant (Weng et al., 2018). Mineral supplementation might have some effect on milk

production and composition; however, its effects on animal welfare and whether or not mineral supplementation truly mitigates heat stress is up for debate and further research.

Vitamin A and vitamin B_3 have been studied to determine their effects on heat stress mitigation. Reproductive efficiency, immune function, and somatic cell count in milk were all reduced by vitamin A supplementation (Lawrence et al., 2004; De et al., 2014). Reproductive efficiency was improved through reduction of oocyte defects and immune function improvement included increased activity of neutrophils and Il-8 concentrations (Lawrence et al., 2004; De et al., 2014). Vitamin B_3 (niacin) actually mitigates heat stress effects by increasing vasodilation and blood flow from visceral organs to peripherals (Di et al., 1997; (Maciejewski Lenoir et al. 2006; Cheng et al. 2006) as well as increasing sweating rate and loss of heat load through evaporation (Di et al., 1997). In 2010, 2012, and 2013, Zimbelman et al., as well as Wrinkle et al. (2012), tested the effects of niacin on rectal and vaginal temperature of Holstein cows and found that temperatures decreased with 12g/day of supplementation. However, Rungruang et al. (2014) combated these results by saying that 12 g/day had no effect on temperatures of Holstein dairy cattle. Although the use of niacin makes physiological logic in its mitigation of heat stress, there are conflicting reports on whether or not niacin actually decreases body temperatures of heat stressed dairy cattle.

Utilization of plant extracts may have an effect of heat stress mitigation in dairy cattle. Betaine, *Radix bupleuri,* essential oils, and *Ascophyllum nodosum* have all been explored by researchers (Zhang et al., 2014; Pompeu et al., 2011; Boyd et al., 2011; Pan et al., 2014;). Betaine supplementation of 15 g/day significantly increased feed intake and milk yield for lactating Holstein cattle inhabiting an environment with an average THI of 78.6 units (Zhang et al., 2014) and supplementation with 4g/kg of DM improved lactation performance in dairy goats (Fernández

et al., 2004a; Fernandez et al., 2004b; Fernandez et al., 2009a); however, more studies on the effects of betaine are needed to determine its effects on heat stress (Peterson et al., 2012). Pan et al. (2014) used *R. bupleuri* to determine its effects on body temperature and respiratory rate, and found that respiratory rate and rectal temperature significantly decreased upon supplementation of 0.25g/kg DM, 0.5 g/kg DM, or 1 g/kg DM. Although evaluated, *Ascophyllum nodosum* and essential oils (*capsicum, cinnamaldehyde,* and *eugenol)* treatments were not much different from controls (Boyd et al., 2010; Pompeu et al., 2011), with the exception of *A. nodosum's* potential effect on decreasing the amount of heat gained over a period of time when compared with controls (Pompeu et al., 2011). More research on plant extracts and their effects on heat stress is needed before determining whether they are effective at mitigating the effects of heat stress.

Abatement methods can help to decrease Holstein heat load, reduce the effects of high environmental heat on cattle production, and increase cattle welfare but, these abatement mechanisms rely on electricity, water, land, and time. Electricity is predicted to increase in cost over the next decade, drinkable water is diminishing as global temperatures increase, and land is decreasing as population, urbanization, and sea levels rise. Genetic selection requires several generations to see a significant effect on whether the dairy cow can successfully abate heat without human mitigation. Dietary changes have the potential to reduce heat load; however, these changes also decrease the dry matter intake during a period where dry mater intake is already waning, require more expense and/or research, or can cause further health detriment to the cattle (acidosis). To ensure the future success of the dairy industry, development of novel heat abatement methods for dairy cattle are needed.

Exercise as a Method to Increase Thermoregulatory Capabilities

Thermoregulatory Adaptations to Exercise

Exercise in hot, humid environments has been reported to increase sweat output and capillary density, as well as redistribute and hypertrophy skeletal muscle towards fiber types that are more capillary dense (Périard et al., 2015; Sawka et al., 1996; Sawka et al., 2011). Acclimation to heat has occurred in human and equine exercise studies, presumably due to the continuous and consistent deviations in body temperature during regular bouts of exercise in a sweltering environment (Geor & McCutcheon, 1998; Taylor, 2014; Periard et al., 2015; Sawka et al., 2019). Regular deviations in body temperature caused by exercise in a hot environment, have been shown to increase the shear force on blood vessel walls and increase angiogenic and angioadaptive blood markers (Chaar et al., 2015). Through angiogenesis and angioadaptation, new blood vessels are created within muscle and skin. Increased capillary density and more efficient structure of capillaries are presumed to improve cooling capabilities by allowing space for more heated blood from the viscera towards the body's surface for abatement. In addition, capillary growth after exercise trials are often accompanied by changes in skeletal muscle, with increases in the amount of type I and IIA fibers and their respective cross-sectional surface areas (Tyler et al., 1998; Rivero et al. 1995; Sinha et al., 1993; Schiaffino & Reggiani, 2011; Hudlicka et al., 1992). These changes have the potential to be protective against heat stress (Periard et al., 2016).

There are many types of exercise; however, the two forms investigated in this thesis are low-intensity and high-intensity exercise. Low-intensity exercise is defined as exercise that utilizes mostly aerobic muscle fibers and occurs below the species-specific lactate threshold. Low-intensity exercise induces a heart rate that is approximately 40% of the maximum heart rate (Alansare et al., 2018). High-intensity exercise is defined as exercise at or above the species-

specific lactate threshold and utilizes mostly anaerobic muscle fibers. High-intensity exercise induces a heart rate that is approximately 90% of the maximum heart rate (Alansare et al., 2018). During all exercise intensities, heat is generated by skeletal muscles, and core body temperature (CBT) will rise. To counterbalance this rise in CBT, blood flow to the skin, heart rate, respiratory rate, and sweat secretion increase during exercise.

At the onset of exercise, CBT rises expeditiously. Shortly thereafter, CBT increases at a rate that is slower than the initial temperature spike (Sawka et al., 1993). Neilsen (1938) found that the core temperature elevation is proportional to the metabolic rate of the individual engaging in the exercise and that the sudden increase in initial CBT can be even more rapid when exercise is occurring during greater ambient temperatures. In other words, thermoregulation during exercise becomes increasingly more difficult at greater ambient temperatures. However, mammals can acclimate to exercise at high ambient temperatures. Acclimatization will improve the efficiency with which they manage and maintain CBT.

The acclimatization of homeotherms to high environmental temperatures requires repeated exposure to a natural, sweltering, outdoor environment, the temperature and humidity of which prevent sufficient heat abatement from the body (Boyles et al., 2011). This puts stress upon the circulatory and respiratory systems. As the individual acclimates to the change in environment, its body "learns" how to more efficiently respond to heat stress and improve its ability to release heat into the environment (Geor & McCutcheon, 1998). The benefits of heat acclimatization are achieved by improved sweating, peripheral blood flow responses, cardiovascular stability, and fluid-electrolyte balance (Périard et al., 2015; Sawka et al., 1996; Sawka et al., 2011).

Acute heat stress changes the way that the body functions. The body responds to acute heat stress by altering blood pressure, blood flow, and blood volume. In addition, the body experiences

upregulation of heat shock proteins. The increase in blood pressure and flow allow for more rapid delivery of heated, visceral blood to the skin for convective heat loss into the dermis and to the respiratory tract for eventual evaporative cooling. In contrast, blood flow to the renal and splanchnic systems are reduced in proportion to the exercise intensity (Rowell, 1986). The hypothalamus regulates distribution of blood flow by targeting effectors that constrict blood flow to the kidneys, liver, digestive tract, mammary glands, and reproductive organs, while simultaneously dilating other vessels for more blood delivery to contracting muscles and to the skin for heat dissipation.

During exercise, blood volume increases as a result of the multi-step conversion of angiotensinogen to angiotensin II (Chaar et al., 2015). Angiotensin II increases reabsorption of sodium, chloride, and water from the kidney. Rather than being excreted as urine, these electrolytes return to the bloodstream, increasing blood volume (Nussey & Whitehead, 2001). This provides for increased water excretion through the respiratory tract and sweat glands for evaporative heat abatement.

Both blood pressure and blood flow increase during exercise. These surges in pressure and volume increase the amount of shear force that is applied to the endothelial cells that line the vasculature. This increase in shear force on the endothelium results in the emission of nitric oxide (NO), a chemical stimulator of blood vessel morphogenesis (Kolluru et al., 2010). NO release can also be stimulated by the phosphorylation effect of heat shock proteins activated in times of cellular strain (Sun & Liao, 2004). The driving effect of NO release is morphogenesis of the body's blood vessels.

Generation of new endothelial cells via NO pathways and the subsequent branching, elongation, and remodeling of existing blood vessels, is referred to as angiogenesis (Adair &

Montani, 2010). Vasculogenesis is the de novo generation of new endothelial cells from nitric oxide-stimulated angioblasts; vasculogenesis does not require newly generated blood vessels to form off existing blood vessels (Drake, 2003). The continuous state of blood vessel restructuring, breakdown, and generation is referred to as angioadaptation (Zakrzewicz et al., 2002).

Newly created, branched, and re-directed capillaries allow blood to be carried throughout the body, but preferentially to the skin's surface, thereby increasing the efficiency of heat transfer from the body to the environment. The ability to drive more warm blood to the dermis and respiratory tract for dissipation increases the homeotherm's ability to thermoregulate in high ambient temperatures. The morphogenesis of blood vessels can be attributed largely to exercise alone; however, exposure to environmental heat plays a significant role in the strength and speed of angiogenesis (Geor & McCutcheon, 1998).

Animals may also acclimate to the environment by altering sweat gland activity, such that they exhibit increased sweating at lower environmental temperatures (Bouno & Sjoholm, 1988). Sweat gland adaptions of animals with a majority of their sweat glands inactive, such as dairy cattle, are not known to have been studied up to this point in time.

Skeletal Muscle Adaptations to Exercise

Fiber Type Proportion Changes

Skeletal muscle changes can result in increased thermoregulatory capabilities. Skeletal muscle is comprised of large numbers of fibers, the numbers of which vary depending on the muscle type and species. According to Picard et al. (1998), there appeared to be three main types of muscle fibers in dairy cattle: slow-twitch oxidative fibers (I), fast-twitch oxidative (IIA), and

fast-twitch glycolytic (IIX or IIB, depending on literature). There are also hybrids of these fibers, such as I/IIA and IIA/IIX; these occur as a transitionary phase between fiber types.

Type I fibers aerobically contract and exist in excess numbers within the muscles of endurance athletes and horses (Hinchcliff et al., 2008; Wilson et al., 2012). Type I fibers contract slowly compared to type IIA and IIX fiber types. They also have large quantities of mitochondria and capillaries. The former determines the aerobic capacity of the type I fibers, while the latter helps to meet the mitochondria's demand for glucose and oxygen. Type I fibers are sometimes referred to as "red" muscle fibers due to their high capillary content and high oxidative capacity (Scott et al., 2001).

Table 1.2 Summary: Exercise-Induced Fiber Type Count Changes in Humans

Fiber Type	Exercise Intensity	Change
I	HI	NC
IIA	HI	↑
IIX	HI	↓
I	LI	NC
IIA	LI	↑
IIX	LI	NC

Type IIA fibers have quicker contraction speeds and, though they have fewer mitochondria and capillary supplies than type I fibers, are relatively fatigue resistant. Type IIA fibers are often discussed as intermediary fibers between type I and type IIX fibers.

Type IIX fibers contract quickly and with great force, but being completely glycolytic, fatigue quickly, can only be utilized for short periods of time without rest, and produce high quantities of lactate. Type IIX fibers, compared to I and IIA fibers, have more glycogen storage to breakdown for energy when contracting (Kocsis et al., 2014).

Animals are genetically predisposed to have certain fiber type ratios (Ahmetov et al., 2012). However, despite this genetic programming, skeletal muscle fiber composition may change.

For example, exercise is able to induce changes in equine skeletal muscle fiber composition (Hinchliff et al., 2008).

Percentages change for certain human fiber types during various exercise intensities with fibers changing differently for each muscle type (Bagley et al., 2012; Williamson et al., 2001). Most fiber type proportion changes have been studied on predominately fast twitch muscles, such as the quadriceps, soleus, and gastrocnemius, with the indication that, after anaerobic and aerobic endurance exercise, there was an overall shift from the glycolytic fiber type to oxidative fiber types (Bagley et al., 2012).

Table 1.3 Summary: Exercise-Induced Fiber Type Count Changes in Horses

Fiber Type	Exercise Intensity	Change
I	HI	↓
IIA	HI	↑
IIX	HI	↓
I	LI	NC
IIA	LI	↑
IIX	LI	↓

According to a review by Bagley et al. (2012), there were marked changes in fiber type composition upon evaluation of muscle responses to exercise intensity. Human muscle response to high-intensity, sprint interval cycling caused IIX fibers decreased in count, IIA fibers increased in count, and I fibers fluctuated depending on the length of the exercise trial (Aagaard et al., 2011; Simoneau et al., 1985). Andersen & Henricksson (1977), as well as Kraemer et al. (1996), found that, in response to high-intensity exercise, IIX fibers decreased in count, IIA fibers increased, and I fiber counts remained relatively constant. Pette and Staron (1997), as well as Ricoy et al. (1998), found that IIA fibers increase with endurance training in humans. Ricoy et al. (1998) found that there was no significant evidence for endurance exercise to induce a transformation of type IIA and IIX fibers to type I fibers in humans.

In horses, low-intensity training increases the number of type IIA fibers and decreases the proportion of IIX fibers while high-intensity interval training provides similar results, with IIA fibers increasing in count and IIX fiber proportions decreasing (Tyler et al., 1998; Miyata et al., 1999; Gondim et al., 2005; Rivero et al., 1995; Serrano & Rivero, 2000; Serrano et al., 2000; Rivero et al., 2001; Lopez-Rivero et al., 1989; Yamano et al., 2002; Kim et al. 2005; Rivero et al., 2000; D'Angelis et al., 2005; Essen-Gustavvsson & Lindholm, 1985; Essen-Gustavsson et al., 1989; Lopez-Rivero, 1991; Sinha et al., 1993); however, high-intensity exercise also induced a decrease in type I fibers (Rivero et al., 2002; Lovell & Rose, 1991).

Fiber Type Cross-Sectional Area, Capillary Density, and Oxidative Capacity Changes

In addition to fiber type proportion changes, exercise modifies fiber cross-sectional surface area, capillary density, and fiber oxidative capacity (Seene et al., 2017). Highly oxidative fiber types (type I fibers) are generally smaller in surface area than their glycolytic counterparts (IIA and IIX) (Seene et al., 2017). In humans, muscle hypertrophy increases for all fiber types during aerobic and anaerobic exercise, thus increasing the cross-sectional surface area of utilized muscle fibers (Harber et al., 2012; Kraemer et al., 1996).

It is still controversial as to whether cross-sectional area of muscle fibers increases for equine models (Hinchcliff et al., 2008). Horses bred for endurance, such as Thoroughbreds and Standardbreds, do not show a marked increase in cross-sectional surface area of all fiber types (Henckel, 1983; Rivero et al., 1996; Lindholm et al., 1983; Foreman et al., 1990); this is different for endurance-bred Arabian horses, as they show an increase in type I and IIA fiber cross-sectional surface areas after training (D'Angelis et al., 2005).

High-intensity exercise in horses causes an increase in cross-sectional area of type II fibers (Heck et al., 1996). High-intensity exercise has also been linked to an increase in cross-sectional

surface area of type I and IIA fibers (Tyler et al., 1998; Lopez-Rivero et al., 1992; Rivero et al., 1995; Serrano et al., 2000; Rivero et al., 2001; Yamano et al., 2002; Gottlieb et al., 1989; D'Angelis et al., 2005).

The capillary density of fibers after protracted exercise trials varies among fiber type and muscle types. Terjung (1995), and Laughlin & Roseguini (2008), found that capillary density increases relative to fiber size after long-term, aerobic, interval sprint training *and* endurance training. Capillary density is often tied to oxidative capacity of muscle fibers; the more oxygen and mitochondrial nutrients needed for fiber contraction, the greater the quantity of blood flow required (Terjung et al., 2002). In rats that underwent high-intensity training, there was significant NO release to stimulate angiogenesis; however, the authors reported no significant changes in capillary density of muscle fibers (Karimian et al., 2015). McCall et al. (1996) stated that fiber type hypertrophy is the main cause for increased capillary density; the ratio of fiber cross-sectional area to capillary density remains the same after low-intensity training.

The total oxidative capacity of muscle, specifically of type I and IIA fibers, increases after exercise due to an increase in the mitochondrial volume; however, the mechanisms by which mitochondrial density increases and to what extent it increases, varies among different muscles (Turner et al., 1997). Turner and others (1997) identified that, while oxidative capacity increases after endurance training, the muscle volume remained approximately the same in highly utilized muscles (such as the gastrocnemius and biceps femoris). However, in less utilized muscles (such as the rectus abdominus), mitochondrial density, and thus oxidative capacity, increased in addition to an increased muscle volume. Exercise should increase the oxidative capacity of all muscle fibers, specifically within endurance or low-intensity training (Scott et al., 2001).

Skeletal muscle composition contributes to thermoregulatory capabilities of homeothermic animals. In addition to the contributions of shear force to blood vessel morphogenesis, fiber type composition can contribute to the modification of blood vessel properties for heat dissipation improvement. Increasing I and IIA fiber counts and cross-sectional surface areas can increase the capillary density of the muscle, improve blood flow, and improve the efficiency of capillary arrangement for heat abatement.

Chapter 2 - Effects of Multiple Exercise Intensities on Holstein Heifer Skeletal Muscle

Introduction

Heat stress affects a variety of physiological parameters in dairy cattle and causes increased cattle morbidity and decreased milk production and reproductive efficiency. In 2003, the dairy industry experienced a net loss of $1 billion due to heat stress (St-Pierre et al., 2003). Using present-day milk pricing, losses to the dairy sector due to heat stress are estimated to exceed $2 billion annually by 2099 (Mauger et al., 2015). The economic impact of heat stress is expected to increase as global temperatures increase (Mauger et al., 2015). While there are existing management strategies that are effective in reducing the impacts of heat stress on dairy cattle production, environmentally sustainable and cost-effective methods of heat stress abatement are still needed to solidify the most effective option for minimizing the effects of heat stress on dairy cattle.

The most common dairy breed in the United States is the Holstein Friesian; however, their large size, high milk-yield, and high metabolic rate predisposes the breed to heat stress (Spiers et al., 2004). A mature, lactating dairy animal can undergo effects of heat stress when ambient

temperatures exceed the upper critical temperature (UCT) of 25°C (Berman et al., 1985). Collier et al. (2011), Kingma (2012), and Noordhuizen (2015) demonstrated breed, age, stage of lactation, sex, stage of adaptation, and the thickness and length of hair on the cow's body affected the upper critical temperature and range of acceptable ambient temperatures. Heat stress results in physiological changes in body temperature, respiration rate, heart rate, all of which have been linked to decreases in fertility (de Rensis & Scaramuzzi, 2003), 600-900 kg/lactation losses in milk yield (West, 2003), 0.85 kg less dry matter intake per degree (°C) increase in ambient temperature (West, 2003), and immune dysfunction (Latcetera et al., 2005; Latcetera et al; 2006; Do Amaral et al., 2009; Elvinger et al., 1991; Kamwanja et al., 1994; Do Amaral et al., 2010; Do Amaral et al., 2011; Tao et al., 2012a; Laporta et al., 2017; Nardone et al., 1997; Tao et al., 2019). Increases in cow mortality rates also occur as a result of heat stress (Elvinger et al., 1991; Bishop-Williams, 2015; Monty & Racowsky, 1987).

Due to the over $1 billion-dollar economic impact and the effects of heat stress on animal welfare, methods to facilitate heat abatement for Holstein dairy cattle have been utilized. Hansen et al. (2001) and Jordan (2003) both describe the use of fans, misters, *ad libitum* access to water, air conditioning, and free-standing shade (trees, buildings, etc.) as viable solutions to mitigate heat stress; however, these methods, while proven to be useful, are resource- and financially-dependent (Trenberth, 2011).

In addition to direct heat mitigation techniques, indirect techniques such as dietary changes have also been tested. Vitamin and mineral supplementation, as well as low carbohydrate diet formulations, have both been studied to determine their effects on heat stress mitigation; however, both methods have proven to be unfruitful towards reducing impacts of heat stress while maintaining DMI levels and overall cattle health. Genetic selection, and cross-breeding of

Holstein cattle have also been studied, but both methods are variable in results and could take decades to see a significant effect (Reodecha et al., 2002; Chanvijit et al., 2005; Da Costa et al., 2015; De Paula Xavier de Andrade et al., 2017; Khongdee et al., 2006; Khongdee et al., 2010). Considering temperature predictions for the next decade and the current and future cost associated with heat stress, it is imperative a viable heat stress treatment be determined as soon as possible. As global temperatures and the prevalence of heat stress increase, novel heat abatement methods must be investigated in conjunction with improving established methods.

One novel method is to exercise Holstein cattle in high ambient humidity and temperature. During and after exercise in humid environments above the UCT, blood vessel generation (angiogenesis) and restructuring (angioadapation) occurs from an increase in shear force on blood vessel walls (Pries et al., 2016). Angiogenesis and angioadaptation of capillaries due to low- and high-intensity exercise induced change in skeletal muscle and improved thermoregulatory capabilities (Périard et al., 2015; Sawka et al., 1996; Sawka et al., 2011).

