EFFECT OF ACUTE INFLAMMATORY EVENTS AND PARITY ON LENGTH OF

TELOMERES IN BRAHMAN CATTLE

A Thesis

by

SYDNEY ELIZABETH O'DANIEL

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee,   Thomas H. Welsh, Jr.
Co-Chair of Committee,   Ronald D. Randel
Committee Members,   Jason P. Banta
                         David G. Riley
                         Francis M. Rouquette, Jr.
Head of Department,   G. Clifford Lamb

August 2019

Major Subject: Physiology of Reproduction

Copyright 2019 Sydney E. O'Daniel

ABSTRACT

Two experiments were conducted to evaluate the effects of acute inflammation

(Experiment 1) and parity (Experiment 2) on peripheral blood leukocyte telomere length

(TL) in Brahman cattle. Experiment 1 utilized 11 heifers (20-21 mo of age) grouped into

either control (n =6) or lipopolysaccharide (LPS) treatment (n = 6) groups. Heifers were

fitted with vaginal temperature probes 7 d prior to treatment. Heifers were weighed, BCS

recorded, and bled by jugular venipuncture prior to each treatment. The LPS doses

increased on corresponding treatment days (d 0, 0.25 µg/kg; d 7, 0.50 µg/kg; d 14, 0.75

µg/kg). Sickness behavior was observed post treatment. In both experiments,

quantitative PCR methods were utilized to determine quantity of telomere sequences and

bovine β-2-globulin gene. Absolute standard curves were created using serial dilutions

of double-stranded DNA oligonucleotides of bovine telomere and β-2-globulin genes.

Statistical analyses were conducted utilizing mixed linear models with the MIX

procedure of SAS. The LPS treatment did elicit a febrile response observed as increased

maximum vaginal temperature (VT) (P = 0.0429), increased change in VT (P < 0.0001),

and decreased time to maximum VT (P < 0.0001). No relationship was seen between TL

for treatment (P = 0.3554), day (P = 0.7338), or the interaction of treatment and day (P =

0.9441). A trend was observed for increased days of age and decreased TL (P = 0.0715).

Experiment 2 involved 19 cows [primiparous (n = 8) and multiparous (n = 11)] sampled

on d -28, d + 7, and d + 28 pre- and post-calving. Cows were weighed and BCS recorded

on sample days as well as observed for duration of labor and calving ease. A reduction in

TL (9224.5 copies of telomere) was observed between parity 1 and 2 (P = 0.0223), but no differences were seen in sample day (P = 0.2315) or the interaction of parity and day (P = 0.7696). A greater degree of endotoxin exposure than utilized in this study may be needed to adequately assess TL response to acute inflammation. Parity was negatively associated with peripheral blood leukocyte TL in Brahman cows.

DEDICATION

I would like to dedicate this work to my Grandpa Ken (Ken Killion '55). Your lifelong

passion for agriculture and animals has shaped me into the person I am today. I am so

grateful to have shared my experience at Texas A&M with you and make you proud.

ACKNOWLEDGEMENTS

I would like to thank my committee chair and co-chair, Dr. Welsh and Dr. Randel, as well as my committee members, Dr. Riley, Dr. Banta, and Dr. Rouquette, for their guidance and support throughout the course of this research. I would also like to include Don Neuendorff for his assistance in my research and sharing his lifetime of knowledge and experience with cattle. A special thanks also goes out to Kelli Kochan and everyone at the TIGSS lab for assisting me with the lab portion of my thesis.

Thanks also go to my friends and colleagues and the department faculty and staff for making my time at Texas A&M University a great experience.

Finally, thanks to my parents for their continual support and encouragement.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a thesis committee consisting of Dr. Thomas Welsh Jr. and Dr. Ronald Randel and Dr. David Riley, Dr. Jason Banta of the Department of Animal Science as well as Dr. Rouquette of the Department of Soil and Crop Science.

All work conducted for the thesis was completed by the student independently.