Low-intensity exercise is defined as exercise that utilizes mostly aerobic muscle fibers and occurs below the lactate threshold; this type of exercise is typically methodical and low-speed. High-intensity exercise is defined as exercise at or above the lactate threshold and utilizes mostly anaerobic muscle fibers; this type of exercise is high-speed for intermittent periods of time. Low- and high-intensity exercise on equine, human, and canine subjects increased capillary density in muscle tissue (Terjung, 1995; Laughlin & Roseguini, 2008). During both low- and high- intensity exercise, heat is generated by skeletal muscles, and core body temperature (CBT) rises. To slow/lower the rate of CBT increase, blood flow to the skin, heart rate, respiratory rate, shear force on blood vessel walls, and sweat secretion increase. These responses to exercise, specifically the increase in shear force on vessel walls, have been shown to function together to have facilitated

angiogenesis and angioadaptation of capillaries (Dopheide et al., 2017; Casey et al., 2017; Gustafsson & Kraus, 2001). Angioadaptation and angiogenesis ultimately moved greater quantities of blood more efficiently through viscera and muscles and towards the surface of the skin for abatement (Gustafsson & Kraus, 2001). Exercise while in a hot, humid environment was found to have escalated the efficiency of angiogenic and angioadaptive responses (Kuhlenhoelter et al., 2016).

Increases in capillary density have been directly proportional with increases in muscle cell mitochondria (Gavin, 2013; Brodal & Hermanson, 1977). When capillary density increases, so does the quantity of mitochondria present in a muscle fiber; new capillaries and mitochondria generate new muscle fibers or modify pre-existing fibers from one type to another. According to Picard et al. (1998), there appeared to be three main types of muscle fibers in dairy cattle: slow-twitch oxidative fibers (I), fast-twitch oxidative (IIA), and fast-twitch glycolytic (IIX or IIB, depending on literature). During exercise periods, hybrids of these fibers, such as I/IIA and IIA/IIX have occurred in humans as a transitionary phase between fiber types (Pette & Staron, 1997). Type I fibers have the most mitochondria and the largest blood supply, type IIX fibers have the least, and IIA fibers are intermediate between I and IIX. During equine exercise trials, it was possible for fiber types to change, depending on the type of exercise. High-intensity exercise caused increases in IIA fibers and decreases in IIX (Tyler et al., 1998; Miyata et al., 1999; Gondim et al., 2005; Rivero et al., 1995; Serrano & Rivero, 2000; Serrano et al., 2000; Rivero et al., 2001; Lopez-Rivero et al., 1989; Yamano et al., 2002; Kim et al. 2005; Rivero et al., 2000; D'Angelis et al., 2005; Essen-Gustavvsson & Lindholm, 1985; Essen-Gustavsson et al., 1989; Lopez-Rivero, 1991; Sinha et al., 1993; Rivero et al., 2002; Lovell & Rose, 1991), while low-intensity exercise in humans caused increases in IIA fibers and decreases in IIX fibers (Pette & Staron, 1997; Ricoy et

al., 1998). Capillaries, in conjunction with mitochondria, increase in muscle fibers after high- and low-intensity exercise, causing a shift from type IIX fibers to the capillary- and mitochondria-rich type I and IIA fibers. The genesis and restructuring of capillaries increase the efficiency of blood flow from visceral tissues to peripherals; as vessel density increases and more blood moves through the muscles towards the skin, convective cooling of the muscle increases (Sokolnicki et al., 2008; Charkoudian, 2003; Johnson & Proppe, 1996; Rowell, 1983).

Changes in capillary density and structure and the associated redistribution of skeletal muscle fiber types, have the potential to increase the heat dissipation ability of a dairy cow. We hypothesize that low- and high- intensity exercise will change the skeletal muscle composition of Holstein heifers towards fiber types that are more capillary dense and thus more conducive towards heat abatement, such as type I and IIA fibers. This shift in fiber types could, in future research, indicate an increase in capillary density and cooling capabilities. Moving forward, we anticipate this research to be utilized to determine if exercise increases cooling capabilities of Holstein heifers.

Materials and Methods

Experimental Design

The Kansas State University Institutional Animal Care and Use Committee approved all protocols for experimental use of live animals (Protocol #3710).

Holstein heifers between 7 and 9 months of age were obtained from the Kansas State University Dairy Teaching and Research Center and were separated into 2, 8-week trials. Trial 1 (*N = 22*) took place between May 16th, 2016 and July 18th, 2016 and trial 2 (*N* = 21) took place between July 25th, 2016 and September 27th, 2016.

Table 2.1 Diet for SED and LI heifers before and during experiment

Feed Name	Dry Matter, kg
Corn Gluten Feed	2.02
Corn Grain, Ground	0.97
Grass Hay, Cool	2.83
Limestone	0.08
Vitamin Mix, (0.2%)	0.04
Triticale Silage	1.35

Housing and Care of Heifers

During the months preceding each trial, heifers were either housed in drylot pens with access to a single, sloped-roof shelter (trial 1) or in a non-shaded multi-acre pasture (trial 2). Thus, housing and handling conditions were somewhat different for heifers used for each trial in terms of prior exercise/activity and comfort around humans.

During the exercise trials, heifers were housed in a drylot and provided *ad libitum* water and a total mixed ration (TMR). The TMR (Table 1.1) was delivered twice-daily and was comprised of approximately 28% corn gluten feed on a dry matter (DM) basis, 13% ground corn grain (DM), 39% cool season grass hay (DM), 1% limestone (DM), 1% vitamin mix (0.2%) (DM), and 19% triticale silage (DM).

Exercise Treatments

Heifers that underwent treatment were stratified by age, as well as the estimated ratio of black to white in their coat color. Two people independently estimated black to white ratios and the results were averaged before stratifying for this variable. Heifers were then randomly assigned to 1 of 3 treatments: Sedentary (SED; no exercise treatment applied), low-intensity (LI) exercise, and high-intensity (HI) exercise. Low-intensity and high-intensity exercise regimens were determined in Wilson (2018). The time periods, speed, and amount of repetitions used for treatments 2 and 3 of both trials are listed in Table 2.2.

Table 2.2 Exercise Treatments for Trial 1 and 2

Week	High-Intensity Treatment	Low-Intensity Treatment
W1	15 min walk (4.5 km/h)	15 min walk (4.5 km/h)
W2	10 min (5.47 km/h)	20 min (4.5 km/h)
W3 & W4	2X: 2 min (6.11 km/h) 1 min (3.21 km/h) 2 min (6.11 km/h) 1 min (3.21 km/h) 2 min (6.11 km/h) 5 min (3.21 km/h)	30 min (4.5 km/h)
W5 & W6	3X: 1 min (6.76 km/h) 2 min (3.21 km/h) 1 min (6.76 km/h) 2 min (3.21 km/h) 1 min (6.76 km/h) 5 min (3.21 km/h)	45 min (4.5 km/h)
W7 & W8	3X: 1 min (7.40 km/h) 2 min (3.21 km/h) 1 min (7.40 km/h) 2 min (3.21 km/h) 1 min (7.40 km/h) 5 min (3.21 km/h)	60 min (4.5 km/h)

Exercised heifers were transported via trailer to an 8-panel free walker (Priefert, 8-horse exerciser, Mount Pleasant, TX) for treatment (Fig. 2.1). After arrival at the exerciser, one to two heifers were placed into each segment of the exerciser. The exerciser was pre-programmed for appropriate speed and duration relative to the treatment being applied; an exercise treatment commenced as soon as all heifers were loaded into the exerciser. The panels of the exerciser were occasionally electrified to discourage heifers from stopping, but this feature was used minimally. Sedentary heifers were transported for approximately five minutes in a trailer to mimic travel stress occurring for the exercised group of heifers; otherwise, sedentary heifers were kept in their pen during the exercise periods and penned away from food but provided water ad libitum.

Trailer loading and transportation of exercised heifers began at approximately 0530 on Monday, Wednesday, and Friday. Start time of exercise varied depending on the treatment being applied, but the first exercise treatment typically occurred around 0600. Time of treatment application alternated for low- and high-intensity groups; if LI heifers underwent exercise first on one treatment day, HI heifers would undergo treatment first on the subsequent treatment day.

Figure 2.1 Priefert 8-Panel Exerciser

The exerciser consisted of 8 panels with the ability to move clockwise or counterclockwise. All exercised heifers were trailered to the exerciser and one to two heifers were loaded into each segment of the exerciser. The speed and duration of panel movement were pre-programmed into an attached computer that controlled panel movement. Computer programming for speed and duration was dependent on the week and the treatment being applied.

Biopsy Sample Collection

Biopsy samples from the right semitendinosus muscle were obtained from all heifers 1 week before exercise commenced (pre-exercise biopsy) and again one week after 8 weeks of exercise (post-exercise biopsy). The pre-exercise biopsy was taken approximately 1 cm lateral to the midline of the semitendinosus muscle. The post-exercise biopsy was also from the

semitendinosus muscle but, to avoid any scar tissue from the previous biopsy site, was taken 1 cm medial to the midline of the semitendinosus muscle. For all biopsies, hair was removed over the biopsy site, which was subsequently prepared for surgery with betadine (Betadine Surgical Scrub, Doctors Fosters and Smith©, Rhinelander, WI) and 70% ethanol. A sterile piercing needle was utilized to puncture the dermal layers and was immediately followed by the use of a 10 G × 5-cm long Quick-Core Biopsy Needle (Cook Medical, Chicago, IL) to obtain approximately 5, 2 mm × 1.5 cm biopsy cores.

The biopsy sample was covered with OCT (optimal cutting temperature) compound (Thermo Fisher Scientific, Kalamazoo, MI), subsequently placed into a supercooled isopentane solution, and stored at −80°C until analysis.

Immunohistochemistry and Histology

Immunohistochemistry and traditional histology techniques were utilized for muscle fiber morphometric and succinate dehydrogenase staining intensity, respectively. The staining procedure documented in Phelps et al. (2014a) was used for immunofluorescence of muscle fiber morphometrics. Five micrometer-thick cryosections were collected on positively charged microscope slides (Diamond White Glass; Globe Scientific Inc., Paramus, NJ) using a Microm™ HM 550 Cryostat (Thermo Fischer Scientific, Kalamazoo, MI). Three to 4 cryosections per sample were placed approximately 1 mm apart and heat-fixed by placing the slides directly onto the surface of a heated, digital dry bath. Slides were removed from the dry bath surface after 10 minutes and then left to cool for 3 to 5 minutes at room temperature (~23°C). Cryosections were incubated in 10% equine serum and 0.2% TritonX-100 in a 1× phosphate buffer solution (1×-PBS) for 30 min to block all nonspecific binding sites. Cryosections were incubated for approximately 12-16 hours at room temperature with a primary antibody solution consisting of the blocking

solution, 1:500 α-dystrophin (Thermo Fischer Scientific, Waltham, MA), 1:10 supernatant myosin heavy chain, slow, IgG2b (BA-D5; Developmental Studies Hybridoma Bank, Iowa City, IA), and 1:10 supernatant anti-myosin all but IIX myosin heavy chain, IgG1 (BF-35; Developmental Studies Hybridoma Bank). Muscle cryosections were washed 3 times for 5 minutes with 1×-PBS. After the last wash, cryosections were incubated for 30 minutes with secondary antibodies in blocking solution containing 1:1000 AlexaFlour 633 goat-anti-mouse IgG2b (Life Technologies, Carlsbad, CA) for BA-D5, 1:1000 Alexa-Flour 594 goat-anti-rabbit heavy and light chains (Life Technologies) for BF-35, 1:1,000 Alexa-Flour 488 goat-anti-rabbit heavy and light chains (Life Technologies) for α-dystrophin, and 1:1000 DAPI (Thermo Fischer Scientific, Waltham, MA). After incubation with secondary antibody, sections were washed in 1×-PBS 3 times for 5 minutes each and were coverslipped for image processing. Cryosections were stored in the dark at room temperature (~23°C) until visual analysis and photographing.

The methods of Noel et al. (2016) were followed for succinate dehydrogenase (SDH) staining as follows: 20 μm-thick cryosections processed as described above were incubated for 1.75 hours in a pre-warmed (37°C) solution containing 20 ml of nitro blue tetrazolium solution (.02 g of nitro blue tetrazolium in 20 ml of Milli-Q water), 10 ml of a phosphate buffer solution (.195 g of potassium phosphate monohydrate and 10.99 g disodium hydrogen phosphate in 100 ml of Milli-Q water; solution was replaced every 30 days), and 10 ml of sodium succinate solution (2.7014 g sodium succinate dibasic hexahydrate in 100 ml Milli-Q water). Immediately following incubation, cryosections were washed in Milli-Q water 3 times for 1 minute each and subsequently protected with a glass coverslip for imaging. Muscle sections were kept in the dark at room temperature (~23°C) until ready for visual analysis and photographing.

Image Processing

All cryosections for fiber type and SDH analysis were photographed utilizing a 10x apochromatic objective within a Nikon Eclipse TI-U inverted microscope (Nikon Instruments Inc., Melville, NY). Fiber type and SDH microphotographs were taken at 100× magnification with a Nikon DS-QiMC digital camera (Nikon Instruments Inc.) and a Nikon DS-Fil color digital camera (Nikon Instruments Inc.), respectively. All photographic analyses were performed using the NIS-Elements Imaging software (Basic Research, 3.3; Nikon Instruments Inc.). Approximately 6,000 fibers from trial 1 and approximately 13,000 fibers from trial 2 were imaged and subsequently analyzed for fiber type and fiber cross-sectional area. For muscle fiber typing, fibers that stained positive for BA-D5 and BF-35 were labeled type I and fibers that stained for BF-35 only were labeled type IIA fibers. Fibers that were not stained by both BA-D5 and BF-35 antibodies were labeled as type IIX fibers (Moreno-Sanchez et al., 2008; Schiaffino et al., 1989). The cross-sectional area of muscle fibers was determined as the area within the dystrophin border (stained red).

To determine the SDH staining intensity of each section, approximately 5,000 fibers were analyzed for fiber type. Once fiber type was determined, each type of fiber (I, IIA, or IIX) was analyzed for average white light intensity (kept constant for SDH analysis) by the NIS-Elements Imaging Software. The software provided stain intensity values of 0 for the most intensive (black) staining and a value of 250 for the least intensive (white) staining; the more intensive (darker) the staining, the more mitochondria present in the muscle fiber. To create easier to read graphics, the inverse of oxidative capacity was calculated so that values closer to 0 denoted lesser oxidative capacity and values closer to 1 denoted greater oxidative capacity.

Statistical Analysis

All data were combined for trials 1 and 2 and analyzed using the International Business Machine (IBM), Inc.'s Statistical Package for the Social Sciences (SPSS), version 26. A two-way repeated measures ANOVA with randomized complete block design was analyzed to determine the effect of sedentary, low-intensity, and high-intensity exercise over time on body weight, fiber type percentage, cross-sectional surface area, and oxidative capacity data (oxidative capacity data were inversed before analysis to ease visualization). If the ANOVA results were significant, a Sidak post-hoc test was evaluated to determine differences between simple main effects of treatments and time. If the ANOVA results were not significant, main effects (treatment and time) were determined. A 95% confidence interval was utilized with P-values less than 0.05 denoting significance.

Results

Heifer Weights

The treatments did not elicit a change in body weight over time ($P = 0.962$); therefore, the main effects of treatment and time were analyzed (Table 3.2 and Figure 3.2). There were no differences caused by treatment on body weights ($P = 0.525$). There were differences caused by time on body weights ($P = 0.000$), with week 1 similar to weeks 2 and 3($P > 0.05$), week 4 similar to weeks 5 and 6 ($P > 0.05$), week 6 similar to week 7 ($P > 0.05$), and week 7 similar to week 8 ($P > 0.05$).

Table 2.3 Body weights over weeks 1-8 of trials 1 and 2

Week	Sedentary Treatment	Low-Intensity Treatment	High-Intensity Treatment	*P*-Value		
				Time	Treatment	Treatment x Time
1	322.73 ± 11.6	323.94 ± 8.7	333.84 ± 11.2	*0.000*	0.525	0.962
2	321.90 ± 11.0	325.75 ± 8.9	331.65 ± 11.4			
3	324.85 ± 12.0	328.55 ± 10.2	337.17 ± 10.7			
4	335.96 ± 12.9	340.50 ± 8.6	346.02 ± 13.0			
5	340.50 ± 10.5	342.99 ± 8.1	350.02 ± 12.4			
6	345.11 ± 11.4	352.44 ± 8.1	356.75 ± 12.8			
7	350.02 ± 10.4	357.96 ± 8.0	361.59 ± 12.8			
8	359.02 ± 12.5	368.77 ± 8.1	373.16 ± 9.6			

Treatment values are mean weight (kg) ± the standard error of the mean

Average Body Weight of Heifers Per Treatment Per Week for Trials 1 and 2

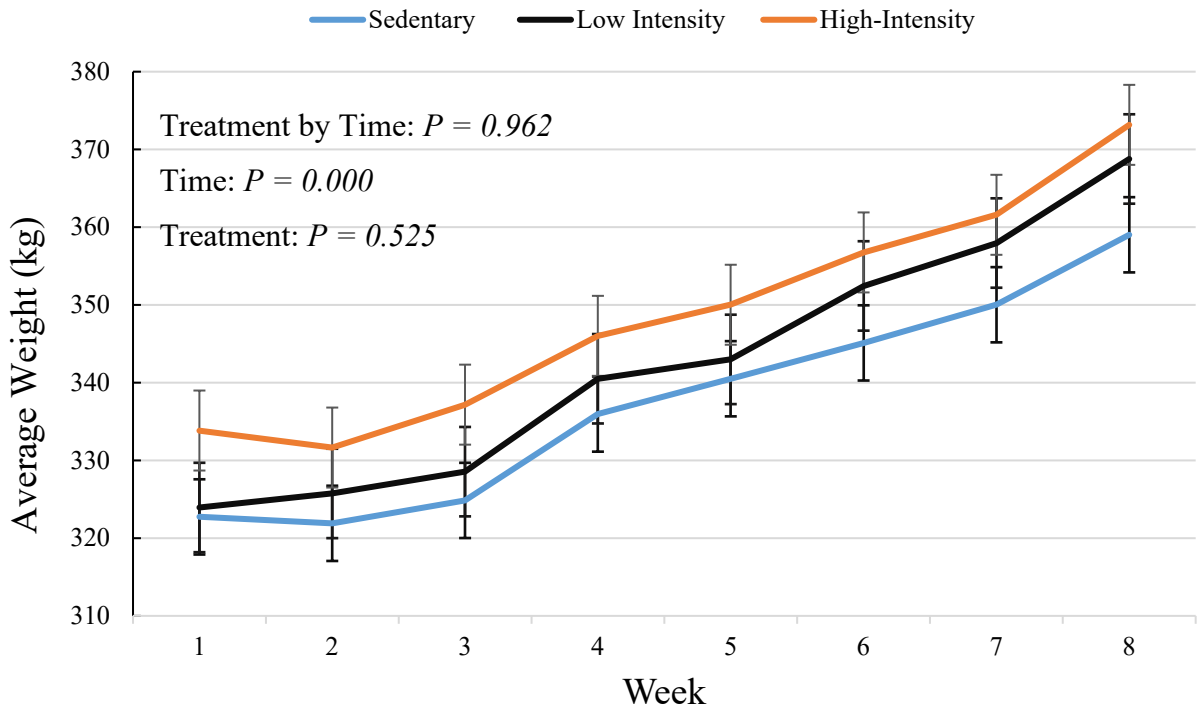


Figure 2.2 Heifer weights combined for trials 1 and 2.

Heifers in each treatment group were weighed weekly throughout the 8-week treatment period in both trial 1 (early to mid-summer) and trial 2 (mid to late-summer); trial data were combined for analysis. Heifers were either sedentary (n = 14; taken to the exerciser but not exercised), exercised at low intensity (n = 15), or exercised at high intensity (n = 13). Treatment x time, time, and treatment P values are as indicated within the graph.

Effects of Exercise Intensity on Muscle Fiber Type Percentage

The exercise treatments did not elicit change in type I fiber percentages over time (*P = 0.362*); therefore, main effects were analyzed for type I fiber percentages (Table 2.4). There were no differences caused by treatment or time (*P = 0.486* and *P = 0.101*, respectively) on percentage of type I fibers (Figures 2.3 and 2.4).

As for type IIA fiber percentages, the exercise treatments did not elicit change in type IIA fiber percentages over time (*P = 0.582*); therefore, main effects were analyzed for type IIA fiber percentages (Table 2.4). There were no differences caused by treatment (*P = 0.734*) on percentage of type IIA fibers (Figures 2.3 and 2.4); however, there were differences caused by time (*P = 0.025)*. The differences were due to the increase in type IIA fiber percentages for all three treatments between week 0 and week 8 (mean difference of 5.334).

Additionally, the exercise treatments did not elicit changes in type IIX fiber percentages over time (*P = 0.205*); therefore, main effects were analyzed for type IIX fiber percentages (Table 2.4). There were no differences caused by treatment (*P = 0.862*) on percentage of type IIX fibers (Figures 2.3 and 2.4); however, there were differences caused by time (*P = 0.009)*. The differences were due to the decrease in type IIX fiber percentages for all three treatments between week 0 and 8 (mean difference of -6.755).

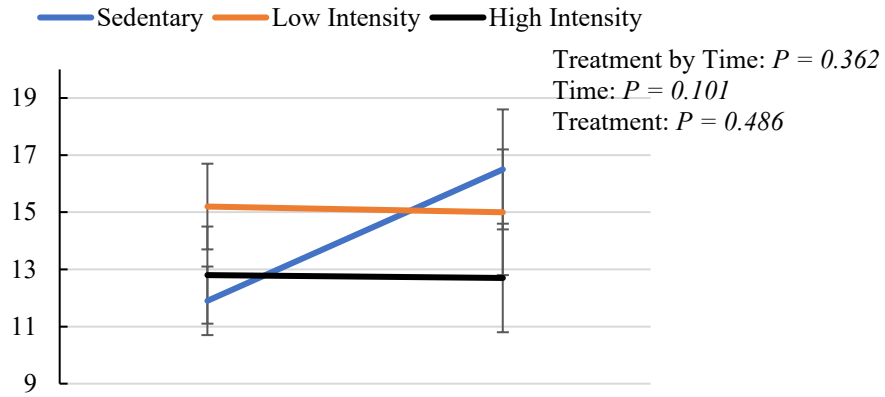
Table 2.4 Average fiber type percentages for sedentary, low-intensity, and high-intensity treatments between week 0 and week 8

	Sedentary Treatment		Low-Intensity Treatment		High-Intensity Treatment		*P*-Value		
Fiber type	Week 0	Week 8	Week 0	Week 8	Week 0	Week 8	Time	Treatment	Treatment x Time
I	11.9 ± 1.2	16.5 ± 2.1	15.2 ± 1.5	15.0 ± 2.2	12.8 ± 1.7	12.7 ± 1.9	0.101	0.486	0.362
IIA	26.8 ± 1.6	33.0 ± 1.2	24.4 ± 2.0	31.1 ± 3.4	27.8 ± 2.0	30.8 ± 2.9	*0.025*	0.734	0.582
IIX	61.3 ± 0.7	50.5 ± 2.0	60.4 ± 2.6	53.9 ± 4.1	59.5 ± 3.1	56.5 ± 3.9	*0.009*	0.862	0.205

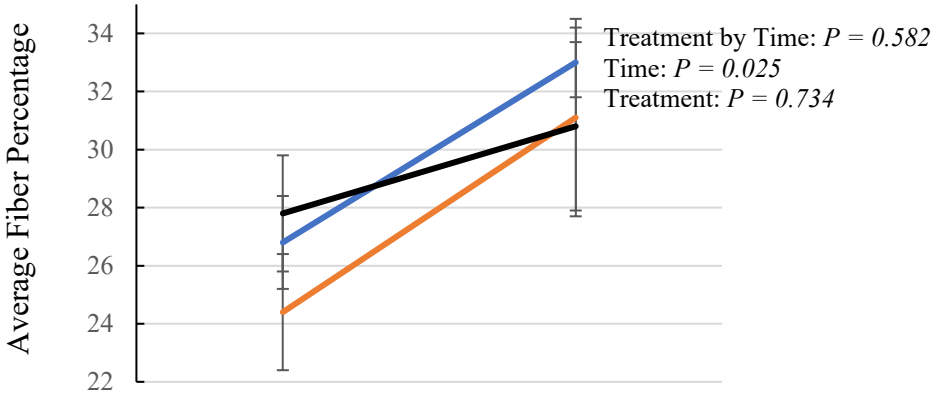
Treatment values are mean percentage (%) ± standard error of the mean

Average Fiber Percentages Between Treatments and Time

A) Average Type I Fibers



B) Average Type IIA Fibers



C) Average Type IIX Fibers

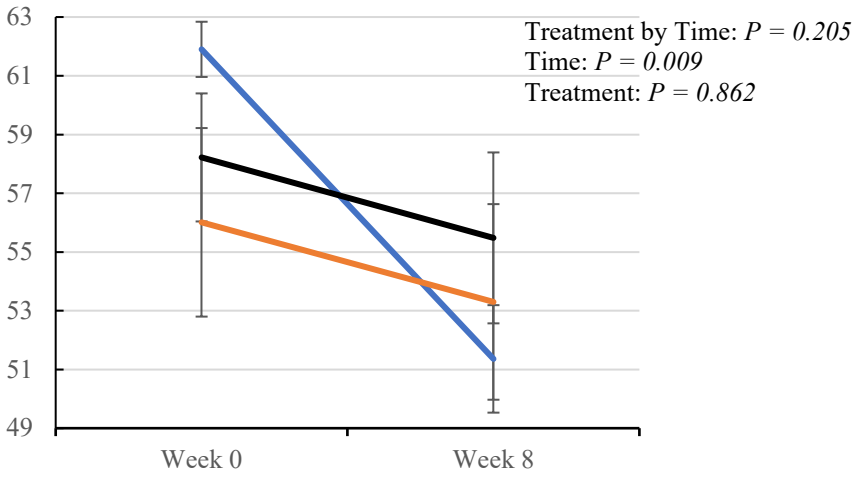


Figure 2.3 Average fiber type percentages between treatments at week 0 and 8

Muscle biopsies were collected from the right semitendinosus of Holstein heifers at week 0 and week 8 of each of the 8-week exercise trials. Trial 1 and 2 data were combined for analysis. Results from immunofluorescence analysis are expressed as the average number of fibers per section per treatment for type I (panel A), IIA (panel B), and IIX (panel C) fibers. Treatment by time interaction, time, and treatment P values are indicated in Panels A-C.

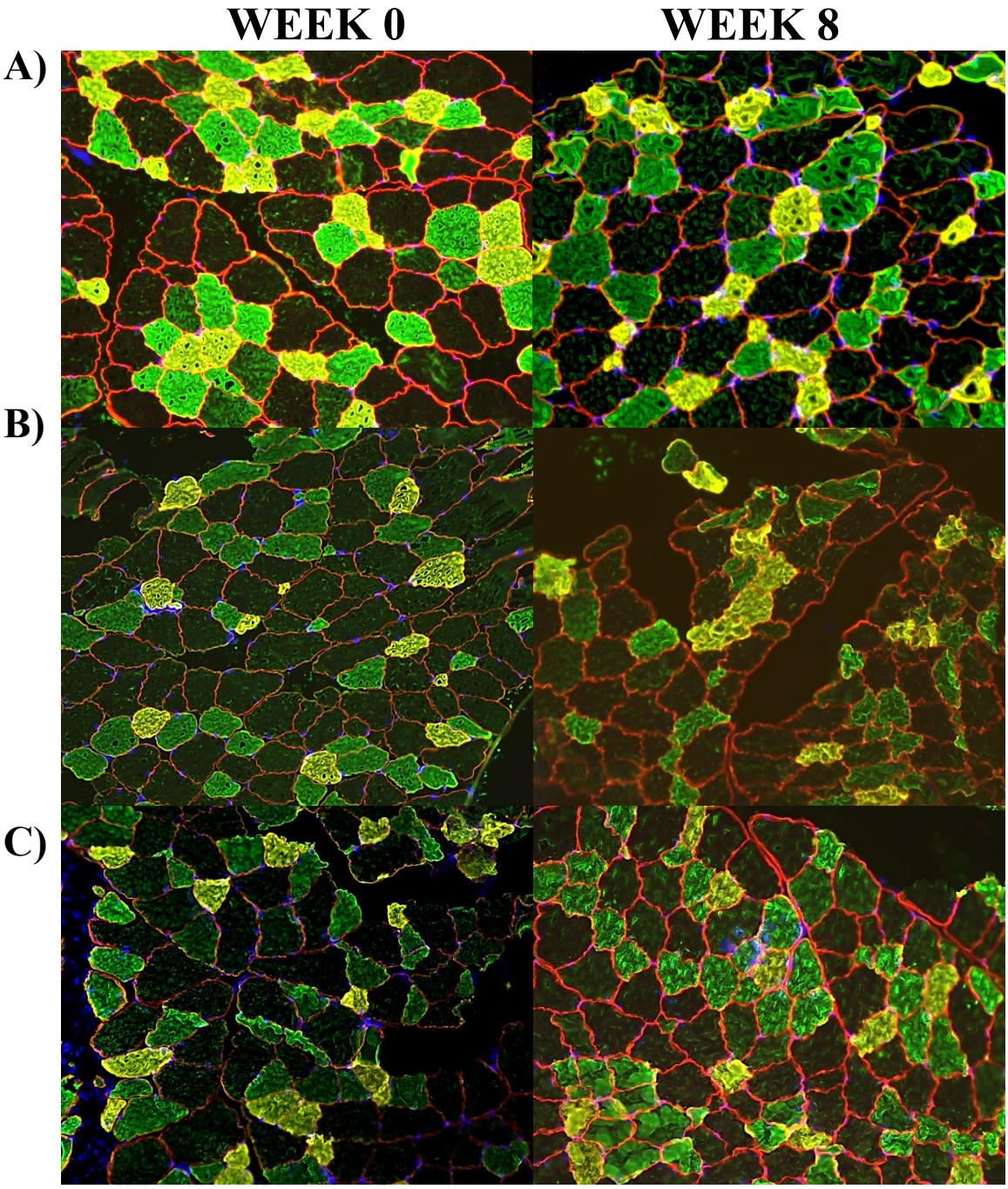


Figure 2.4 Immunofluorescence staining for fiber type and surface area at weeks 0 and 8

Representative images of fiber type composition obtained from biopsies of the semitendinosus muscle for sedentary (Panel A), low-intensity (Panel B), and high-intensity (Panel C) treatment groups prior to treatment (week 0) and after 8 weeks of treatment (week 8). Red boundaries represent the sarcolemma of the muscle fibers; the sarcolemma circumference was measured to determine cross-sectional surface area of muscle fibers. Yellow indicates a type I, slow-twitch oxidative fiber. Green indicates a type IIA, fast-twitch oxidative glycolytic fiber. Colorless fibers indicate a type IIX, fast-twitch glycolytic fiber.

Effects of Exercise Intensity on Muscle Fiber cross-sectional area

The exercise treatments did not elicit changes in type I, IIA, or IIX fiber cross-sectional surface areas over time (*P = 0.839, P = 0.938,* and *P = 0.532,* respectively); therefore, main effects were analyzed (Table 2.5). There were no differences caused by treatment or time (*P = 0.339* and *P = 0.181*, respectively) on type I fiber cross-sectional surface area (Figure 2.5). There were no differences caused by treatment (*P = 0.366*) on type IIA fiber cross-sectional surface area; however there were differences caused by time, as all heifers in all treatment groups had increased surface area of type IIA fiber types between weeks 0 and 8 (*P = 0.021)*. There were no differences caused by treatment or time (*P = 0.679* and *P = 0.136*, respectively) on type IIX fiber cross-sectional surface area.

Table 2.5 Average fiber cross-sectional surface areas at week 0 and week 8 for all treatments

	Sedentary Treatment		Low-Intensity Treatment		High-Intensity Treatment		*P*-Value		
Fiber type	Week 0	Week 8	Week 0	Week 8	Week 0	Week 8	Time	Treatment	Treatment x Time
I	2715.6 ± 233.8	3029.7 ± 143.9	3142.8 ± 311.8	3366.0 ± 431.4	2664.5 ± 223.2	3218.8 ± 149.8	0.181	0.339	0.839
IIA	3073.9 ± 210.0	3674.1 ± 120.2	3522.6 ± 369.3	3961.6 ± 328.2	3124.3 ± 364.1	3777.8 ± 246.9	*0.021*	0.366	0.938
IIX	4970.4 ± 395.3	5340.1 ± 268.0	5192.6 ± 565.2	5979.7 ± 379.6	5301.8 ± 547.5	5269.1 ± 366.2	0.136	0.679	0.532

Treatment values are mean fiber cross-sectional surface area (μm) ± the standard error of the mean

Average Fiber Cross-Sectional Surface Area Between Treatment and Time

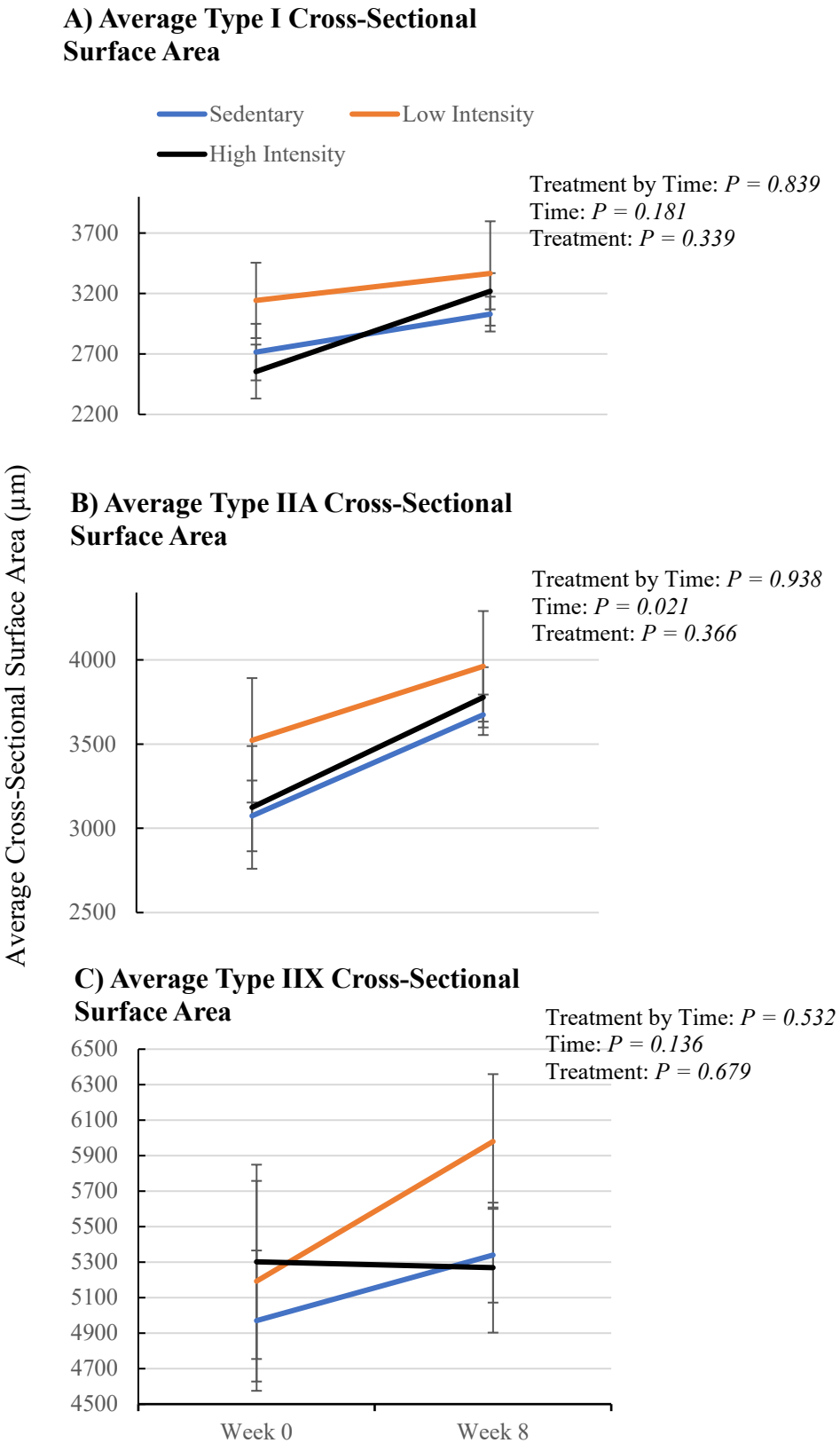


Figure 2.5 Average fiber type cross-sectional surface areas in response to exercise.

Muscle biopsies were collected from the right semitendinosus of Holstein heifers at week 0 and week 8 of both 8-week exercise trials. Trial 1 and 2 data were combined for analysis. Results from immunofluorescence analysis are expressed as the average cross-sectional surface area per fiber per section per treatment for type I (panel A), IIA (panel B), and IIX (panel C) fibers. Treatment by time interaction, time, and treatment P values are indicated in Panels A-C.

Effects of Exercise Intensity on Oxidative Capacity of Fiber Types

The exercise treatments did not elicit change in type I, IIA, or IIX fiber oxidative capacity over time (*P = 0.228, P = 0428,* and *P = 0.331,* respectively); therefore, main effects were analyzed (Table 2.6). There were no differences caused by time (*P = 0.431*) on type I fiber oxidative capacity; however, there was a difference caused by treatment (*P = 0.020)* on type I fiber oxidative capacity, as high-intensity treatment caused a more intense staining than the low-intensity treatment (mean difference of 0.004) (Figures 2.6 and 2.7). There were no differences caused by time (*P = 0.891*) on type IIA fiber oxidative capacity; however, there was a difference caused by treatment (*P = 0.043)* on type IIA fiber oxidative capacity, as low and high-intensity treatments caused a higher staining intensity at week 8 than the sedentary treatment (mean difference of 0.003). There were no differences caused by treatment or time (*P = 0.067* and *P = 0.248*, respectively) on type IIX fiber oxidative capacity.

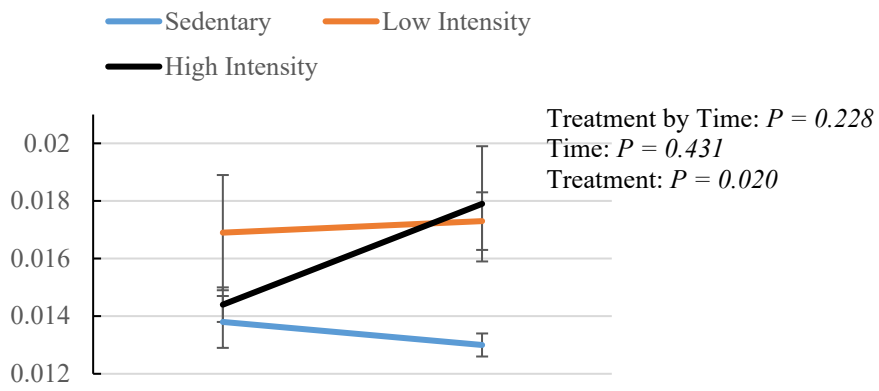
Table 2.6 Average fiber oxidative capacities between treatments at week 0 and week 8.

	Sedentary Treatment		Low-Intensity Treatment		High-Intensity Treatment		*P*-Value		
Fiber type	Week 0	Week 8	Week 0	Week 8	Week 0	Week 8	Time	Treatment	Treatment x Time
I	0.0138 ± 0.0009	0.0130 ± 0.0004	0.0169 ± 0.0020	0.0173 ± 0.0010	0.0144 ± 0.0006	0.0179 ± 0.0020	0.431	*0.020*	0.228
IIA	0.0097 ± 0.0007	0.0089 ± 0.0002	0.0109 ± 0.0009	0.0104 ± 0.0006	0.0102 ± 0.0005	0.0112 ± 0.0009	0.891	*0.043*	0.428
IIX	0.0070 ± 0.0003	0.0069 ± 0.0001	0.0075 ± 0.0004	0.0079 ± 0.0005	0.0069 ± 0.0001	0.0078 ± 0.0004	0.248	0.067	0.331

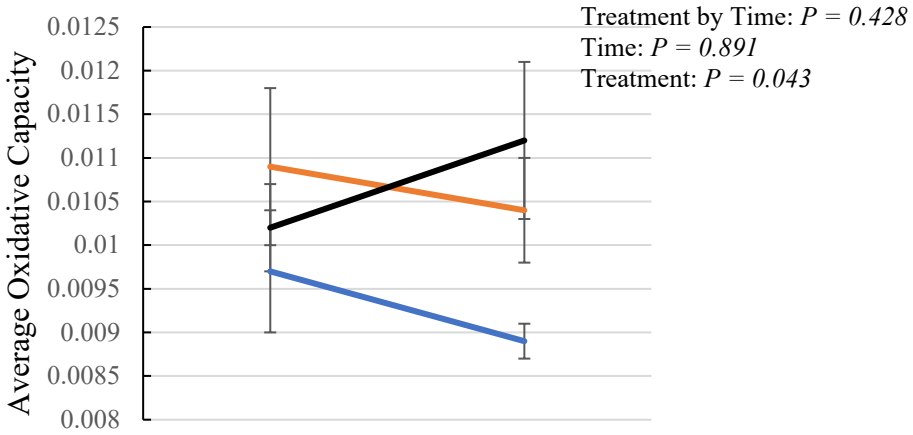
Treatment values are the means of the inverse of the staining intensity provided by NIS-Elements Imaging software ± the standard error of the mean

Average Fiber Oxidative Capacity Between Treatment and Time

A) Average Type I Oxidative Capacity



B) Average Type IIA Oxidative Capacity



C) Average Type IIX Oxidative Capacity

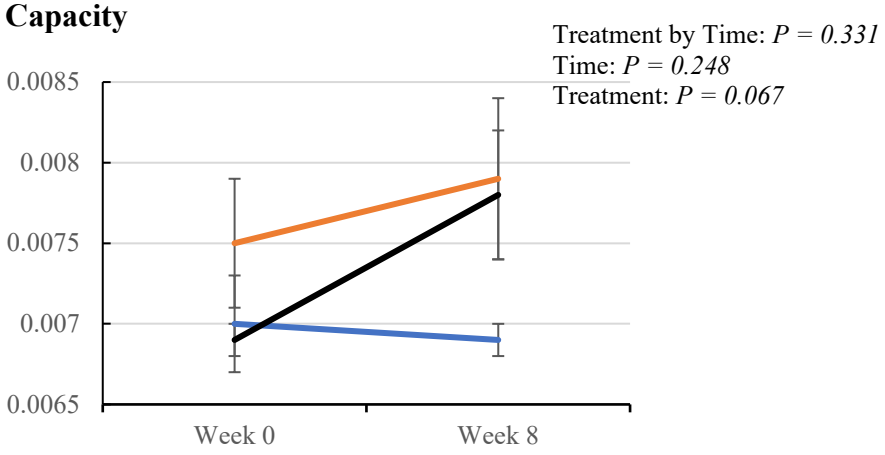


Figure 2.6 Average fiber type oxidative capacities between treatments and time.