Funding Sources

NOMENCLATURE

TL	Telomere Length
dsDNA	Double Stand DNA
ssDNA	Single Strand DNA
TRF1	Telomeric Repeat Binding Factor 1
TRF2	Telomeric Repeat Binding Factor 2
POT1	Protection of Telomere Protein 1
RAP1	TRF2-Interacting Protein 1
TPP1	Adrenocortical Dysplasia Protein Homolog
TIN2	TRF1-Interacting Factor 2
DDR	DNA Damage Response
DSB	Double Strand Break
ROS	Reactive Oxygen Species
HTERT	Telomerase Catalytic Subunit
HRT	Telomerase RNA Component
HSC	Hematopoietic Stem Cell
ALT	Alternative Lengthening of Telomeres
11βHSD	11-β-Hydroxysteroid Dehydrogenase
HPC	Hematopoietic Progenitor Cell
HPA	Hypothalamus-Pituitary-Adrenal Axis
CRH	Corticotropin Releasing Hormone

LPS	Lipopolysaccharide
RCF	Relative Centrifugal Force
WBC	White Blood Cell
RBC	Red Blood Cell
qT	Quantity of Telomere
B2G	Beta-2-Globulin

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION

Cow longevity is considered by cow-calf producers to be one of the most
economically important traits. Longevity refers to the total duration of life; however,
when referencing beef cattle production, longevity refers to the total duration of the
productive segment of a cow's life until she must be removed from the herd. However,
as with most species, increased age is linked to many degenerative diseases and
disorders which may be considered "aging." Aging phenotypes in cattle such as
structural unsoundness, reproductive failure, or a general decline in productivity are
some of the most common culling criteria for today's beef cattle producers. Ultimately, a
cow is expected to be culled when she is no longer profitable to the producer.

Finding a biological marker for productive longevity is an important topic in the
dairy industry and is becoming an important emergent concept in the beef industry.
Longevity is a difficult trait to quantify but it is believed to be influenced by genetic
factors which in turn may be influenced by environmental stress factors. Discerning
cattle longevity from closely related production traits that have been altered by
environmental influences such as nutritional mismanagement or poor production
practices makes finding a selection criterion for productive longevity difficult. Due to
this difficulty, many producers have adopted to culling cows at an arbitrary age based on
the probability that the next year that female will fail to raise a calf. One potential
biological marker for longevity is the length of telomeres (TL). Telomeres are a highly

plastic nucleic sequence responsible for protecting the ends of chromosomes during DNA replication and are representative of an organism's immune history and biological age. The measurement of telomeres may be a useful tool in determining cow longevity.

A variety of stressors have been found to significantly impact TL. Environmental stressors such as prenatal stress, perceived stress, and disease pressure as well as genetic factors and congenital disorders are some of the most well documented influencers of TL. Environmental stressors appear to impact cell proliferation and mechanisms of the DNA replication process, when telomeres are most vulnerable to damage. The reduction of TL is associated with acceleration of phenotypic aging and at critical telomere lengths pathogenesis of disease. Prenatal, perceived stress, and disease stress are present in beef cattle production in all segments from cow-calf production through the feedlot phase as well as impacting breeding herd soundness.

At the inception of this project, there did not appear to be research involving TL as a selection factor for beef cow longevity and there appears to be demand for further investigation of telomere dynamics involving common production stressors. This thesis project assessed the relationship of Exp. 1) acute inflammation on heifer TL over time and Exp. 2) parity on cow TL. Use of an endotoxin mimicked an acute inflammatory disease stress response in heifers. Number of parities, duration of labor, and raising a calf represent an acute physiological and perceived stress. Results of this experiment provide valuable information on longevity and health in cattle subjected to common stressors found in modern beef cattle production.

CHAPTER II

LITERATURE REVIEW

Defining Telomeres

Upon the discovery of the DNA replication process, Russian biologist Alexey

Olovnikov and American molecular biologist James Watson individually identified what

was eventually coined as the "End Replication Problem" (Olovnikov, 1971; Watson,

1972). It was found that DNA replication is innately flawed due to the inability of the

final RNA primer on the 3' lagging strand to be replaced with transcribed nucleotides by

DNA Polymerase 1. This results in the remaining Okazaki fragment to be cleaved and

ultimately leads to the net loss of DNA per replication cycle. Within the same decade, it

was noted by Leonard Hayflick, that cultured cells could only survive a limited number

of divisions, which became the Hayflick hypothesis of why physiological functions

breakdown as organisms age (Hayflick and Moorhead, 1961). Questions related to both

of these studies remained uncertain until the discovery of tandem, repetitive sequences at

the ends of the genome of *Tetrahymena thermophila* (Blackburn and Gall, 1978). The