Muscle biopsies were collected from the right semitendinosus muscle of Holstein heifers at week 0 and week 8 of each of the 8-week exercise trials. Trial 1 and 2 data were combined for analysis. Results from immunofluorescence analysis are expressed as the average oxidative capacity per fiber per section per treatment for type I (panel A), IIA (panel B), and IIX (panel C) fibers. Treatment x time, time, and treatment P values are indicated in each panel.

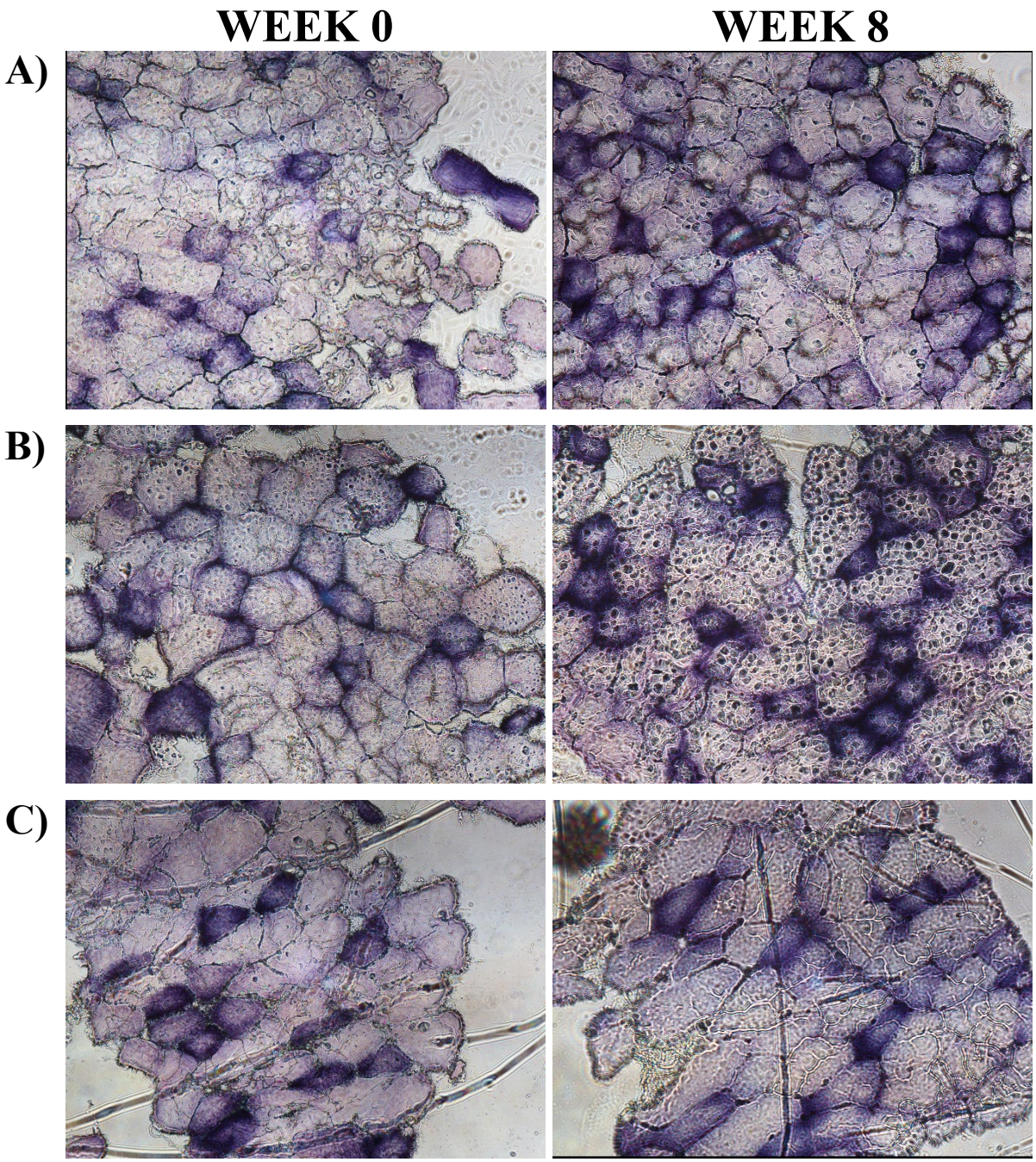


Figure 2.7 Immunofluorescence staining for fiber oxidative capacity at weeks 0 and 8

Representative images of fiber type oxidative capacity obtained from biopsies of the semitendinosus muscle for sedentary (Panel A), low-intensity (Panel B), and high-intensity (Panel C) treatment groups prior to treatment (week 0) and after 8 weeks of treatment (week 8) for trial 1. Succinate dehydrogenase activity was determined based on staining intensity from a range of 0 (most intense staining) to

250 (least intense staining). Type I fibers are stained a darker purple and have more oxidative capacity (mitochondrial density), type IIA fibers have purple staining around the cell border, and type IIX fibers are light purple or white.

Discussion

To date, no studies exist that have analyzed the effects of exercise on fiber type composition in Holsteins. We compared muscle fiber type percentages, cross-sectional area, and oxidative capacity before and after two, 8-week treatment periods between Holstein heifers that were sedentary, exercised at low intensity, or exercised at high intensity. Although two trials were performed and, although there were differences in pre-trial housing (trial 1 heifers were housed on a pasture before the treatment period while trial 2 heifers were housed on a drylot) and date of trial (early summer versus late summer), data were combined among trials for analysis. Data integration was performed to increase sample size and, as we did a randomized complete block design, was considered robust to any differences between trials. Ultimately, we found that our treatments did not cause significant change to fiber percentages, surface area, or oxidative capacity over time.

Tyler (1998), Rivero (1995), Sinha (1993), Schiaffino & Reggiani (2011), Hudlicka (1992), Turner et al. (1997), and Scott et al. (2001) found that low- and high-intensity exercise caused increases in type I and IIA fiber percentages; however, in this study, exercise did not affect fiber type percentage for type I, IIA, or IIX fibers. The discrepancies between our data and previous research is potentially due to differences between the muscles utilized for biopsy analyses, as well as differences between the sexes. Most of the muscles used in the previous studies were from the vastus lateralis and costal diaphragm; however, this study utilized semitendinosus muscle. Suzuki et al. (1999) indicated that the muscle biopsies obtained from vastus lateralis and semitendinosus were very different regarding fiber type composition in pigs and, not only are the muscles themselves different regarding fiber composition, but specific regions (cranial versus caudal and

lateral versus medial) in the muscle have different fiber proportions as well. The medial vastus lateralis contained 19.4% type I fibers while the lateral vastus lateralis contained 5.2%; the semitendinosus contained 4.2% caudally and 42.6% cranially (Suzuki et al., 1999). It is likely that, because muscle biopsy type and obtainment location were different, previous research is not indicative of the changes that exercise induced in fiber type percentage.

Interestingly, most of the studies mentioned in the last two paragraphs used male subjects in their research, with the exception of Simoneau et al. (1985) who used 40% women and 60% men, and Howald et al. (1985) who used 50% men and 50% women. Staron et al. (2000) reported a difference of fiber proportions in men and women, with quantity of IIA fibers>type I fibers>type IIX fibers in men and quantity of type I fibers>type IIA fibers>type IIX fibers in women. Others have also stated that differences between the sexes exist in rabbits (Eason et al., 2000 and English et al., 1999, respectively). We assume that this is because of hormonal differences between males and females. Therefore, it is possible that sex has influenced how fiber types change after exercise in females; exercise results that have been reported from male subjects might not be equivocal to exercise results from female cattle.

Additionally, there was an effect of time on fiber type percentage; however, this is likely due to growth of heifers over time since treatment had no impact. Muscle fiber type in relation to growth and development has seemed to vary between species and the muscle analyzed. Wicks et al. (2019) reported that muscle fibers in newborn calves were more oxidative (type I or IIA) than glycolytic (IIX) but, that as cattle aged, glycolytic fibers increased in quantity and oxidative fiber types decreased; however, this was opposite of what we witnessed in our heifers, as there was an increase in IIA fiber percentage and a decrease in IIX fiber percentage over time. As Wicks et al. (2019) measured changes in beef cattle, there could be an effect of breed on growth's impacts on

muscle fiber composition. Additionally, Wicks et al. (2019) did not dictate which muscles were utilized for their research, so there is the possibility that some muscle types become more oxidative through growth and activity versus others becoming more glycolytic.

In previous studies on humans, high-intensity exercise treatments increased the surface area of type I and IIA fiber types (Tyler et al., 1998; Lopez-Rivero et al., 1992; Rivero et al., 1995; Serrano et al., 2000; Rivero et al., 2001; Yamano et al., 2002; Gottlieb et al., 1989; D'Angelis et al., 2005). After low intensity exercise in humans, Mitchell et al. (2012) found an approximate 23% increase in the surface area of type I and an approximate 16% increase in IIA fibers. In Arabian horses that engaged in low- to moderate-intensity exercise, surface area of type I and IIA fibers also increased (D'Angelis et al., 2005). We did not see the increase in surface area of type I and IIA fibers from low and high-intensity exercise and this was possibly due to differences between sex (the previously indicated research was done on both males and females versus just females). Staron et al. (2000) concluded that cross-sectional surface area between women and men is different, so the possibility of sex being a determining factor may exist. There was an effect of time on the surface area of type IIA fibers, as IIA fibers increased in surface area over the 8-week trial. Heifers were continuously growing over the treatment periods and it is likely that this growth contributed to the hypertrophy of IIA fiber types over the span of 8 weeks (Wicks et al., 2019).

Oxidative capacity also did not change from time and treatment. Increases in oxidative capacity are associated with a concurrent increase of mitochondria (Schwerzmann et al., 1989). Turner et al. (1997), Bishop et al. (2014), and MacInnis and Gibala (2016) reported that the mechanisms by which mitochondrial density (and thus oxidative capacity) increases and to what extent it increases, varies between species and among different muscles. Others have reported increases in oxidative capacities of type I and IIA fibers after low- and high-intensity exercise in

humans, specifically in muscles such as the vastus lateralis and diaphragm (Powers et al., 1992; Jansson & Kaijser, 1977). Differences between muscle characteristics could occur due to some muscles being preemptively more glycolytic or oxidative before exercise trials. A muscle that is more glycolytic at the beginning of an exercise trial might show greater increases in oxidative capacity than a muscle that is already optimized for oxidative capacity. Mattson et al. (2002) performed a study using hamsters and found relative oxidative capacities of muscles were different: the semitendinosus (used in our previous study) and biceps femoris (used herein) were similar in oxidative capacity (citrate synthase activity [indicative of oxidative capacity] of 13.5 versus 17.9, respectively), while the vastus lateralis and costal diaphragm had a greater oxidative capacity (citrate synthase activity 45.6 and 64.6, respectively). Due to differences between oxidative capacity of different muscle groups, results from this chapter may not be comparable with others' results.

One potential issue with exercising Holstein heifers is that more energy is used for the activity and less is available for growth, resulting in slower growth rates during the exercise period. In our study, however, body weight was unaffected by either high or low intensity exercise and was also similar for sedentary heifers over the 8 weeks of the study. While the steady weight gain was expected over time, the growth could have impacted the skeletal muscle composition of the heifers. Muscle fiber type in relation to growth and development has varied between species and the specific muscle. Wicks et al. (2019) reported that muscle fibers in newborn calves were more oxidative (type I or IIA) than glycolytic (IIX) but, that as cattle aged, glycolytic fibers increased in quantity and oxidative fiber types decreased. Wegner et al. (2000) further demonstrated fiber type proportions in the semitendinosus changed, but only between birth and 4 months of age. Wegner et al. (2000) also found type I, IIA, and IIX fiber quantity did not change in Holstein

semitendinosus muscle between 4 and 12 months of age and that the surface area of muscle fibers all increased consistently for Holstein cattle between birth and 24 months of age. Thus, while it is possible that growth impacted fiber type percent distribution, the probability is likely low due to the age of our heifers (7-9 months of age). Additionally, we used a group of heifers that underwent sedentary treatment as comparison; this allowed us to determine if fibers changed significantly between the exercised and non-exercised groups regardless of the effects of growth.

For future studies we would suggest the use of continuous body temperature measurements, potentially with a vaginal thermometer or ear sensor, to determine if there is a correlation between exercised-induced skeletal muscle changes and the ability of a heifer to cool herself. Additionally, analysis of low-intensity and high-intensity exercise on capillary density may prove an interesting supplement to this study. It would also be useful to more precisely examine the amount and duration of exercise necessary to cause acclimations, because, while human acclimation to heat can be seen in less than 2 weeks due to increases in eccrine sweat glands (Pandolf, 1998; Sawka et al., 1996), dairy cattle, due to the inactivity of their eccrine sweat glands and thus decreased likelihood for evaporative cooling improvements, may have an altered responsiveness to repeated deviations in body temperature. Non-esterified fatty acids could be analyzed to determine the effects of exercise on liver health since increased lipid accumulation in the liver is a common occurrence in heat stressed dairy cattle (Skibiel et al., 2018). Fatty liver disease has been shown to decrease in exercised humans (van der Windt et al., 2018) and thus it is possible that repeated bouts of exercise improves the ability of the liver to process fatty acids via beta-oxidation, because the release of adrenal medullary catecholamines during exercise causes decreased triglyceride storage and thus increased circulating fatty acids.

To conclude, there are many avenues for exploration on dairy cattle exercise. There were no effects of time and treatment on fiber type percentage, surface area, or oxidative capacity, potentially indicating that exercise does not impact improvements in thermoregulatory cooling of heifers any more than a mostly sedentary lifestyle does. Further research should be conducted on capillary density and thermoregulatory changes after exercise treatment to determine other health and heat protective benefits of exercise in Holsteins.

Chapter 3 - Effects of Low-Intensity Exercise on Holstein Dairy Heifers' Thermoregulatory Capabilities and Skeletal Muscle Composition

Introduction

Exercise in hot, humid environments increased sweat output and capillary density, as well as redistributed and hypertrophied skeletal muscle towards more capillary dense fiber types (Sawka et al., 1996; Sawka et al., 2011; Périard et al., 2015). Acclimation to heat has occurred in human and equine exercise studies, presumably due to the continuous and consistent deviations in body temperature during regular bouts of exercise in the heat (Geor & McCutcheon, 1998; Taylor, 2014; Periard et al., 2015). Regular deviations in body temperature caused by exercise in a hot environment, have been shown to increase the shear force on blood vessel walls and increase angiogenic and angioadaptive signals (Chaar et al., 2015) to increase capillary density within the skin and skeletal muscles. In addition to capillary growth after exercise, type I and IIA muscle fibers and their respective cross-sectional surface areas have also been reported to increase (Hudlicka et al., 1992; Sinha et al., 1993; Rivero et al. 1995; Tyler et al., 1998; Schiaffino &

Reggiani, 2011). Because of the effects of exercise in a hot, humid environment, utilization of exercise may prove beneficial in the prevention and/or abatement of heat stress in Holsteins.

In a previous study (Chapter 2) we examined the effects of low- and high- intensity exercise on fiber type count, cross-sectional surface area, and oxidative capacity of semitendinosus biopsies from Holstein heifers. Ultimately, we found that the three treatments did not influence skeletal muscle changes, either due to the type of exercise or in comparison to peers that remained sedentary.

In this study we compare sedentary and low-intensity exercise treatments to further examine muscle changes in response to exercise. Regular deviations in body temperature caused by exercise in a hot environment, have been shown to increase the shear force on blood vessel walls and increase angiogenic and angioadaptive blood markers (Chaar et al., 2015). Through angiogenesis and angioadaptation, new blood vessels are created within muscle and skin. Increased capillary density and more efficient structure of capillaries are presumed to improve cooling capabilities by allowing space for more heated blood from the viscera towards the body's surface for abatement. In addition, capillary growth after exercise trials are often accompanied by changes in skeletal muscle, with increases in the amount of type I and IIA fibers and their respective cross-sectional surface areas (Hudlicka et al., 1992; Sinha et al., 1993; Rivero et al. 1995; Tyler et al., 1998; Schiaffino & Reggiani, 2011).

In addition, we wanted to determine if, through continuous measurement of body temperature, body temperature trends for cattle indicated improved cooling capabilities and thus potential physiological acclimation to hot environments. We also wanted to know if CowManager ear tag sensors could be utilized to effectively determine changes in temperatures after exercise and on days of rest.

Our hypothesis was that low-intensity exercise during high ambient temperatures, would, through regular and repeated deviations in body temperature, cause an increase in capillary density (and potentially a concurrent increase in type I and IIA fiber types) that would improve efficiency of convective cooling and thus potentially improve thermoregulation of Holsteins in hot ambient environments.

Materials and Methods

Experimental Design

The Kansas State University Institutional Animal Care and Use Committee approved all protocols for the experimental use of live animals (Protocol #3710).

Housing and Care of Heifers

Between June 12 and August 7 of 2017, 28 Holstein heifers between 7 and 9 months of age were obtained from the Kansas State University Dairy Teaching and Research Center for exercise treatment. Before and during the exercise trial, heifers were housed together in a drylot pen with access to a single, sloped-roof shelter. Water was provided *ad libitum* and a total mixed ration (TMR) was delivered to the heifers twice a day. The TMR (Table 3.1) was comprised of approximately 28% corn gluten feed on a dry matter (DM) basis, 13% ground corn grain (DM), 39% cool season grass hay (DM), 1% limestone (DM), 1% vitamin mix (0.2%) (DM), and 19% triticale silage (DM).

Table 3.1 Diet for SED and LI heifers before and during trial

Feed Name	Dry Matter, kg
Corn Gluten Feed	2.02
Corn Grain, Ground	0.97
Grass Hay, Cool	2.83
Limestone	0.08
Vitamin Mix, (0.2%)	0.04
Triticale Silage	1.35

Exercise Treatments

Heifers were stratified by weight and then randomly assigned to 1 of 2 treatments: Sedentary (SED; no exercise treatment applied; *n = 14)*, and low-intensity (LI) exercise (*n = 14)*. The time periods, speed, and amount of repetitions used for low-intensity treatment are listed in Table 3.2.

Table 3.2 Low-Intensity Exercise Treatment

Week	Time (Speed)
W1	15 min walk (4.5 km/h)
W2	20 min (4.5 km/h)
W3 & W4	30 min (4.5 km/h)
W5 & W6	45 min (4.5 km/h)
W7 & W8	60 min (4.5 km/h)

Heifers in the low-intensity group were transported via trailer to an 8-panel free walker (Priefert, 8-horse exerciser, Mount Pleasant, TX) for exercise (Fig. 2.1). After arrival at the exerciser, one to two heifers were placed into each segment. The exerciser was pre-programmed for appropriate speed and duration relative to the treatment being applied; exercise activities commenced as soon as all heifers were loaded into the exerciser. The panels of the exerciser were occasionally electrified to discourage heifers from stopping, but this feature was used minimally. Sedentary heifers were transported for approximately five minutes in a trailer to mimic travel stress occurring for the exercised group of heifers; otherwise, sedentary heifers were kept in their pen during the exercise periods and penned away from food but provided water *ad libitum*.

Trailer loading and transportation of exercised heifers began at approximately 1630 on Monday, Wednesday, and Friday. Start time of exercise varied due to weather and/or transportation challenges, but typically occurred approximately at 1700 on Monday, Wednesday, and Friday.

Figure 3.1 Priefert 8-Panel Exerciser

The exerciser consisted of 8 panels with the ability to move clockwise or counterclockwise. All exercised heifers were trailered to the exerciser and one to two heifers were loaded into each segment of the exerciser. The speed and duration of panel movement were pre-programmed into an attached computer that controlled panel movement. Computer programming for speed and duration was dependent on the week and the treatment being applied.

Biopsy Sample Collection

Biopsy samples from the right biceps femoris muscle were obtained from all heifers 1 week before exercise commenced (pre-exercise biopsy) and again 9 weeks later, after 8 weeks of exercise (post-exercise biopsy). The pre-exercise biopsy was taken approximately 1 cm lateral to the midline of the biceps femoris muscle. The post-exercise biopsy was also from the biceps femoris muscle but, to avoid any scar tissue from the previous biopsy site, was taken 1 cm medial to the midline of the biceps femoris muscle. For all biopsies, hair was removed over the biopsy site, which was subsequently prepared for surgery with betadine (Betadine Surgical Scrub, Doctors Fosters and Smith©, Rhinelander, WI) and 70% ethanol. An injection of 0.5 ml of lidocaine was administered to the biopsy site via a 5 ml syringe with a 20 G needle. A sterile piercing needle was utilized to puncture the dermal layers and was immediately followed by the use of a 10 G × 5 cm

long Quick-Core Biopsy Needle (Cook Medical, Chicago, IL) to obtain approximately 5, 2 mm × 1.5 cm biopsy cores.

The biopsy sample was covered with OCT (optimal cutting temperature) compound (Thermo Fisher Scientific, Kalamazoo, MI), subsequently placed into a supercooled (via liquid nitrogen) isopentane solution, and stored at −80°C until analysis.

Immunofluorescence Histology

Immunofluorescence histology was utilized to distinguish between different fiber types (I, IIA, and IIX), determine the relative succinate dehydrogenase levels of fiber types, and identify the circumference of muscle fiber cross-sections. The staining procedure documented in Phelps et al. (2014a) was used for immunofluorescence of muscle biopsies. Biopsy samples were sectioned using a Microm™ HM 550 Cryostat (Thermo Fischer Scientific, Kalamazoo, MI) and then placed on positively charged microscope slides (Diamond White Glass; Globe Scientific Inc., Paramus, NJ). Three to 4 cryosections per sample were placed approximately 1 mm apart and heat-fixed by placing the slides directly onto the surface of a heated, digital dry bath. Slides were removed from the dry bath surface after 10 minutes and then left to cool for 3 to 5 minutes at room temperature (~23°C). Muscle sections were incubated in primary staining solution within 6 hours of heat fixing the samples to the slides. Two slides were created for each muscle sample: 1 slide for fiber type (5 μm cut thickness) and 1 slide for SDH analysis of oxidation (20 μm cut thickness). Three to 4 slides were placed into a staining box covered with aluminum foil to exclude light. Water was added to the bottom of the staining box to increase the humidity and prevent desiccation of biopsy sections. A Liquid Blocker Super PAP Pen (Daido Sangyo Co. Ltd., Tokyo, Japan) was used to draw a square around the cryosections. All photosensitive reagents were kept at 4°C and in a dark container when not in use.

Cryosections for fiber typing were incubated in 10% equine serum and 0.2% TritonX-100 in a 1X phosphate buffer solution (1X-PBS) for 30 min to block all nonspecific binding sites. The blocking solution was removed at the end of 30 minutes. Cryosections were then incubated for approximately 12-16 hours at room temperature with a primary antibody solution consisting of the blocking solution, 1:500 α-dystrophin for staining the cell's sarcolemma (Thermo Fischer Scientific, Waltham, MA), 1:10 supernatant myosin heavy chain, slow, IgG2b (BA-D5; Developmental Studies Hybridoma Bank, Iowa City, IA), and 1:10 supernatant anti-myosin heavy chain, IgG1, which allows for sole visualization of type IX fibers (BF-35; Developmental Studies Hybridoma Bank, Iowa City, IA). Muscle cryosections were then washed 3 times for 5 minutes each with 1X-PBS. After the last wash, cryosections were incubated for 30 minutes with secondary antibodies in blocking solution containing 1:1000 AlexaFlour 633 goat-anti-mouse IgG2b (Life Technologies, Carlsbad, CA) for BA-D5, 1:1000 Alexa-Flour 594 goat-anti-rabbit heavy and light chains (Life Technologies, Carlsbad, CA) for BF-35, 1:1,000 Alexa-Flour 488 goat-anti-rabbit heavy and light chains (Life Technologies, Carlsbad, CA) for α-dystrophin, and 1:1000 DAPI (Thermo Fischer Scientific, Waltham, MA). After incubation with secondary antibody, sections were washed in 1X-PBS 3 times for 5 minutes each and were protected with a glass coverslip for image processing. Cryosections were stored in the dark at room temperature (~23°C) until visual analysis and photographing. Stained sections were viewed and photographed within 1 to 2 days.

The methods of Noel et al. (2016) were followed for succinate dehydrogenase (SDH) staining as follows: Cryosections were incubated for 1.75 hours in a pre-warmed (37°C) solution containing 20 ml of nitro blue tetrazolium solution (.02 g of nitro blue tetrazolium in 20 ml of Milli-Q water), 10 ml of a phosphate buffer solution different than what was utilized for the fiber-type staining procedure (.195 g of potassium phosphate monohydrate and 10.99 g disodium

hydrogen phosphate in 100 ml of Milli-Q water; solution was replaced every 30 days), and 10 ml of sodium succinate solution (2.7014 g sodium succinate dibasic hexahydrate in 100 ml Milli-Q water; solution was replaced every 30 days). Immediately following incubation, cryosections were washed in Milli-Q water 3 times for 1 minute each and subsequently protected with a glass coverslip for imaging. Muscle sections were kept in the dark at room temperature (~23°C) until ready for visual analysis and photographing.

The methods of Noel et al. (2016) were used for capillary density staining histology with one modification: the Platelet Endothelial Cell Adhesion Molecule (PECAM) used in Noel et al. (2016) would not luminesce the bovine blood vessels involved in this study, therefore a different PECAM antibody was selected for use. Capillary density cryosections were incubated in 10% horse serum and 0.2% TritonX-100 in a 1X phosphate buffer solution (1X-PBS) for 30 min to block all nonspecific binding sites. The blocking solution was removed at the end of 30 minutes. Cryosections were then incubated for approximately 12-16 hours at 4°C with a primary antibody solution consisting of the blocking solution, 1:500 α-dystrophin for staining the cell's sarcolemma (Thermo Fischer Scientific, Waltham, MA), and 1:50 PECAM-1, 10G9 SC-13537 (Santa Cruz Biotechnology, Dallas, TX). Following the primary incubation period, cryosections were washed 3 times for 5 minutes each with 1X-PBS. Post-wash, cryosections were incubated for 30 minutes with secondary antibodies in blocking solution containing 1:1000 Alexa-Flour 594 goat-anti-rabbit IgG_1 (Life Technologies, Carlsbad, CA) for conjugation with the α-dystrophin, and 1:1,000 Alexa-Flour 488 goat-anti-rabbit heavy and light chains (Life Technologies, Carlsbad, CA) for conjugation with the PECAM-1. Muscle sections were thereupon washed in 1X-PBS 3 times for 5 minutes each and were protected with a glass coverslip for imaging. Cryosections were stored in

the dark at room temperature (~23°C) until visual analysis and photographing. Biopsy sections were viewed and photographed within 1 to 2 days post capillary density staining.

Image Processing

Images of cryosections for fiber type and SDH analysis were taken with a 10x apochromatic objective within a Nikon Eclipse TI-U inverted microscope (Nikon Instruments Inc., Melville, NY). Cryosections for capillary density analysis were photographed with a 20x apochromatic objective within a Nikon Eclipse TI-U inverted microscope (Nikon Instruments Inc., Melville, NY). Fiber type and capillary density images were photographed with a Nikon DS-QiMC digital camera (Nikon Instruments Inc., Melville, NY), while SDH sections were photographed with a Nikon DS-Fil color digital camera (Nikon Instruments Inc., Melville, NY). All photographic analyses were performed using the NIS-Elements Imaging software (Basic Research, 3.3; Nikon Instruments Inc.).

Approximately 25,000 fibers were imaged and subsequently analyzed for fiber type and fiber cross-sectional area. For muscle fiber typing, fibers that stained positive for BA-D5 and BF-35 were labeled type I (yellow) and type IIA (green), respectively, and fibers that were not stained by both BA-D5 and BF-35 antibodies were labeled as type IIX fibers (black/colorless) (Moreno-Sanchez et al., 2008; Schiaffino et al., 1989). The cross-sectional area of muscle fibers was determined as the area within the dystrophin border (stained red), and areas were recorded in units of μm^2.

Approximately 5,300 fibers were estimated to be imaged and subsequently analyzed for capillary density. The α-dystrophin cell border was stained green from Alexa-Fluor 594 conjugation, with capillaries staining red from Alexa Fluor 488 conjugation. A red stain was only counted as a capillary if it was lying within the dystrophin border since muscle capillaries only

fuse between fibers and not within. Total fibers within each section were recorded to determine the fiber to capillary ratio of the section.

To determine the succinate dehydrogenase staining intensity of each section, approximately 25,000 fibers were analyzed for fiber type. Once fiber type was determined, each type of fiber (I, IIA, or IIX) was analyzed for average white light intensity (kept constant for SDH analysis) by the NIS-Elements Imaging Software. The software provided intensity values of 0 for the most intensive (most oxidized) staining and a value of 250 for the least intensive (least oxidized) staining. To create easier to read graphics, the inverse of oxidative capacity was calculated so that values closer to 0 denoted lesser oxidative capacity and values closer to 1 denoted greater oxidative capacity.

Temperature and Weight Collection

Both sedentary and low-intensity heifers were fitted with bioinformatic ear tags approximately 1 week before trial commencement. Ear tags were placed into the heifers' left pinnae. Ear tag temperature data were sent digitally to an offsite database, were recorded in Celsius, and were obtained every hour.

Heifer weights, as well as skin and core body temperatures, were manually recorded each Thursday (a day where exercise did not occur) during the exercise period. Data collection started with the first heifer at approximately 16:00 and finished with the last heifer at approximately 17:50. Skin and core body temperatures were recorded while heifers were on the scale. Skin temperatures were taken with an infrared thermometer approximately 6 inches away from the skin surface at the masseter, gluteobiceps, paralumbar fossa, and cranial surface of the left pinnae at approximately the midpoint. Core body temperature was obtained by utilizing a rectal thermometer for approximately 1 minute or until the temperature reading stabilized. Additionally, core body

temperatures and skin surface temperatures were recorded on Monday of week 4 and 8 immediately after the exercise treatment.

Statistical Analysis

All data were analyzed using the International Business Machine (IBM), Inc.'s Statistical Package for the Social Sciences (SPSS), version 25.

A two-way repeated measures ANOVA with randomized complete block design was analyzed to determine the effect of sedentary and low-intensity exercise over time on body weight, fiber type percentage, cross-sectional surface area, and oxidative capacity data (oxidative capacity data were inversed before analysis to ease visualization). If the ANOVA results were significant, a Sidak post-hoc test was evaluated to determine differences between treatments and time. If the ANOVA results were not significant, simple main effects (treatment and time) were determined. A 95% confidence interval was utilized with P-values less than 0.05 denoting significance.

The temperature humidity index (THI) was calculated for each hour during the 8-week exercise period. The hourly THI was calculated using the following formula: (*1.8 x Ambient Temperature (°C) + 32) - [(0.55 - (0.0055 x Ambient Humidity)) x (1.8 x Ambient Temperature – 26)].* Ambient temperature (T_A) and ambient humidity (H_A) were obtained from the Kansas State University Mesonet (KSU Mesonet). The KSU Mesonet utilizes inversion-based temperature/humidity sensors atop towers in the Manhattan, Kansas area. THI was subsequently included within temperature graphs for trend comparison with CowManager Sensor, core body, and mean skin temperatures.

The value for mean skin temperature was obtained by averaging the masseter, gluteobiceps, paralumbar fossa, and left pinnae temperatures that were manually recorded each Thursday. The mean skin temperature was subsequently calculated as a ratio to the core body temperature

(MST:CBT) that was simultaneously obtained by rectal thermometer. Week 4 was omitted from the MST:CBT dataset due to a large rainstorm during temperature acquisition that substantially impacted the obtained temperatures.

A one-way ANOVA was utilized to determine the differences between the CowManager Sensors, skin of the right ear (infrared thermometer), and core body temperature (rectal thermometer) within week 4 and week 8. An independent samples t-test was used to determine differences between weeks and treatments for CowManager Sensors, skin of the right ear, and core body temperature. A 95% confidence interval was utilized with values less than 0.05 denoting significance.

Ear tag temperature data in general were analyzed via the General Linear Model on SPSS to determine within-subject time x treatment interactions. More specifically, cooling and heating rates were evaluated by determining periods of time where ear temperatures were trending towards decreasing or increasing, respectively. A weekly cooling period was determined to be Sundays between midnight and 0400. Sundays between the hours of 1300 and 1700 was determined to be a heating period. Differences between treatments were analyzed by GLM with repeated measures. Significant differences between means were further analyzed by a Sidak Post-Hoc test to determine differences in each treatment between weeks 0 and 8. A 95% confidence interval was utilized with values less than 0.05 denoting significance.

Results

Heifer Weights

The treatments did not elicit a change in body weight over time ($P = 0.582$); therefore, main effects were analyzed (Table 3.3 and Figure 3.2). There were no differences caused by treatment on body weights ($P = 0.771$). Body weights changed over time ($P = 0.000$), with every

time point being different from one another ($P < 0.05$) except weeks 4 and 5 (mean difference of -3.60, $P = 0.831$) and weeks 7 and 8 (mean difference of 0.786, $P = 1.000$).

Table 3.3 Weights of Heifers Over Weeks 1-8

			P-Value		
Week	Sedentary Treatment	Low-Intensity Treatment	Time	Treatment	Treatment x Time
1	272.14 ± 7.8	270.33 ± 7.2	*0.000*	0.771	0.582
2	277.29 ± 8.3	275.35 ± 6.8			
3	282.83 ± 8.0	283.54 ± 5.8			
4	297.18 ± 7.6	291.58 ± 7.5			
5	299.06 ± 8.4	296.89 ± 7.4			
6	307.97 ± 8.4	307.97 ± 7.5			
7	318.06 ± 7.2	315.23 ± 8.0			
8	316.36 ± 9.2	315.35 ± 8.2			

Treatment values are mean weight (kg) ± the standard error of the mean

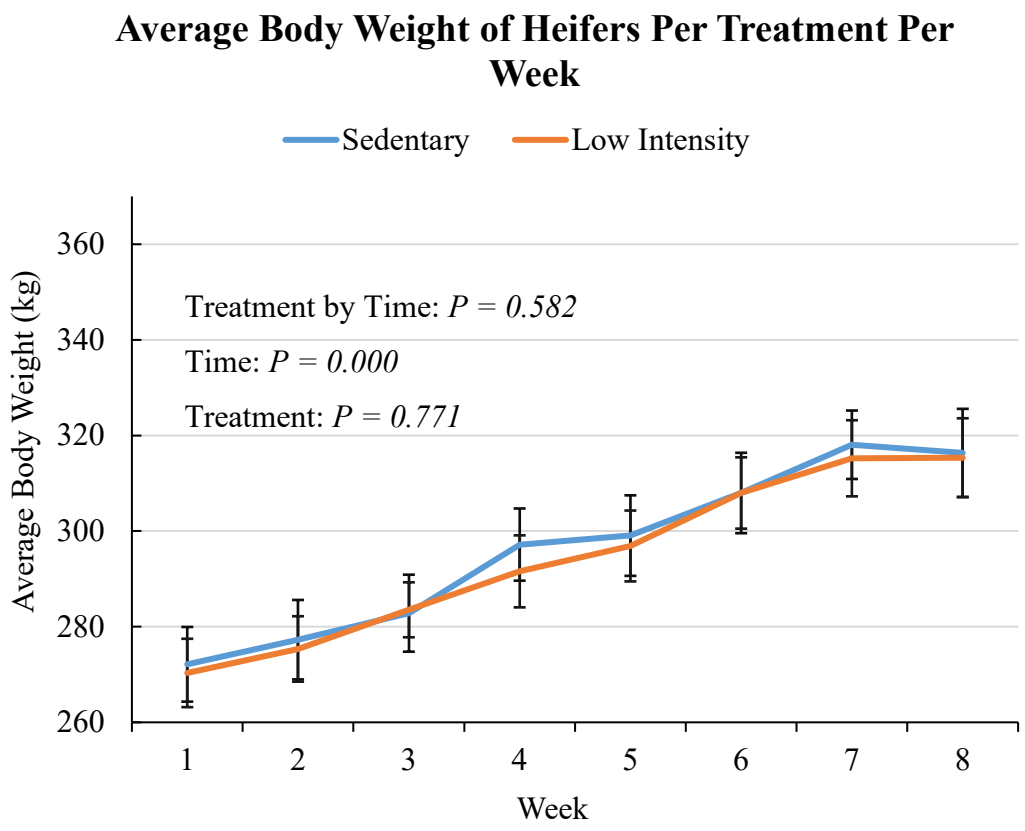


Figure 3.2 Weights of heifers over the 8-week experiment

Heifers in each treatment group were weighed weekly throughout the 8-week treatment period. Heifers were either sedentary (n = 14; taken to the exerciser but not exercised) or exercised at low intensity (n = 14). The average weekly weights of heifers between treatment groups were not significantly different. Treatment x time, time, and treatment P values are indicated in the graph.

Effects of Exercise Intensity on Muscle Fiber Type Composition

The treatments elicited changes in type I fiber percentages over time (*P = 0.030*). Heifers that remained sedentary had a decrease in the percentage of type I fibers over time (mean difference of -6.9%) while heifers that underwent low-intensity exercise had an increase in the percentage of type I fibers over time (mean difference of 2.6%) (Table 3.4; Figures 3.3 and 3.4).

The exercise treatments did not elicit change in type IIA fiber type percentages over time (*P = 0.144*); therefore, main effects were analyzed. There were no differences caused by time (*P = 0.315)* or treatment (*P = 0.744)* for type IIA fibers.

The exercise treatments elicited changes in type IIX fiber type percentages over time (*P = 0.141*). Heifers that remained sedentary had an increase in the percentage of type IIX fibers over time (mean difference of 8.8%) while heifers that underwent low-intensity exercise had a minimal increase in the percentage of type IIX fibers over time (mean difference of 1.7%).

Table 3.4 Average fiber type percentages for sedentary and low-intensity treatments between week 0 and week 8

Fiber type	Sedentary Treatment		Low-Intensity Treatment		*P*-Value		
	Week 0	Week 8	Week 0	Week 8	Time	Treatment	Treatment x Time
I	33.06 ± 3.3	26.24 ± 1.2	29.04 ± 2.0	46.51 ± 2.5	0.494	0.763	*0.030*
IIA	29.52 ± 1.2	39.34 ± 1.5	31.70 ± 1.0	37.55 ± 0.9	0.315	0.744	0.144
IIX	37.42 ± 2.4	46.21 ± 1.2	39.27 ± 1.7	40.94 ± 2.4	0.633	0.524	0.141

Treatment values are mean percentage (%) ± standard error of the mean

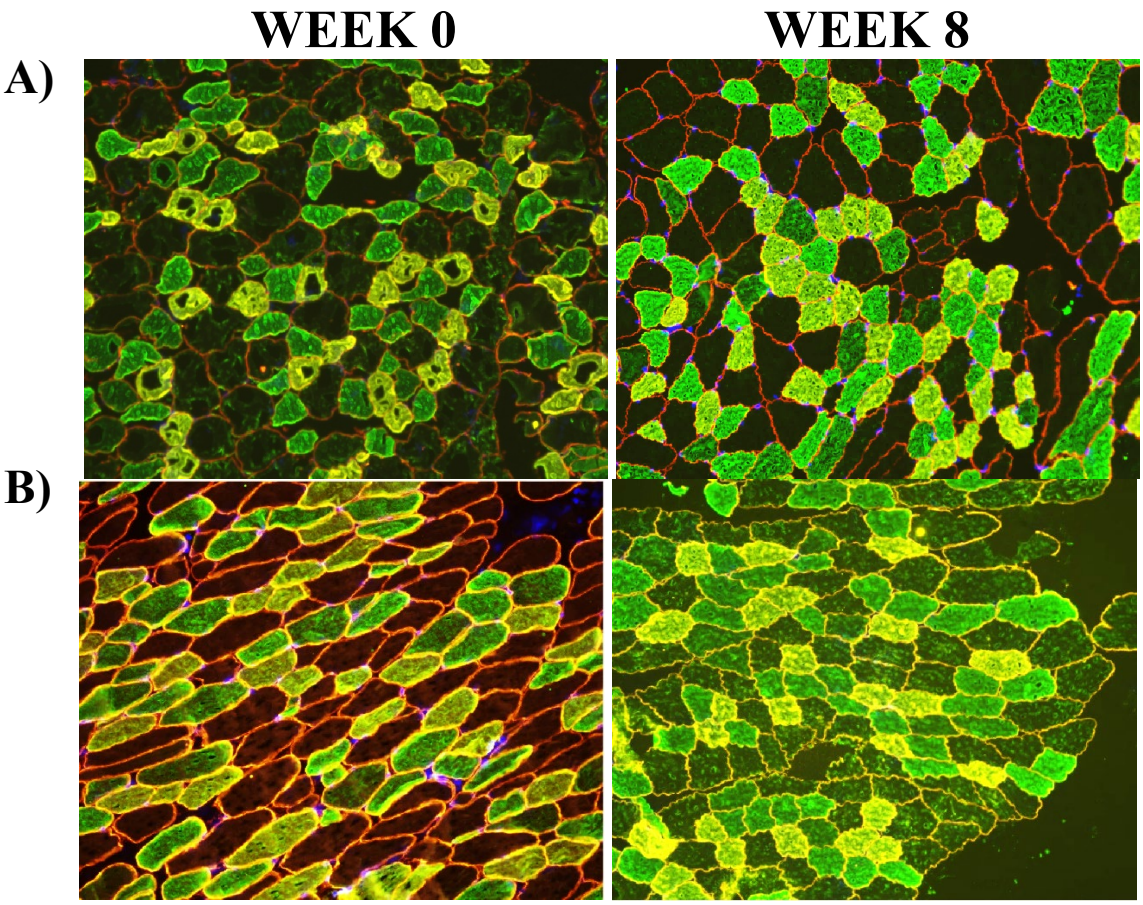
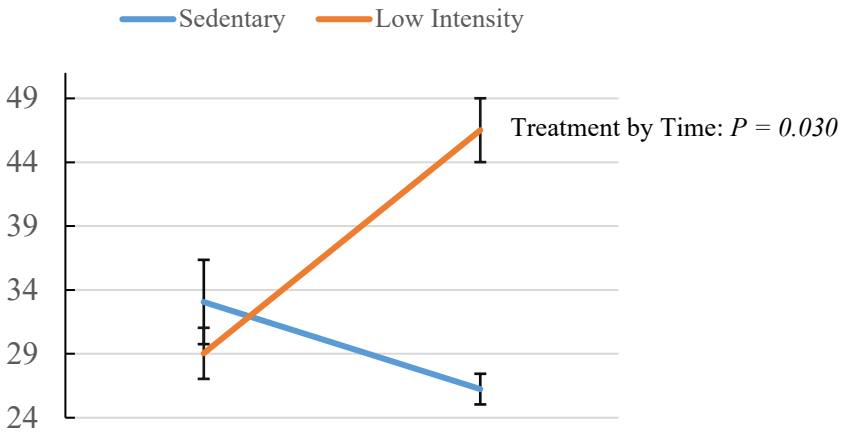


Figure 3.3 Immunofluorescence staining for fiber type and surface area at weeks 0 and 8

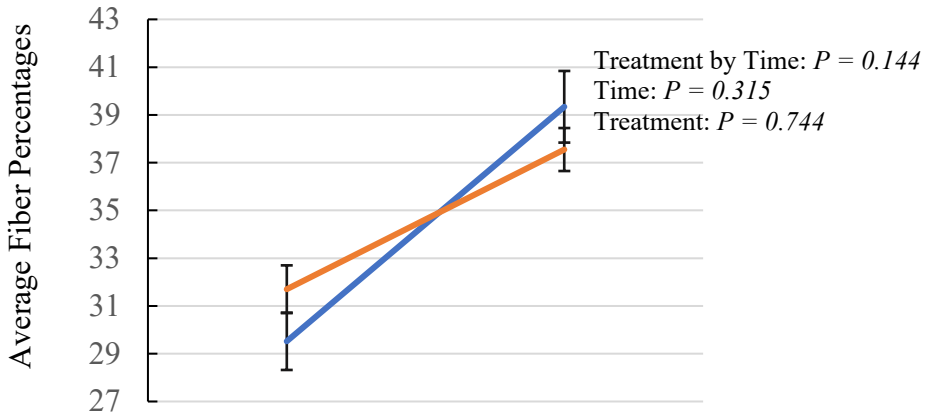
Representative images of fiber type composition obtained from biopsies of the biceps femoris muscle for sedentary (Panel A) and low-intensity (Panel B) treatment groups prior to treatment (week 0) and after 8 weeks of treatment (week 8). Red boundaries represent the sarcolemma of the muscle fibers; the sarcolemma circumference was measured to determine cross-sectional surface area of muscle fibers. Yellow indicates a type I, slow-twitch oxidative fiber. Green indicates a type IIA, fast-twitch oxidative glycolytic fiber type. Colorless fibers indicate a type IIX, fast-twitch glycolytic fiber type.