Latin term "telomere" was used to describe these sequences, "telo" meaning "end" and

"mere" meaning "piece". Blackburn further elucidated that telomere function was

transferrable between species (vertebrates found to contain 5' – TTAGGG – 3') and

that an enzyme existed to mitigate base pair (bp) loss between replications, eventually

termed as "*telomerase*" (Szostak and Blackburn 1982; Greider and Blackburn, 1985,

1987, 1989). Initially, the focal point of telomere/telomerase research was the role of

telomerase activation in cancerous tumor cells until the observation of telomerase in adult stem cells and germ cells (Mantell and Greider, 1994; Broccoli et al., 1995). Telomeres and telomerase present in stem cell lines have allowed for the mechanisms of aging to be increasingly better understood by the scientific community. The establishment of telomeres as a possible quantifiable measurement of cellular aging has popularized the study of telomere length in humans and became a topic of interest for improving livestock efficiency.

Telomere Biology

Structure

The telomere sequence in eukaryotic organisms is canonically composed of short G-rich sequences oriented toward the chromosome terminus (Teasley and Stewart, 2016). Telomeres in vertebrates are comprised of (5' – TTAGGG – 3') sequences; however, the sequence varies between invertebrates and plant species (McEachern et al., 2000). Since all eukaryotic genomes are organized into linear chromosomes, these tandem repeats act as the protective ends of chromosomes and maintain genetic integrity during DNA replication as previously stated. Telomeres are predominantly comprised of double stranded DNA (dsDNA) with a comparably short 3' single strand DNA (ssDNA) overhang of the G-rich strand (Sfeir, 2012). These single strand overhangs allow telomeres to form secondary telomeric loop structures (t-loops) by the invasion of the 3' ssDNA into the dsDNA of the telomere, which in turn forms a small ssDNA displacement loop (d-loop) (de Lang, 2004).
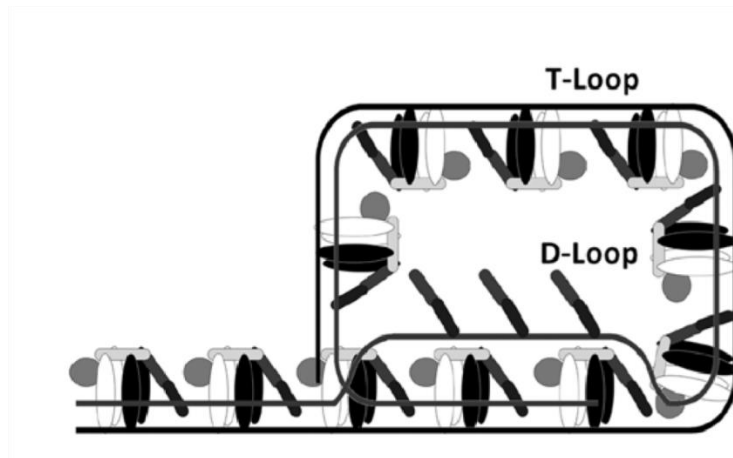
Figure 1. A schematic representation of a telomere in a T-loop configuration with a displacement loop (Reprinted with permission from Spandidos Publications Ltd; Gomez et al., 2012).

Currently, the function of t-loops is the prevention of end-to-end chromosome fusion but the mechanisms of this are poorly understood. Exposed ssDNA of the 3' overhang as well as ssDNA of the G-strand may also form G-quadruplexes. The phenomenon of telomeres to form G-quadruplexes results in the inhibition of both semiconservative DNA replication and telomerase-mediated telomere lengthening (Paechke et al., 2011). These secondary structures, while necessary for telomere stability, pose a problem for the continuity of DNA replication and must be regulated by alternate mechanisms within the cell.