Average Fiber Type Percentages Between Treatments and Time

A) Average Type I Fibers



B) Average Type IIA Fibers



C) Average Type IIX Fibers

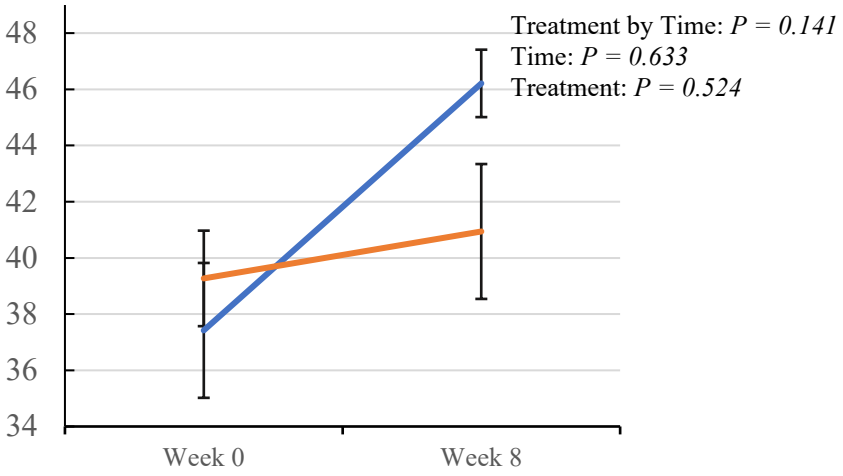


Figure 3.4 Average fiber type counts between treatments at week 0 and 8

Muscle biopsies were collected from the right biceps femoris of Holstein heifers at week 0 and week 8 of the 8-week trial for heifers that remained sedentary (n = 14) or for heifers that underwent low intensity exercise (n = 14). Results from immunofluorescence analysis are expressed as the average number of fibers per section per treatment for type I (panel A), IIA (panel B), and IIX (panel C) fibers. P values for treatment x time and the main effects of time, and treatment are shown for each fiber time in each respective panel. Main effects were not analyzed when the interaction of treatment and time was significant.

Effects of Exercise Intensity on Muscle Fiber Cross-Sectional Surface Area

The exercise treatments did not elicit changes in type I, IIA, or IIX fiber cross-sectional surface areas over time (*P = 0.127, P = 0.997,* and *P = 0.226,* respectively); therefore, main effects were analyzed (Table 3.5; Figure 3.5). There were no differences caused by treatment or time (*P = 0.093* and *P = 0.876*, respectively) on type I fiber cross-sectional surface area. There were no differences caused by treatment or time (*P = 0.207* and *P = 0.502*, respectively) on type IIA fiber cross-sectional surface area. There were no differences caused by treatment or time (*P = 0.534* and *P = 0.770*, respectively) on type IIX fiber cross-sectional surface area.

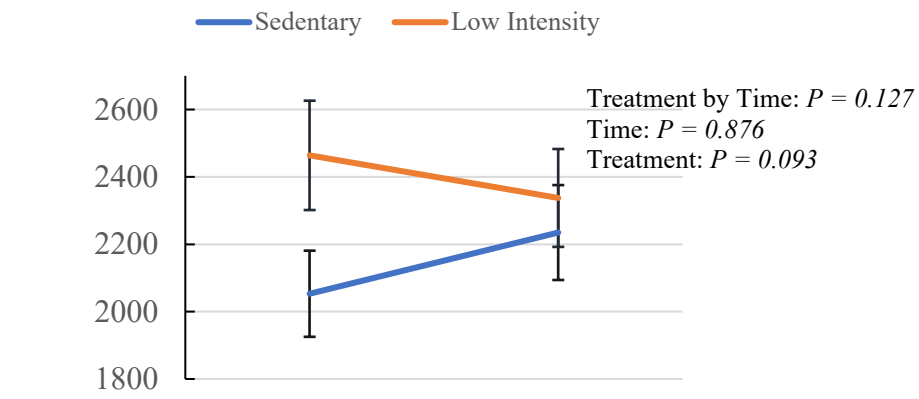
Table 3.5 Average fiber cross-sectional surface area between week 0 and week 8 for sedentary and low-intensity treatments

	Sedentary Treatment		Low-Intensity Treatment		*P*-Value		
Fiber type	Week 0	Week 8	Week 0	Week 8	Time	Treatment	Treatment x Time
I	2053.1 ± 127.9	2234.8 ± 140.9	2463.9 ± 162.3	2337.5 ± 145.3	0.876	0.093	0.127
IIA	2517.3 ± 119.9	2456.7 ± 98.5	2711.2 ± 115.2	2649.7 ± 118.7	0.502	0.207	0.997
IIX	3651.4 ± 205.8	3377.5 ± 166.3	3539.1 ± 136.3	3714.2 ± 206.9	0.770	0.534	0.226

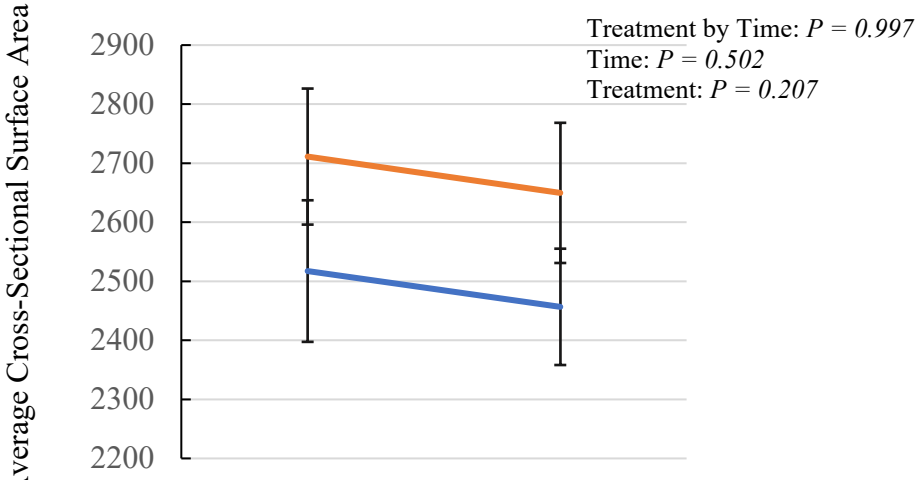
Treatment values are mean fiber cross-sectional surface area (μm) ± the standard error of the mean

Average Fiber Cross-Sectional Surface Area Between Treatment and Time

A) Average Type I Cross-Sectional Surface Area



B) Average Type IIA Cross-Sectional Surface Area



C) Average Type IIX Cross-Sectional Surface Area

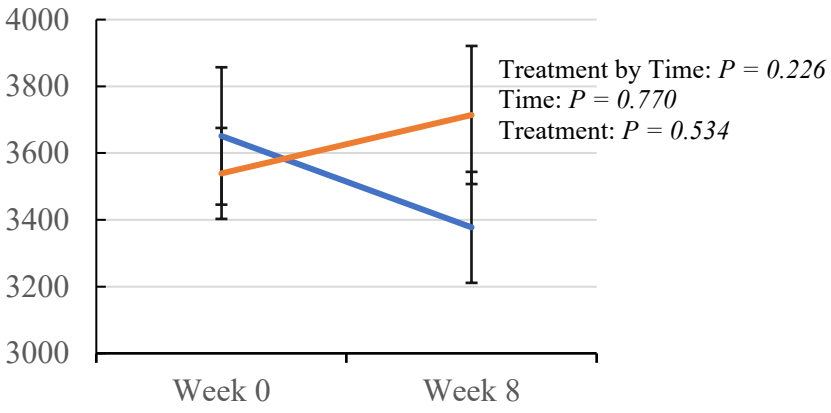


Figure 3.5 Fiber type cross-sectional surface area between treatments and time

Muscle biopsies were collected from the right biceps femoris of Holstein heifers at week 0 and week 8 of the 8-week exercise trial. Results from immunofluorescence analysis are expressed as the average cross-sectional surface area per fiber per section per treatment for type I (panel A), IIA (panel B), and IIX (panel C) fibers. Treatment x time, time, and treatment P values are indicated in panels A-C.

Effects of Exercise Intensity on Oxidative Capacity of Fiber Types

The exercise treatments did not elicit changes in type I, IIA, or IIX fiber oxidative capacity over time (*P = 0.221, P = 0.156,* and *P = 0.648,* respectively); therefore, main effects were analyzed (Table 3.6; Figures 3.6 and 3.7). There were no differences caused by time or treatment (*P = 0.551* and *P = 0.453*, respectively) on type I fiber oxidative capacity. There were no differences caused by time or treatment (*P = 0.460* and *P = 0.465*, respectively) on type IIA fiber oxidative capacity. There were no differences caused by time or treatment (*P = 0.123* and *P = 0.182*, respectively) on type IIX fiber oxidative capacity.

Table 3.6 Fiber oxidative capacity between treatments at week 0 and week 8

	Sedentary Treatment		Low-Intensity Treatment		*P*-Value		
Fiber type	Week 0	Week 8	Week 0	Week 8	Time	Treatment	Treatment x Time
I	0.0172 ± 0.002	0.0167 ± 0.001	0.0177 ± 0.001	0.0163 ± 0.001	0.575	0.733	0.304
IIA	0.0109 ± 0.0004	0.0112 ± 0.0006	0.0111 ± 0.0003	0.0111 ± 0.0006	0.460	0.465	0.296
IIX	0.0068 ± 0.0001	0.0070 ± 0.0003	0.0069 ± 0.0002	0.0072 ± 0.0001	0.123	0.182	0.886

Treatment values are the inverse of the mean stain intensity provided by NIS-Elements Imaging software ± the standard error of the mean

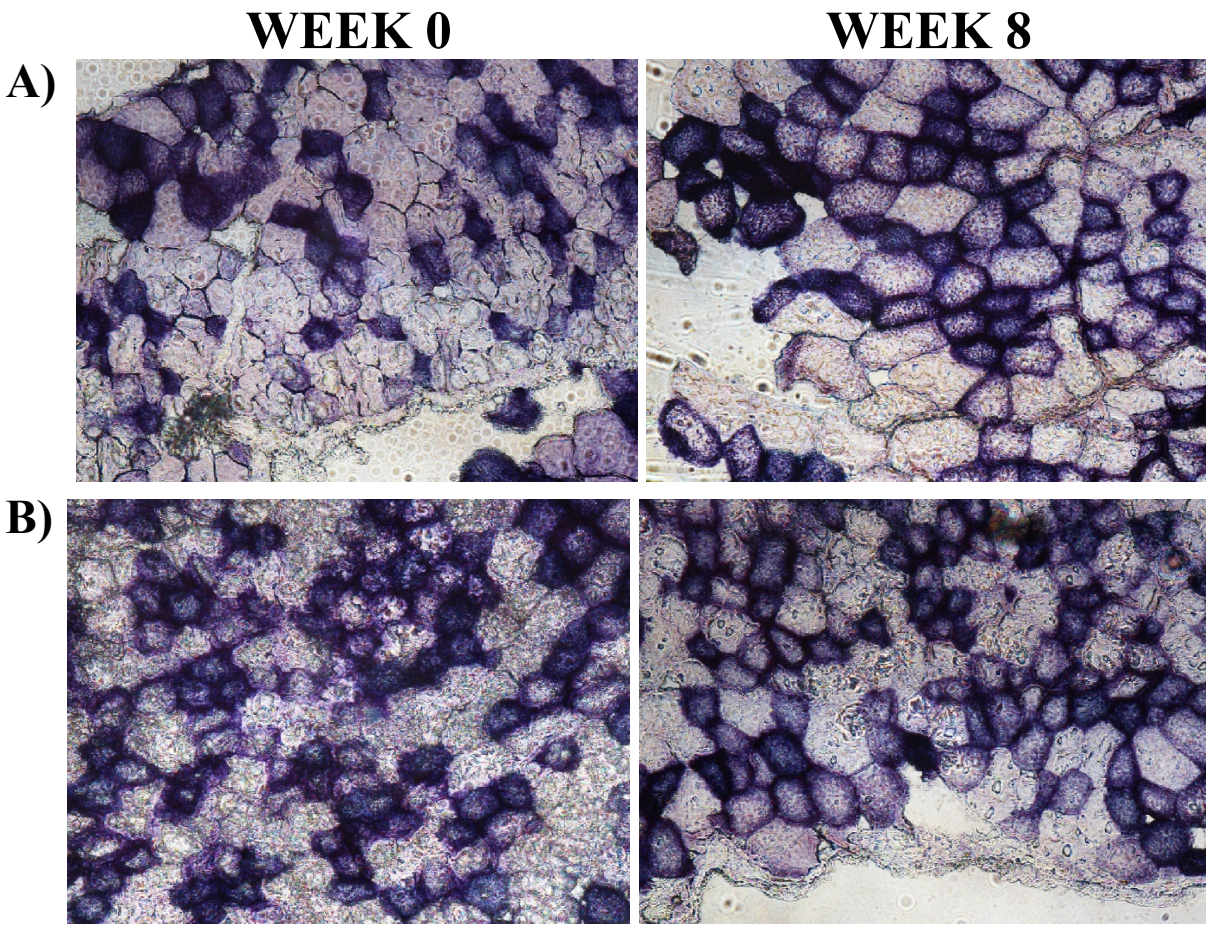
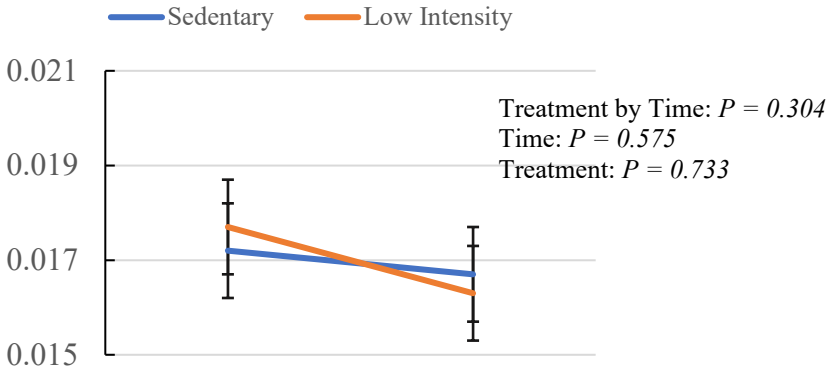


Figure 3.6 Immunofluorescence staining for fiber oxidative capacity at weeks 0 and 8

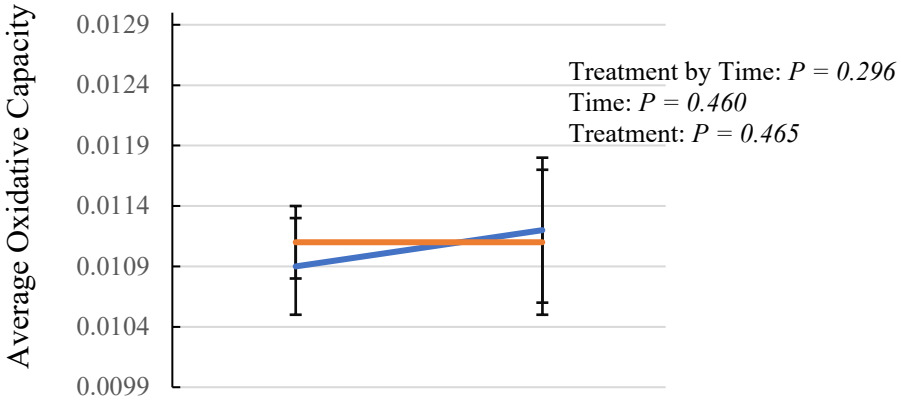
Representative images of fiber type oxidative capacity obtained from biopsies of the biceps femoris muscle of heifers from the sedentary (Panel A) or low-intensity (Panel B) treatment groups prior to treatment (week 0) and after 8 weeks of treatment (week 8). Succinate dehydrogenase activity was determined based on staining intensity from a range of 0 (most intense) to 250 (least intense). Fibers that had the greatest oxidative capacity were intensely stained (darker purple) and indicated a lower intensity value closer to 0. Type I fibers are stained a darker purple and have more oxidative capacity, type IIA fibers have purple staining around the cell border, and type IIX fibers are light purple or white.

Average Fiber Oxidative Capacity Between Treatment and Time

A) Average Type I OC



B) Average Type IIA OC



C) Average Type IIX OC

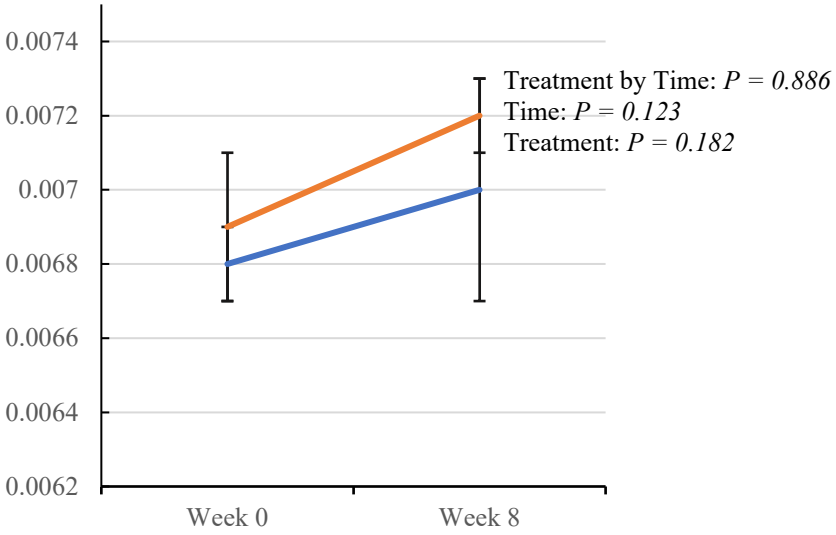


Figure 3.7 Fiber type oxidative capacity between treatments and time

Muscle biopsies were collected from the right biceps femoris of Holstein heifers at week 0 and week 8 of the 8-week exercise trial. Results from immunofluorescence analysis are expressed as the average oxidative capacity per fiber per section per treatment for type I (panel A), IIA (panel B), and IIX (panel C) fibers. The line in Panel B is dark orange to indicate that the lines overlapped; oxidative capacity data were similar for type IIA fibers. Treatment x time, time, and treatment P values are indicated in panels A-C.

Effects of Exercise on Capillary Density of Skeletal Muscle

Exercising heifers at low intensity did not elicit a change in the capillary density to fiber ratio over time in comparison to heifers that remained sedentary (*P = 0.418*); therefore, main effects were analyzed (Table 3.7; Figures 3.8 and 3.9). There were no differences caused by treatment or time (*P = 0.498* and *P = 0.453*, respectively) on the capillary density to fiber ratio.