Sheltrin Complex

A number of proteins are associated with telomeres that are crucial for correct maintenance and function. Six telomere-specific proteins exist to form the Shelterin Complex: TRF1 (telomeric repeat-binding factor 1), TRF2 (telomeric repeat-binding factor 2), POT1 (protection of telomeres protein 1), Rap1 (telomeric repeat-binding factor 2-interacting protein 1), TPP1 (adrenocortical dysplasia protein homolog), and

5

TIN2 (TRF1-interacting factor 2) (de Lang, 2005). TRF1, TRF2, and POT1 directly

interact with telomeric sequences to aid in replication fork progression by preventing the

DNA damage response (DDR) and the p53 apoptosis activation pathway as well as

preventing chromosome end-to-end fusion and mitigating telomere elongation by

competitively binding to telomerase binding sites (Sfeir, 2012; Longhese et al., 2012).

While RAP1, TPP1, and TIN2 cannot bind directly to telomeres, they still play an

important role in the Sheltrin Complex. Rap1 acts with Pot1 to negatively regulate

telomere length, TIN2 functions as a bridging protein to connect TRF1 and TRF2, while

TPP1 aids in the recruitment of POT1 to the telomere due to a lack of binding affinity of

POT1 to ssDNA (Sfeir, 2012). Together these six proteins stabilize telomeres, regulate

telomerase activity, and most importantly prevent telomere ends from being recognized

as DNA double strand breaks leading to cell cycle arrest or DNA damage response

(DDR).

Figure 2. A schematic interpretation of telomeric binding proteins (Sheltrin Complex; Reprinted with permission from Spandidos Publications Ltd; Gomez et al., 2012).

End Replication and Chromosomal Instability

Despite many mechanisms in place to aid in telomere stabilization, DNA replication still poses a problem due to the "end replication problem." Landsorp (2005) estimated, utilizing several animal models, that approximately a 10 bp loss can be attributed to incomplete DNA replication. However, other estimates have found approximately 25-100 bp are lost from the 3' ends of chromosomes after each DNA replication cycle (Morrison et al., 1996). As previously discussed, telomeres protect the coding segments of DNA found in the interior region of the chromosome from being exposed or lost during cellular replication. In the absence of lengthening mechanisms, critical telomere attrition activates p53/pRb-dependant apotosis and/or senescence pathways (Frias et al., 2012). It is well-established that senescent cells secrete inflammatory cytokines and have multiple roles in promoting degenerative diseases and pathology (Campisi et al., 2011). Telomere length ultimately determines cells "Hayflick Limit" or the total amount of divisions a cell can make within its lifetime before achieving senescence (Hayflick, 1965). However, in some cases telomeres will continue to degrade eventually exposing essential gene coding segments. Unprotected chromosome caps lead to chromosome instability, fusion, and cell death often referred to as telomere crisis (Blackburn, 2001; McEachern et al., 2000). Chromosome instability and fusion associated with telomere crisis (end-to-end fusion between chromosomes followed by breakage due to critically short telomeres or "breakage-fusion-bridge cycle") can produce oncogene amplification, tumor suppressor loss of function, and gene fusions that can lead to rapid proliferation and tumorigenesis (Deng et al., 2008).

Telomere crisis is not common and multiple mechanisms exist that inhibit such extreme conditions.

Telomeres play a vital role in maintaining genome stability and prevent the ends of linear chromosomes from being recognized as double strand breaks (DSB). However, the most important telomere function is its role as a tumor suppressive checkpoint and the ability to trigger cellular replicative senescence (Sobinoff and Pickett, 2017). Multiple mechanisms are responsible for these protective properties of telomeres. First, the Sheltrin protein TRF2, prevents nonhomologous end joining as well as preventing DNA damage signaling by initiating t-loop formation and inhibiting downstream DSB signaling cascade (Doksani et al., 2013; Okamoto et al., 2013) The TPP1/POT1 complex also prevents DNA damage signaling at the single strand 3' telomeric overhang (Zimmerman et al, 2014). It is important to note that telomere shortening beyond a critical length results in the loss of TRF2 and protective t-loop formation. Deficiencies in adequate telomere length or in sheltrin protein binding results in activation of the DNA damage response. Telomere attrition DDR activates both the ATM an ATR kinase checkpoints and ultimately activates p53 angiogenesis inhibition pathways (Deng et al., 2012). Activation of p53 may diverge to entry into either cellular senescence or apoptosis (Frias et al., 2012). Cells that escape these anti-proliferative mechanisms in the presence of telomere dysfunction become the agents of many cancers. However, these cells face one more checkpoint before entering oncogenesis. The telomere crisis checkpoint is crucial for cells with continued proliferation in the presence of critically short telomeres. This checkpoint functions because critically short telomeres are inclined

8

to fuse to other chromosome ends, causing aneuploidy and massive genome instability which leads to death in the majority of cells (Deng et al., 2008). However, a small percent of cells (approximately 15%) escape telomere crisis by means of alternative lengthening mechanisms or reactivation of telomerase which will ultimately manifest into premalignant cells (Gomez et al., 2012; Opresko and Shay, 2017).

Allostasis

Three endogenous factors most responsible for influencing TL and its attrition are inflammation, oxidation, and glucocorticoid signaling. Physiological stress is well established to have an integral part in altering the homeostasis of these factors. Allostasis is the adaption of neural, neuroendocrine, and neuroendocrine immune mechanisms to a stressful challenge in order to maintain stability through change (McEwen, 1998; Valsamakis et al., 2018). Allostatic load, or the amount of stimulation caused by stressors, is mediated by glucocorticoids generated by the hypothalamic-pituitary-adrenal (HPA) axis and is functionally linked to the mechanisms regulating telomere dynamics (Angelier et al., 2017; McEwen, 2013). Allostasis is an essential component of maintaining homeostasis, however, frequent activation of allostatic systems or failure to shut off allostatic systems after stress can lead to disease over time (McEwen, 1998). Glucocorticoids are known to enhance portions of the innate and adaptive immune response during short term stimulation, but pathological maintenance of elevated glucocorticoid concentrations is associated with suppression innate and adaptive arms of the immune system (Martin, 2009). Chronic inflammation associated with injury or disease, is one of the main sources of glucocorticoid signaling and reactive oxygen

species (ROS) which have the ability to act directly on telomeres (Barnes et al., 2018).

Activation of immune cells by glucocorticoid signaling produces inflammatory

cytokines (IL-1, IL-2, IL-6, TNF-α, etc) and ROS (OH, O_2, and H_2O_2) in the event of

injury or disease. While the exact mechanism of ROS on telomeres is not definite, the

sensitivity of guanine nucleotides to oxidative stress is thought to be the main cause of

injury (Reichert and Stier, 2017). Barnes et al. (2018), theorized that oxidative stress

induces apoptosis/senescence of somatic cells and the corresponding tissues responds

with increased proliferation of surviving cells, which leads to the shortening of telomere

length. Repeated clonal expansion driven by antigenic stimulation makes peripheral

leukocytes an attractive cellular model for the effects of proliferation on telomere length.

This rapid proliferation associated with immune response is the basis of many aging and

disease epidemiological studies (Effros et al., 2005). However, a secondary theory more

relevant to tissues outside the immune system, suggests that ROS induces SSB in

telomeres directly, creating lesions and ultimately causing replication fork collapse and

loss of telomere sequences (von Zglinicki, 2002). This would agree with the propensity

of guanine to oxidize to 8-oxoguanine, a more sensitive purine that is the substrate for

hydantoin lesions on telomeric DNA (Fleming and Burrows, 2017; Luo et al., 2001).

Hydantoin lesions halt DNA polymerase 1 progression and if not excised are thought to

lead to telomere dysfunction (Barnes et al., 2018; Redrejo-Rodriguez et al., 2011).

Several studies have shown that chronic inflammation and oxidative stress are associated

with telomere length attenuation. Of these studies, pathogenesis of diabetes, arthritis,

cardiovascular atherosclerosis, ulcerative colitis, Barrett's esophagus, hepatitis, asthma,

chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis, as well as autoimmune disease resulted in shortening of telomeres in subjects (Aikata et al., 2000; Armanios and Blackburn, 2012; Nzietchueng et al., 2011; Rey et al., 2017; Colmegna et al., 2008). The correlation between chronic inflammation and reduced telomere length is well established but less is known about acute inflammation. Kliment and Oury hypothesize that effects of "sporadic disease" may be similar to the proliferation/ROS theory of chronic inflammation. However, studies of acute psychological stress found that increases in cortisol, oxidative stress markers, and changes in distribution of immune cells actually upregulated immune function and telomerase activity (Dhabhar and McEwen, 1998; Gidron et al., 2006). This upregulation of immune function during acute stress shows mixed effects on TL and seems to be complex and highly variable on individual immune response (Angelier et al., 2017; Epel et al., 2010). While many factors contribute to telomere shortening, mechanisms exist to prevent loss of telomeres and extend cellular longevity.