Table 3.7 Capillary count to fiber ratio between week 0 and week 8 for sedentary and low-intensity treatments

			P-Value		
Treatment	Week 0	Week 8	Time	Treatment	Treatment x Week
Sedentary	1.38 ± 0.15	1.32 ± 0.12	0.453	0.498	0.418
Low-Intensity	1.52 ± 0.20	1.29 ± 0.18			

Values are the mean capillary to fiber density ratio per cross-section analyzed ± the standard error of the mean

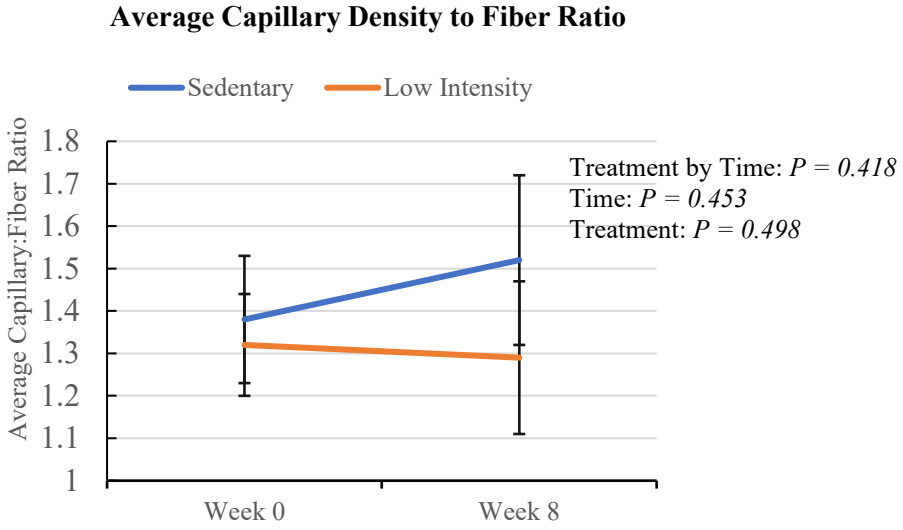


Figure 3.8 Average capillary density to fiber ratio

Muscle biopsies were collected from the right biceps femoris of Holstein heifers at week 0 and week 8 of the 8-week exercise trial. Results from immunofluorescence analysis are expressed as the average capillary to fiber count ratio. Treatment x time, time, and treatment P values are indicated in the graph.

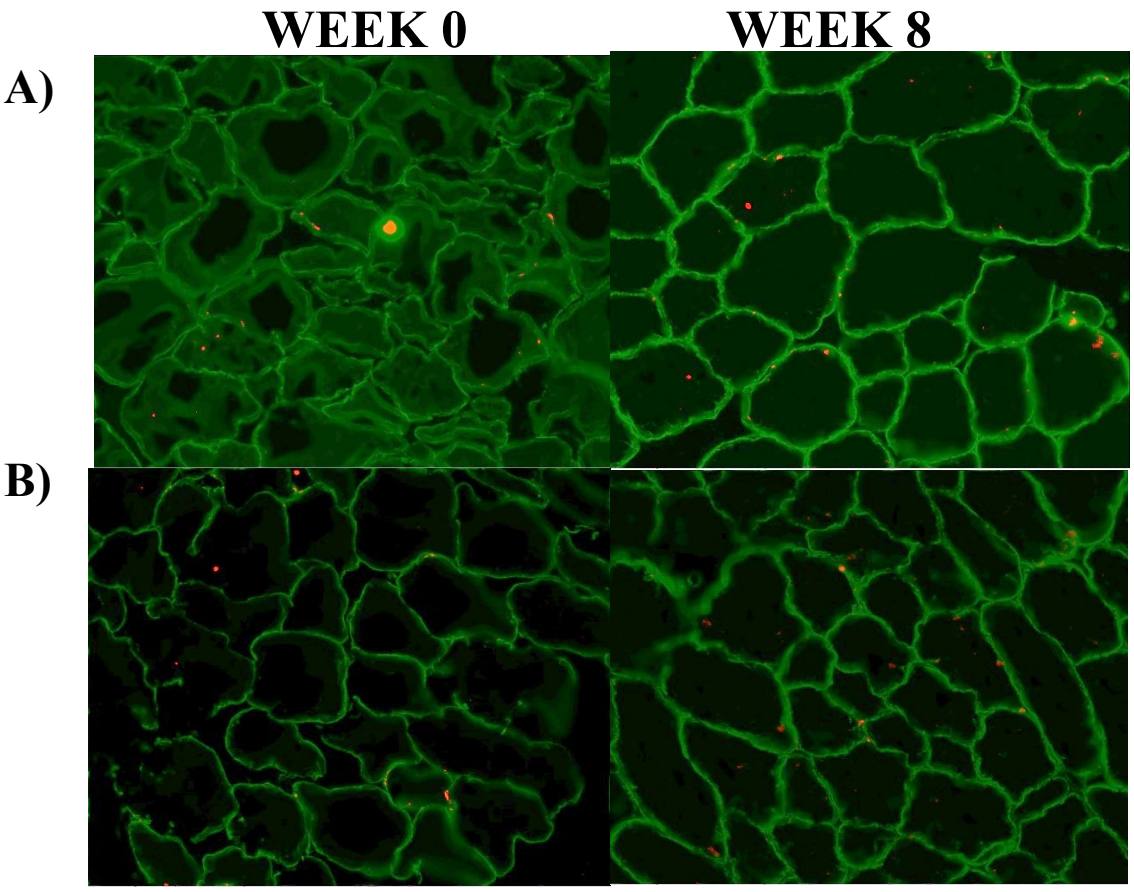


Figure 3.9 Immunofluorescence staining for capillaries at week 0 and 8

Representative images of capillary density obtained from biopsies of the biceps femoris muscle for sedentary (Panel A) and low-intensity (Panel B) treatment groups prior to treatment (week 0) and after 8 weeks of treatment (week 8). Green boundaries represent the sarcolemma of the muscle fibers and red dots along the sarcolemma indicate the presence of a capillary; red dots that appeared to be inside a muscle fiber were not counted as a capillary vessel.

Effects of Exercise on Thermoregulatory Capabilities

Mean Skin Temperature to Core Body Temperature Ratio

The skin temperature was taken and averaged over four different areas and then divided by the core body temperate to generate a ratio of surface to internal body temperature on a rest day throughout the 8 weeks of the treatment period. The exercise treatments did not elicit a change in the MST:CBT ratio over time ($P = 0.485$); therefore, main effects were analyzed (Table 3.8; Figure 3.10). Differences occurred due to time ($P = 0.000$). There were differences between week 1 and weeks 3, 5, 7, and 8, week 3 and weeks 1-2; 6-8, and week 7 and 8 ($P < 0.05$). There were no effects of treatment on the MST:CBT ratio ($P = 0.508$).

Table 3.8 Mean skin temperature to core body temperature ratio among weeks for sedentary and low-intensity treatments.

			P-Value		
Week	Sedentary Treatment	Low-Intensity Treatment	Treatment	Time	Treatment x Week
1	0.94 ± 0.01	0.96 ± 0.01	0.508	*0.000*	0.485
2	1.00 ± 0.01	0.98 ± 0.01			
3	1.03 ± 0.01	1.03 ± 0.01			
5	1.06 ± 0.01	1.05 ± 0.01			
6	0.94 ± 0.01	0.94 ± 0.004			
7	0.88 ± 0.01	0.89 ± 0.01			
8	0.90 ± 0.01	0.91 ± 0.01			

Treatment values are mean the ratio of mean skin temperature to core body temperature (°C) ± the standard error of the mean

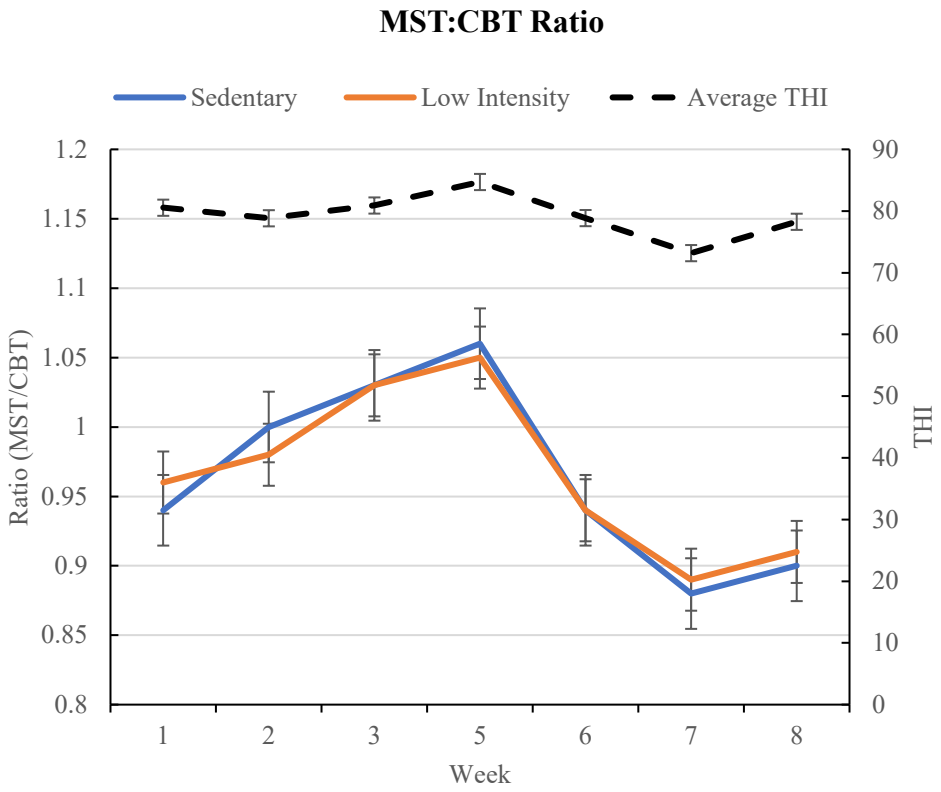


Figure 3.10 Mean skin temperature to core body temperature ratio.
Skin temperatures were obtained on a rest day using an infrared thermometer at four locations: the midpoint between the ileum and ischium, ear, cheek, and paralumbar fossa of heifers from both the sedentary (n=14) and low-intensity (n=14) treatment groups. The temperatures from these areas were averaged to create the mean skin temperature (MST). Core body temperature (CBT) was obtained utilizing a rectal thermometer. MST:CBT ratios were calculated for analysis and are presented as the mean MST:CBT ratio. Average THI (dashed) between the hours of 16:00 and 18:00 (during which CBTs were obtained) is detailed on the right Y axis.

Average Weekly Cooling Period Temperatures from Ear Tags

Temperature sensing ear-tags were used to track body temperature cooling rates during the coolest part of Sunday mornings (a rest day) during the treatment period. The exercise treatments elicited a change in the cooling period temperatures over time ($P = 0.000$); during every week other than weeks 2, 5, and 7, low-intensity treatment caused the temperature to be lower than the sedentary treatment (Table 3.9 and Figure 3.11).

Table 3.9 Mean temperatures obtained from ear tags during the coolest 4 hours of a rest day

			P-Value		
Week	Sedentary Treatment	Low-Intensity Treatment	Treatment	Time	Treatment x Week
1	31.71 ± 0.2	32.23 ± 0.2	0.380	*0.000*	*0.000*
2	30.83 ± 0.2	30.36 ± 0.2			
3	32.83 ± 0.2	32.78 ± 0.2			
4	34.25 ± 0.2	34.12 ± 0.2			
5	34.95 ± 0.1	34.93 ± 0.1			
6	30.08 ± 0.3	29.61 ± 0.4			
7	32.56 ± 0.2	32.64 ± 0.1			
8	26.73 ± 0.3	26.08 ± 0.3			

Treatment values are mean temperature during the cooling period (00:00 to 04:00) for each week (°C) ± the standard error of the mean

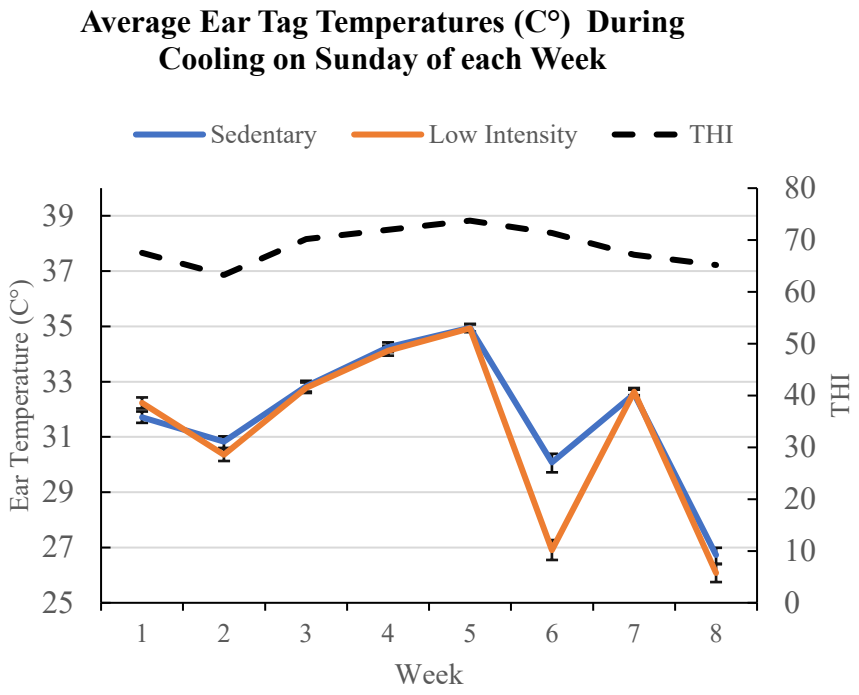


Figure 3.11 Average weekly ear tag temperatures during the coolest 4 hours of a rest day throughout the treatment period.
Ear temperature data were obtained via digital ear tags that were placed in the left ear of the sedentary treated (n=14) and heifers exercised at low-intensity (n=14).

Temperatures were obtained between the hours of 0:00 and 04:00 (previously determined to be the body temperature cooling period; data not shown) on Sundays of the treatment period and averaged throughout the four-hour sampling period. Average THI during the cooling period is detailed on the right Y axis.

Average Weekly Heating Period Temperatures from Ear Tags

Temperature sensing ear-tags were used to track body temperature warming rates during the hottest part of Sunday mornings (a rest day) during the treatment period. The exercise treatments did not elicit change in the rate of warming over time ($P = 0.860$); therefore, main effects of time and treatment were analyzed (Table 3.10 and Figure 3.12). Differences occurred due to time with all weeks being different from one another ($P < 0.05$) except weeks 3 and 7 ($P = 0.070$). There were no differences caused by treatment for rate of body temperature warming ($P = 0.527$).

Table 3.10 Mean temperatures obtained from ear tags during the hottest 4 hours of a rest day

			P-Value		
Week	Sedentary Treatment	Low-Intensity Treatment	Treatment	Time	Treatment x Week
1	30.94 ± 0.2	30.79 ± 0.2	0.527	*0.000*	0.860
2	30.34 ± 0.2	30.17 ± 0.2			
3	33.29 ± 0.2	33.24 ± 0.2			
4	35.42 ± 0.2	35.22 ± 0.3			
5	35. 97 ± 0.2	35.96 ± 0.2			
6	34.35 ± 0.2	34.38 ± 0.2			
7	33.04 ± 0.2	32.88 ± 0.2			
8	29.38 ± 0.2	29.35 ± 0.2			

Treatment values are mean temperature during the heating period (13:00 to 17:00) for each week (°C) ± the standard error of the mean

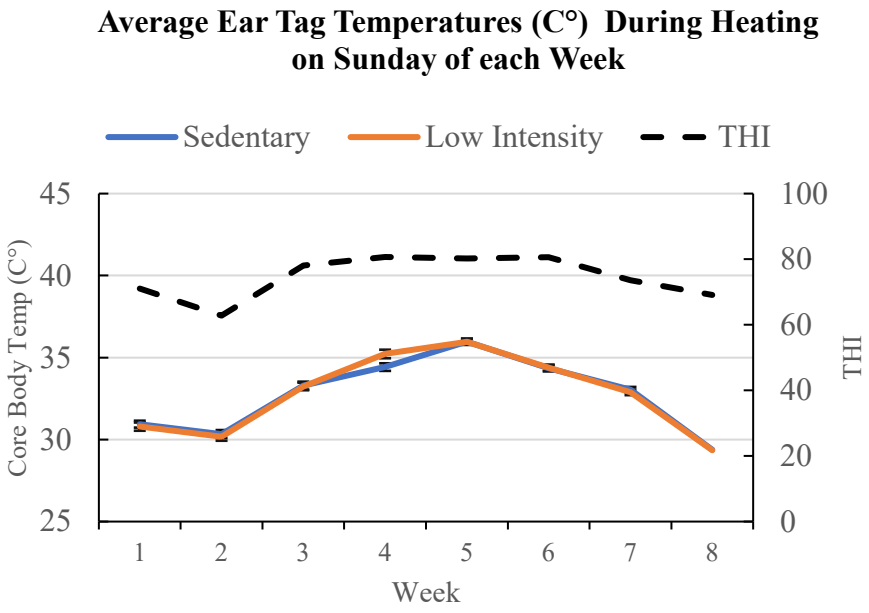


Figure 3.12 Average weekly ear tag temperatures during the hottest 4 hours of a rest day throughout the treatment period.
Temperature data were obtained via digital ear tags that were placed in the left ear of sedentary treated heifers (n=14) and low-intensity exercise treated heifers (n=14). Temperatures were obtained between the hours of 13:00 and 17:00 (considered the heating period) on Sundays of the treatment period. Average THI during the heating period is detailed on the right Y axis.

Comparison of Ear Tag Temperatures, Ear Temperatures Obtained via Infrared Thermometer, and Core Body Temperatures Obtained via Rectal Thermometer, Mondays and Thursdays

At week 4 and week 8 temperature data from the skin, ear and rectum were collected for heifers within both treatment groups; data were collected immediately after exercise on Mondays for heifers within the exercise group followed shortly thereafter for heifers within the sedentary group. Measurements were obtained to identify if the CowManager ear tags were sufficient in measuring approximate core body temperature during the treatment period.

Temperature trends were similar immediately after exercise, as temperatures decreased between weeks 4 and 8 for ear tag, infrared, and rectal temperatures; however, the temperatures obtained from the 3 methods were different from one another during weeks 4 and 8 (P =

0.000)(Table 3.11 and Figure 3.13). While there was a difference identified between treatments at week 4 for rectal thermometer data (*P=0.000*), this difference between heifers that were sedentary versus heifers treated with low-intensity exercise was not indicated from infrared or ear tag data.

Table 3.11 Comparison: average ear tag temperature versus the average infrared thermometer temperature taken on the right pinnae versus the average core body temperature, Mondays immediately after exercise (17:00-19:00)

	Mean ± SEM		P-value
Ear Tag	**Sedentary**	**Low-Intensity**	
Week 4	37.74 ± 0.23^{a}	37.71 ± 0.27^{a}	0.613
Week 8	31.87 ± 0.21^{d}	31.73 ± 0.21^{d}	0.544
Infrared	**Sedentary**	**Low-Intensity**	
Week 4	38.37 ± 0.57^{a}	37.56 ± 0.25^{a}	0.209
Week 8	34.70 ± 0.45^{e}	34.62 ± 0.37^{e}	0.893
Rectal	**Sedentary**	**Low-Intensity**	
Week 4	40.16 ± 0.10^{b}	40.70 ± 0.08^{c}	0.000*
Week 8	39.42 ± 0.19^{f}	39.36 ± 0.08^{f}	0.774
P-value Week 4	0.000*	0.000*	
P-value Week 8	0.000*	0.000*	

Different superscripts indicate statistically different temperature means.

Superscripts a, b, and c are utilized for the ANOVA mean differences between temperature methods and treatments at week 4 and again used for week 8.

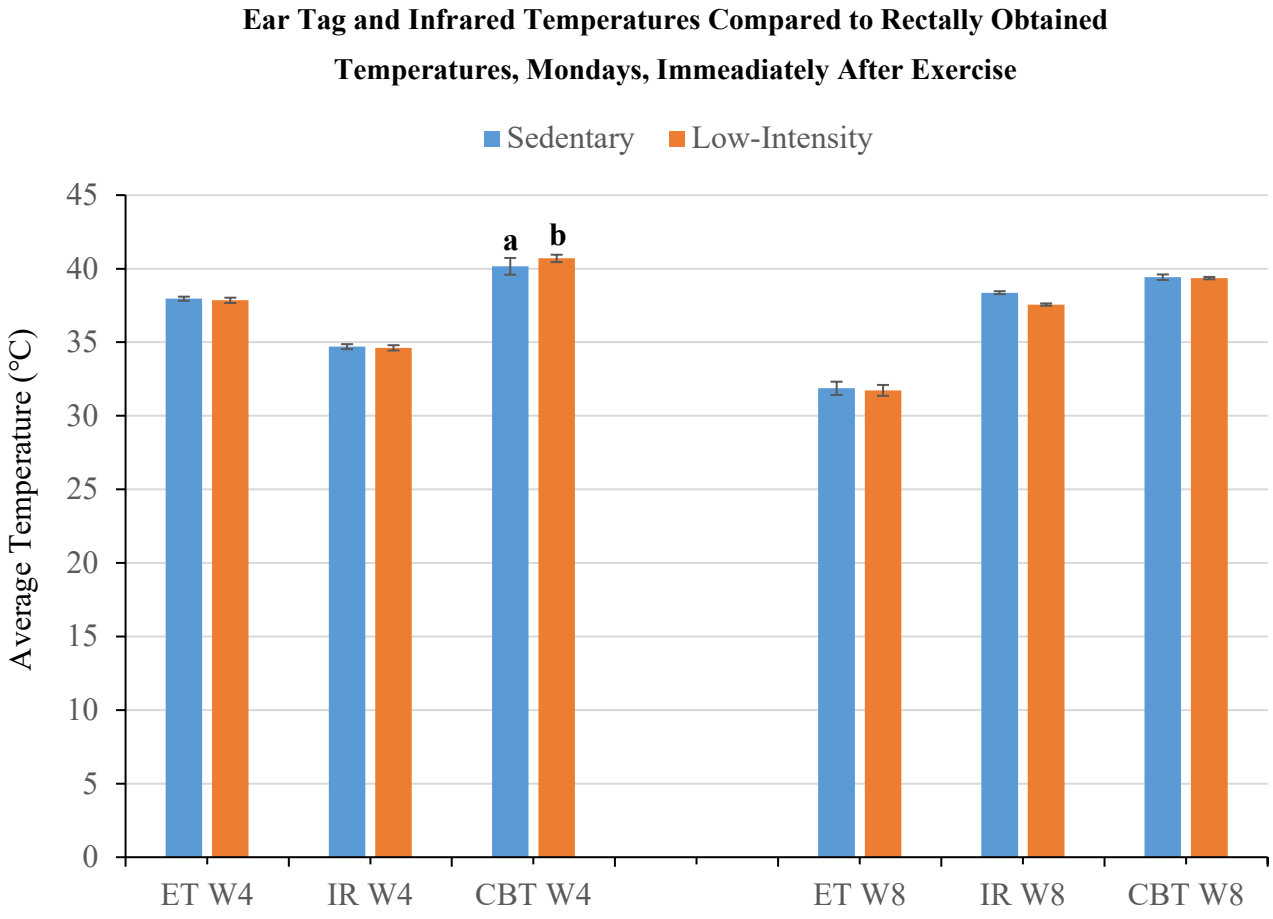


Figure 3.13 Ear Tag and Infrared Temperatures Compared to Rectally Obtained Temperatures, Mondays, Immeadiately After Exercise

Temperature data were obtained immediately after exercise at weeks 4 and 8 on Mondays for infrared temperature (IR), core body temperature (CBT), and ear tag temperature (ET). Measurements were obtained to identify if the ear tags were sufficient in measuring approximate core body temperature immediately after the exercise period. Different letters indicate different means between treatments ($P<0.05$).