Replication and Length Maintenance

DNA replication is a necessary process to ensure genome continuity. Telomeres, as a crucial part of the genome, must be maintained during replication. Telomeres may be replicated primarily by two methods: semiconservative DNA replication or by the actions of the ribonucleoprotein enzyme telomerase. While the primary methods of TL conservation are listed above, other recombinatorial events during replication may result in a net gain of TL or by a process known as alternative lengthening of telomeres (ALT)

(Sobinoff and Pickett, 2017). Together these mechanisms result in telomere replication and maintenance but under certain circumstances can produce telomere lengthening.

Semiconservative Telomere Replication

Replication of telomeres poses unique problems due to its composition and location at the chromosome termini. The hallmark of telomeres is that they are comprised of identical tandem G-rich sequences, which means they lack many of the start and stop codons necessary for facilitating DNA replication. Due to this anomaly, the presence of a sole replication fork at the origin of the telomeric sequences is responsible for copying the entire length of the telomere (Gilson and Geli, 2007). If this replication fork fails or stalls, telomere replication may be incomplete resulting in the net loss of TL. Replication fork failure or collapse is likely due the difficulty of replication of G-rich sequences compared to more variable genomic sequences (Cesare and Karlseder, 2012). In conjunction with replication fork failure, the secondary structures formed with telomeres, such as T-loops and G-quadruplexes, must be aligned into dsDNA posing another challenge for successful DNA replication.

The compounded flaws of telomere replication require robust mechanisms to prevent rapid shortening of TL. DNA replication and support proteins are recruited by the sheltrin complex (Teasley and Stewart, 2016). Many of the sheltrin proteins previously discussed aid in replication fork progression, stabilization, and alleviating topological stress (Sfeir et al., 2009; Ye et al., 2010). However, proteins within the sheltrin complex do not address the problem of telomere secondary structure. To resolve this, RecQ helicases, BLM and WRN, are recruited to unwind G-quadruplexes (Teasley

12

and Stewart, 2016). Despite many proteins in place to safeguard telomere replication, fork stalling and breakdown still occur but additional mechanisms remain in place to initiate fork restart. Few of these mechanisms are well documented and the complete model for fork restart in telomere replication is not fully understood.

Telomerase

Telomeres themselves are maintained by the enzyme telomerase. Telomerase is a ribonucleoprotein that acts as a reverse transcriptase to extend telomeric DNA during S phase and into M phase of DNA replication (Diede and Gottschling, 1999; Marcand et al., 2000). Telomerase is composed of a catalytic subunit, hTERT, and an RNA component, hRT which acts as a template for the addition of the telomeric repeat (5'-TTAAGGG-3') to the 3' end of DNA (Gomez et al., 2012). Telomerase adds telomeric sequence repeats to the telomere termini, thus lengthening the telomere and mitigating the end replication problem. At the telomere termini, the process of telomere elongation occurs in two steps. In step one, the 3' end of the telomere base pairs with the 5' region of hTR and the hTERT active site uses the 3' end of the telomere as a primer to reverse transcribe a telomere repeat using hTR as a template; in the second step, hTR dissociates from the telomeric DNA, translocates, and re-anneals. (Podlevsky and Chen, 2012; Teasley and Steward, 2016). After telomerase has dissociated from the ssDNA template, polymerase α synthesizes the complementary C-rich strand. As previously discussed, many factors can influence the fluidity of telomere elongation. Sheltrin complex proteins POT1-TPP1 complex together to aid in the facilitation of telomere primer binding to telomerase but in some cases can also inhibit telomere binding; the binding of POT1 to

ssDNA is believed to prevent telomerase primer binding (de Lang, 2005). Further research is needed to elucidate the reasoning behind telomerase binding inhibition by sheltrin proteins.