At week 4 and week 8 temperature data from the skin, ear and rectum were collected for heifers within both treatment groups; data were collected on a day without exercise (Thursday) for heifers within the exercise group followed shortly thereafter for heifers within the sedentary group. Measurements were obtained to identify if the CowManager ear tags were sufficient in measuring approximate core body temperature during the treatment period.

Temperature trends were not similar on the analyzed Thursdays, as temperatures decreased between weeks 4 and 8 for infrared and rectal on sedentary heifers but increased for ear tags. For

the low-intensity treatment, ear tag and rectal temperatures decreased between weeks 4 and 8, but infrared temperatures increased. Additionally, the temperatures obtained from the 3 methods were different from one another during weeks 4 and 8 (*P = 0.000*), with rectal temperatures being more than 4.5℃ greater than ear tags and infrared temperatures (Table 3.12 and Figure 3.14). Contrary to the data analyses immediately after exercise, there were no differences between heifers that were sedentary versus heifers treated with low-intensity exercise for either of the three temperature obtainment methods.

Table 3.12 Comparison: average ear tag temperature versus the average infrared thermometer temperature taken on the right pinnae versus the average core body temperature, Thursdays, a non-exercise day

	Mean ± SEM		P-value
Ear Tag	**Sedentary**	**Low-Intensity**	
Week 4	34.84 ± 0.16[a]	34.82 ± 0.20[a]	0.840
Week 8	34.28 ± 0.24[a]	34.29 ± 0.29[a]	0.764
Infrared	**Sedentary**	**Low-Intensity**	
Week 4	34.11 ± 0.35[b]	33.97 ± 0.36[b]	0.786
Week 8	35.00 ± 0.30[b]	35.32 ± 0.31[b]	0.472
Rectal	**Sedentary**	**Low-Intensity**	
Week 4	39.04 ± 0.12[c]	39.18 ± 0.10[c]	0.371
Week 8	39.16 ± 0.14[c]	39.04 ± 0.08[c]	0.466
P-value Week 4	0.000*	0.000*	
P-value Week 8	0.000*	0.000*	

Different superscripts indicate statistically different temperature means.

Superscripts a, b, and c are utilized for the ANOVA mean differences between temperature methods and treatments at week 4 and again used for week 8.

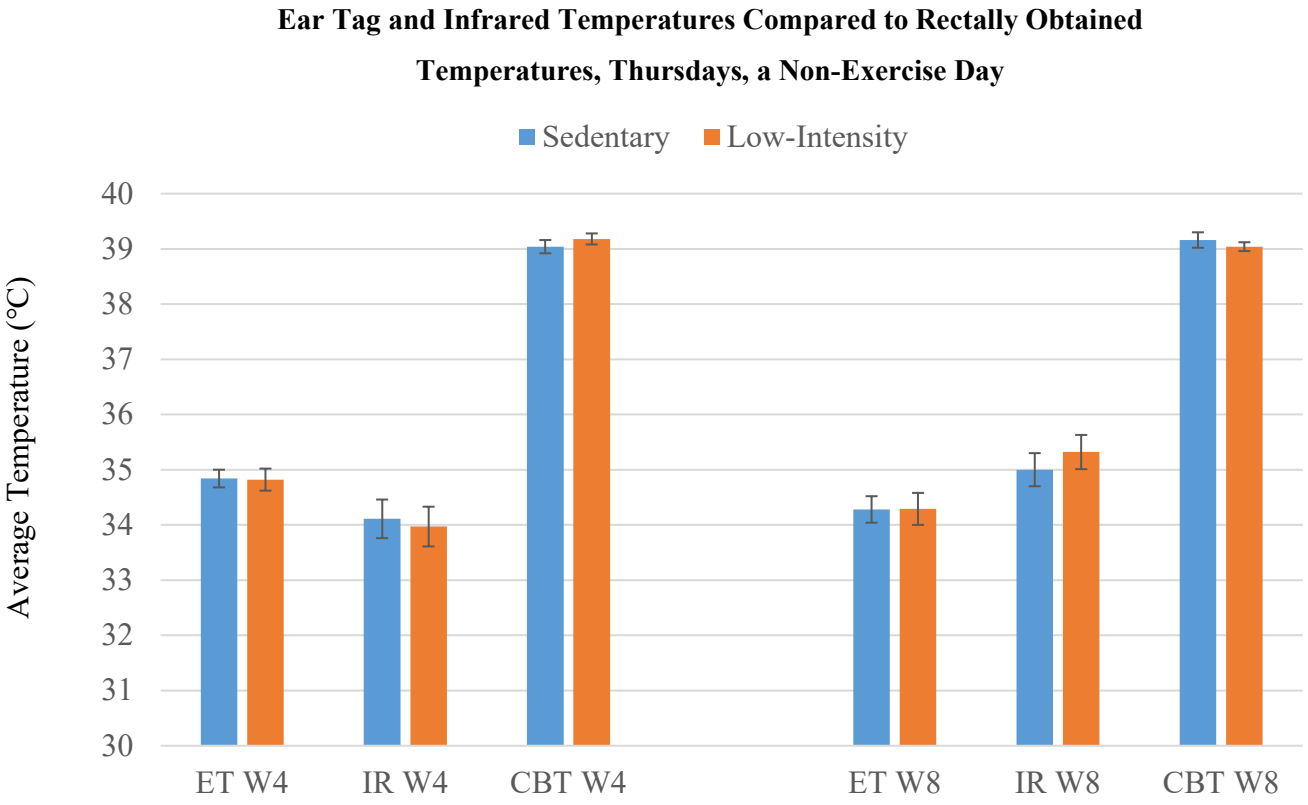


Figure 3.14 Comparison of average ear tag temperatures versus average ear pinnae temperature via infrared thermometer, Thursdays

Temperature data were obtained on a day of rest at weeks 4 and 8 on Thursdays for infrared temperature (IR), core body temperature (CBT), and ear tag temperature (ET). Measurements were obtained to identify if the ear tags were sufficient in measuring approximate core body temperature on days of rest.

Discussion

In this study, we compared muscle fiber type, surface area, capillary density, and oxidative capacity before and after an 8-week treatment period between Holstein heifers that were sedentary or exercised at low intensity. In addition, we examined body temperature trends of the heifers after exercise. We found low-intensity exercise treatment caused a change to the percentage of fiber types in the biceps femoris muscle and potentially promoted quicker cooling of heifers during the morning hours. We also determined that rectal temperatures were increased after an hour of low-

intensity exercise, but that these changes in core body temperatures caused by exercise were not detectable when using the CowManager ear temperature sensing ear tags.

Heifers that underwent low-intensity exercise three times per week for eight weeks showed an increase in type I skeletal muscle fiber percentage. Comparatively, fiber percentages from heifers treated with low-intensity exercise in our previous study (Chapter 2) did not change. There were differences between Chapter 2 and this chapter, namely in how one of the trials in Chapter 2 were housed before exercise treatment. Trial 2 of Chapter 2 was housed on pasture versus the dry lot that this chapter's heifers and trial 1 heifers of Chapter 2 were housed on. This could have impacted the differences between the two chapter's results; however, we expected that Chapter 2 would have shown a change in type I fibers due to increased activity levels before the exercise trial and this did not occur. Although we did not measure activity levels prior to our trials, we assume that, due to the natural grazing behavior of cattle, heifers engaged in walking behaviors on pasture more than in a drylot pen, but this was not indicative from our results. Additionally, we used different muscles for the biopsy sites in Chapter 2 versus this chapter: this chapter used biceps femoris, while Chapter 2 utilized semitendinosus for fiber type analysis. Suzuki et al. (1999) indicated that the muscle biopsies obtained from biceps femoris and semitendinosus were very different regarding fiber type composition in pigs and, not only are the muscles themselves different, but muscle regions are different as well. The cranial portion of the medial biceps femoris contained 27.1% type I fibers while the deep caudal portion of the medial biceps femoris contained 37.1%; the semitendinosus contained 4.2% caudally and 42.6% cranially (Suzuki et al., 1999). Therefore, it is likely that, because muscle biopsy type, as well as obtainment location were different, that dissimilarities existed between the two Chapters.

Our present results for fiber percentage, while contrary to our previous study, seemed to follow previous research in humans and horses. Howald et al., 1985 and Jansson et al., 1978 indicated an increase in the relative proportion of type I fibers after low intensity exercise in humans. This is presumably due to the fatigue-resistance of type I fibers and the requirement for endurance needed when performing low-intensity exercise. Dastmalchi et al. (2007), Gondim et al. (2005), D'Angelis et al. (2008), and Serrano et al. (2000) reported that sedentary behavior either results in decreases or stagnancy of type I fibers; we saw a decrease in type I fiber percentage in growing sedentary heifers. Wicks et al. (2019) reported that muscle fibers in newborn calves were more oxidative (type I or IIA) than glycolytic (IIX) but, that as cattle aged, glycolytic fibers increased in quantity and oxidative fiber types decreased. Wegner et al. (2000) further demonstrated that fiber type proportions in the semitendinosus changed, but only between birth and 4 months of age. Wegner et al. (2000) also found type I, IIA, and IIX fiber quantity did not change in Holstein semitendinosus muscle between 4 and 12 months of age and that the surface area of muscle fibers all increased consistently for Holstein cattle between birth and 24 months of age. Thus, it is likely that inactivity versus growth caused the decrease in type I fibers for sedentary heifers as observed in the present study.

Muscles that contain greater proportions of type I fibers have been documented as having greater quantities of capillary blood supply (Powers & Howley, 2012). It is assumed that, due to the increased vasculature in muscles that are more type I fiber dense, muscles would be more efficient at cooling since increased quantities of blood are available to move through the muscle at any given time. The temperature increase of the blood would activate transient receptor potential channels and subsequently initiate signals within the hypothalamus to facilitate heat loss. The hypothalamic response to high body temperatures includes increased shunting of blood away from

visceral organs and towards the skin surface for heat abatement. Therefore, is can be assumed that increases in type I fibers with a concomitant increase in capillaries could indirectly promote greater hypothalamic shunting of blood to the skin surface for more efficient heat abatement. Thus an important question remained: did low-intensity exercise, with the subsequent increase in type I fibers, increase capillary density in the muscle fibers?

Despite the increase in type I fibers, capillary density did not increase in heifers that underwent low-intensity exercise. Contrary to our results, Joyner & Casey (2015), Andersen and Henriksson (1977), Wagenmakers et al., (2016), and Ingjer (1979), found that low-intensity exercise promoted capillary growth in men. We suspect this discrepancy was due to one of two reasons. Wilson et al. (2018), determined that, at a speed of 4.5 km/hour, heifers should be at 70% of their anaerobic threshold and we used this speed as the basis for the low-intensity exercise treatment and hoped to induce enough vascular shear stress to stimulate angiogenesis, angioadapation, and vasculogenesis in the dermal and muscle layers. However, there is the potential the heifers were not exercising at a high enough intensity to induce shear stress, nitric oxide release, and subsequent capillary changes. Bellman and Gaessser (1991) determined around 72% of the anaerobic threshold was an appropriate marker for low-intensity exercise in humans. However, Robbins et al. (2009) found that while anaerobic threshold was proportional to a capillary density increase in men, women had an inverse proportionality between their anaerobic threshold and capillary density. It is speculated this difference between the sexes occurs due to the utilization of lipid versus carbohydrates; women tend to utilize lipids as their energy source, while men utilize carbohydrates (Tarnopolsky et al., 1990; Carter et al., 2001). Thus, the lack of change in capillary density could be a result of either not meeting the anaerobic threshold or possibly that cattle also have sex differences in response to exercise.

Cross-sectional surface area of fiber types did not change for the heifers in this study, whether due to exercise or simply to growth. This result was expected, as cross-sectional surface area did not change by time and treatment in Chapter 2 either. After low-intensity exercise in humans, Mitchell et al. (2012) found an approximate 23% increase in the surface area of type I and an approximate 16% increase in the surface area of IIA fibers. In Arabian horses that engaged in low- to moderate-intensity exercise, surface area of type I and IIA fibers also increased (D'Angelis et al., 2005). However, in other breeds of horses, such as Thoroughbreds and Standardbreds, low- to moderate-intensity exercise did not induce changes in cross-sectional surface area of I, IIA, or IIX fiber types (Henckel, 1983; Lindholm et al., 1983; Foreman et al., 1990; Rivero et al., 1996). Differences between breeds likely occurred due to genetic variation and subsequent athletic ability and could provide an explanation as to why cross-sectional surface area of fibers from Holsteins did not increase after low-intensity exercise; cattle are likely less genetically inclined towards athleticism and thus may not have the same surface area acclimations as horses or humans.

Oxidative capacity was not impacted by exercise in this chapter or Chapter 2. Increases in oxidative capacity are associated with a concurrent increase of mitochondria (Schwerzmann et al., 1989). Turner et al. (1997), Bishop et al. (2014), and MacInnis and Gibala (2016) reported that the mechanisms by which mitochondrial density (and thus oxidative capacity) increases and to what extent it increases, varies between species and among different muscles. Others have reported increases in oxidative capacities of type I and IIA fibers after low-intensity exercise in humans, specifically in muscles such as the vastus lateralis and diaphragm (Powers et al., 1992; Jansson & Kaijser, 1977). Differences between muscle characteristics could occur due to some muscles being preemptively more glycolytic or oxidative before exercise trials. A muscle that is more glycolytic

at the beginning of an exercise trial might show greater increases in oxidative capacity than a muscle that is already optimized for oxidative capacity. Mattson et al. (2002) performed a study using hamsters and found relative oxidative capacities of muscles were different: the semitendinosus (used in our previous study) and biceps femoris (used herein) were similar in oxidative capacity (citrate synthase activity [indicative of oxidative capacity] of 13.5 versus 17.9, respectively), while the vastus lateralis and costal diaphragm had a greater oxidative capacity (citrate synthase activity 45.6 and 64.6, respectively). Due to differences between oxidative capacity of different muscle groups, our results, both in this chapter and Chapter 2, may not be comparable with others'.

Overall, there was not much change in muscle fiber characteristics outside of the increase in type I fibers over time due to low-intensity treatment. Capillary density and oxidative capacity did not change as expected given the concurrent increase in type I fiber types; however, this could have been due to differences between muscle types utilized for others' research versus this chapter and Chapter 2.

In addition to skeletal muscle characteristics, this study aimed to identify how homeostatic regulation of body temperature was impacted by 8 weeks of exercise. The mean skin temperature to core body temperature ratio (MST:CBT) was analyzed from weeks 1-3 and 5-8 (week 4 had an odd anomaly on the temperature output due to a rainstorm that occurred while measurements were obtained and was thus removed from the results) to determine if, when skin temperature rose due to shunting of blood from internal organs and towards the skin, core body temperature significantly lowered; improved shunting of blood towards the skin is likely indicative of improved blood flow throughout the dermal layers. However, the results did not signal any significant changes in MST:CBT from treatment. The time at which we collected the temperature data affected the ratio,

but this was expected due to fluctuations in temperature and the temperature humidity index from week to week. Regan et al. (1996), Pandolf (1998), Rowell (1986) and Sawka and Wenger (1988) documented the effect of exercising increasing skin temperatures and concurrently decreasing core body temperature after exercise-induced heat acclimation; however, all of these studies were detailed in humans. Humans have greater numbers of active sweat glands and thus increased ability to thermoregulate in hot environments due to evaporative cooling from the skin surface. Dairy cattle, specifically Holsteins, have been documented as not having many active eccrine sweat glands (Gebremedhin et al., 2008) so, despite the potential for increased blood shunting to the skin, they likely cannot dissipate heat as quickly since they have little to no evaporative cooling ability from the epidermal surface and heifers within this study did not have access to sprinklers. In addition, sweat glands that were recorded active were often functionally inhibited by insulative properties of the hair or by the color of the cow (Gebremedhin et al., 2008). Thus, there might not have been differences between treatments for body temperature because of unchanging capillary density, but also potentially due to variables such as defunct sweat glands or coat color.

Additionally, we analyzed the heating rate of treated heifers during a period of time where temperature gradually increased during the afternoon. We compared exercised to sedentary heifers at each week to determine if the ear tag temperatures were different on average, and there were no differences of treatment on the rates at which body temperature increased in response to increasing ambient temperatures.

While the MST:CBT did not change through treatment and neither did the heating period temperatures, cooling period temperatures did change with time and treatment. We analyzed the cooling rate of treated heifers during a period of time where temperature gradually decreased in the early morning. We compared the two treatments at each week to determine if the ear tag

temperatures were, on average, lower for heifers treated with low-intensity exercise than those that were sedentary. Our results indicated that heifers that underwent low-intensity exercise treatment did have significantly reduced average ear tag temperatures at weeks 1-6 and week 8 than their sedentary counterparts. This likely indicates that heifers who were exercised had improved ability to cool themselves during times of reduced ambient temperatures.

We did not utilize in-depth analyses of the data provided by the CowManager ear tags due to variation between rectally obtained temperatures, ear temperatures obtained via infrared thermometer, and the CowManager ear tags. Rectal temperatures were different between treatments at week 4 immediately after exercise; however, the CowManager ear tags did not detect this difference. Additionally, there were differences in the trends obtained between weeks 4 and 8 for the three obtainment methods. Immediately after exercise (Mondays), trends aligned between temperatures obtained through the ear tags, infrared, and rectal temperatures, with all temperatures decreasing over time; however, on non-exercise days (Thursdays), sedentary heifers had an increase in body temperature for infrared and rectal temperatures, but a decrease in body temperature for ear tag temperatures. Thus, we did not find the CowManager ear tags sensitive enough to detect the changes in temperature immediately after exercise or on a day of rest. Multiple factors could have caused the discrepancy between the ear tags and core body temperatures: solar radiation levels, increased wind speed or computational error from the data transition between the actual ear tag and the ear tag database. Others have provided technical notes of the CowManager sensors and recommended its uses for activity monitoring, but not for comparing the sensors' temperature measurements with measurements of core body temperature (Bikker et al., 2014; Borchers et al., 2016; Schirmann et al., 2009; Dolecheck et al., 2015).

Small sample size was a limitation of this study, as we only used 28 heifers for the sedentary and exercise treatments. Despite this limitation, we feel that this research provides valuable insights into how dairy cattle muscle changes from exercise and how it could potentially be used to mitigate heat stress. However, further research is necessary to supplement the results of this study.

Furthermore, we encourage the use of vaginally implanted temperature loggers (held in place by CIDRs) rather than CowManager ear tags to determine if there is a correlation between exercised-induced skeletal muscle changes and the ability of a heifer to cool herself. It would also be useful to more precisely examine the amount and duration of exercise necessary to cause acclimations, because, while human acclimation to heat can be seen in less than 2 weeks due to increases in eccrine sweat glands (Pandolf, 1998; Sawka et al., 1996), dairy cattle, due to the inactivity of their eccrine sweat glands and thus decreased likelihood for evaporative cooling improvements, may have an altered responsiveness to repeated deviations in body temperature. Non-esterified fatty acids could be analyzed to determine the effects of exercise on liver health since increased lipid accumulation in the liver is a common occurrence in heat stressed dairy cattle (Skibiel et al., 2018). Fatty liver disease has been shown to decrease in exercised humans (van der Windt et al., 2018) and thus it is possible that repeated bouts of exercise improves the ability of the liver to process fatty acids via beta-oxidation, because the release of adrenal medullary catecholamines during exercise causes decreased triglyceride storage and thus increased circulating fatty acids. We recommend evaluating non-esterified fatty acids within the blood plasma to determine if fatty acid circulation decreases after periods of exercise treatment.

In conclusion, most of the parameters that we assessed in dairy cattle skeletal muscle did not notably change after low-intensity exercise regarding capillary density, cross-sectional surface

area, and oxidative capacity. Type I fibers did increase after exercise treatment and this increase could relate to the increased ability to cool during periods of rapid temperature decrease. Ultimately, we concluded that more research is needed in the effect of exercise on skeletal muscle composition. We also concluded that the utilization of CowManager ear tags may not be ideal to indicate minute changes in body temperature after a period of exercise. This research, in conjunction with our previous study, provides important foundational information for future studies on the effects of exercise treatments on cattle.

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