The activity level of telomerase is variable between species and within cell types. In most vertebrates', telomeres of somatic cells progressively shorten throughout their lifespan due to absent or low telomerase activity while adult stem cells and germ cells have elevated telomerase activity (Blackburn et al., 2015). Activated lymphocytes in other vertebrate species, have detectable levels of telomerase activity and are commonly used as a model for telomerase behavior (Goronzy et al., 2006). Lin et al., (2010) found that B cells have the greatest level of telomerase activity while CD8 cells have the least activity *in-vivo*. Heterogeneity of telomerase activity in different immune cells is important to note because recruitment of immune cells differs between acute and chronic stressors. Hematopoietic stem cells (HSC), found in the red bone marrow, have a high level of telomerase activity (Elwood, 2004). Hematopoietic stem cells give rise to myeloid and lymphoid cells which ultimately give rise to all white blood cells through hematopoiesis. In a review of the mechanics of blood cell formation, Metcalf (1988) states that approximately 10^9 cells are produced every hour making HSC's highly proliferative. It is important to note that TL in HSC's is a reflection of progenitor cell reserves, and shortened TL in these cells is indicative of diminished reparative capacity (Aviv, 2012). Considering that peripheral leukocytes are produced by HSC for maintenance and during antigenic stimulation of the immune system, it can be assumed that these leukocytes will represent the telomeric status of the HSC themselves.

Progressive shortening of mean TL associated with aging can be observed in peripheral blood mononuclear cells (Iwama et al., 1998) and are representative of biological aging (Entringer et al., 2012b). Telomere erosion can be described as a "mitotic clock" with TL reflecting the replicative history of divisions within individual cells.

Telomerase activity is highly interactive and dependent on multiple exogenous and endogenous factors. T-lymphocytes can up-regulate telomerase activity upon activation by antigens (Weng et al., 1997); however, prolonged activation ultimately leads to loss of telomerase activity and telomeric cellular senescence (Choi et al., 2008). Recent studies have found that telomerase activity is down regulated in peripheral blood leukocytes in mothers undergoing chronic emotional stress (Epel et al., 2004) as well as long term caregivers of patients with Alzheimer's disease (Damjanovic et al., 2007). Choi et al. (2008) found that T lymphocytes had reduced telomerase activity when exposed to cortisol and that CD4 and CD8 subsets had reduced levels of the transcript for hTERT. The catalytic subunit of telomerase is hTERT and without adequate transcription, telomerase cannot catalyze the addition of the telomere sequence during DNA replication. Not only does cortisol have an inhibitory effect on telomerase but long-term antigenic stimulation of CD8+ T cells has a similar response. Akbar and Vukmanovic-Stejic (2007) found that virus specific CD8+ T cells initially have high telomerase activity, but after a year of exposure to viral infection, telomerase activity was un-detectable, and subjects had reduced TL. An in-vivo study of human CD8+ T cell subsets by Effros et al. (2005) found similar telomerase behavior in CD8+ T cells given 3 antigenic stimulations; initial stimulation yielded an increase in telomerase

activity; however, the 3rd stimulation resulted in undetectable telomerase activity and attenuated TL. This models the theory of chronic glucocorticoid exposure on reduced telomerase activity and reduced TL but acute exposure may result in increased lymphocyte telomere length.

Effects of cortisol and antigenic stimulation are not the only endogenous factors influencing telomerase activity. Estradiol and tumor necrosis factor-α have been found to enhance telomerase activity in human T lymphocytes, while interferon alpha and transforming growth factor-β have opposite effects (Effros et al., 2005). Estrogen is known to have antioxidant properties and can directly and indirectly activate telomerase through the hTERT promoter (Kyo et al., 1999). Telomerase activity is a highly plastic mechanism and shows up-regulation and down-regulation depending on the category and duration of the stressor. In addition to telomerase, telomeres may be extended by "Alternative Lengthening of Telomeres" (ALT) which refers to homologous recombination events that result in a net gain of TL (Sobinoff and Pickett, 2017). Telomerase and ALT exist to help mitigate changes in TL but seem to be highly interactive with biotic and abiotic factors.

Plasticity

Telomeres naturally shorten with normal cellular replication, but this process is interactive and can be greatly influenced by outside stressors. Shalev et al. (2013) describe biological/cellular aging to be a progression of prenatal adversity, childhood trauma, adult mental disorders, and age-related disease which may all be influenced by healthy lifestyle factors. Telomere dynamics can be described as highly plastic and while

16

TL shortens with the natural progression of time, the abiotic and biotic events present in an organism's lifetime ultimately determine the rate of attenuation and aging.

Prenatal Stress and Early Adversity

Telomeres can be impacted as early as in fetal life. Prenatal stress from disease as well as physiological stress in the mother has been shown to alter fetal TL (Entringer et al., 2011). Shalev et al (2013) found that conditions during embryonic, fetal, and early postnatal periods of life can interact with the genome of an individual. Entringer et al. (2013, 2012a, 2012b) observed this phenomenon in infants born to mothers with diabetes and preeclampsia as well as mothers who experienced severe psychological stress during pregnancy. Entringer et al. (2011) estimated that infants born to mothers experiencing severe psychological stress had an estimated 3.5 years of additional biological aging. This may be explained by the elevated cortisol concentrations in the mother being passed to the fetus via the maternal blood supply. Cortisol is known to decrease the actions of telomerase in stem cells as described previously (Podlevsky and Chen, 2012). Despite the relationship between cortisol and telomerase, it is well established that cortisol is inactivated by 11β-hydroxysteroid dehydrogenase (11βHSD) to cortisone and acts as a placental "barrier" to glucocorticoids. In an *in-vitro* study, it was observed that 85% of administered cortisol was converted to cortisone by action of 11βHSD in placental tissues (Murphy et al., 1974). However, the fetus is still exposed to 15% of maternal cortisol which may alter the fetal telomere biology. Additionally, decreased placental 11βHSD has been correlated to intrauterine growth restriction (IUGR) and the fetal programming of adult diseases (McMullen et al., 2004). Mulligan

17

et al. (2012) states that fetal tissues are especially sensitive to the intrauterine environment which ultimately determines fetal phenotype.

Telomere length has also been found to be moderately-highly heritable in humans (estimated range 34 - 82%) (Shalev et al., 2013). In an interesting study utilizing Great Reed Warblers, it was found that age of the dam was positively related to offspring TL but offspring TL was not related to paternal age (Ashgar et al., 2015b). This was an interesting contrast to other species including humans that showed offspring TL was positively correlated to paternal age at conception and not maternal age (Njajou et al., 2007). Mangino et al. (2012) notes that although TL has a relatively high heritability, the known genetic variants (GWAS and candidate gene approaches) account for only a small proportion of the variance in TL. Newborn TL is complex and easily influenced by intrauterine conditions and early life experiences.

Stress during post-natal life has the potential to alter an individual's longevity and health into adulthood. Reduced TL was found in children exposed to institutional care (Drury et al., 2011), emotional/physical/sexual abuse (Tyrka et al., 2010; Glass et al., 2010; Kiecolt-Glaser et al., 2011; O'Donovan et al., 2011; Shalev, 2012), and adverse social environments (Kananen et al., 2010). The biological effects on TL of childhood adversity have been well documented. In a study of 4,000 middle aged women, Surtees et al. (2011) found that the more categories of childhood adversity (physical abuse, paternal divorce, unemployment, or drug use) a woman experienced, the shorter her telomeres were later in life. Beyond childhood adversity, telomeres are subjected to stress factors related to traumatic life events and disease.

18

Aging and Disease

A general trend has been observed in vertebrate telomere dynamics with aging. Specifically, during the early growth and development phase, telomeres undergo a rapid attrition followed by a general slow decline through adulthood (Foote et al., 2010; Pauliny et al., 2012; Rattiste et al., 2015). This is supported by the rapid proliferation of cells and tissues associated with growth and development as well as the metabolic modification to support such increases resulting in the production of ROS (Angelier et al., 2017; Smith et al., 2016). The sensitivity of TL during developmental stages could ultimately determine survival probability and lifespan into adulthood. Gradual telomere shortening through adulthood is often the cause of many age-related phenotypes and can be accelerated by chronic stress. The concept of short telomeres determining morbidity and mortality is best supported by the existence of telomeropathies or single mutations in telomere regulatory proteins (TERT, TERC, TINF2, WRAP53, CTC1, DKC1, NHP2, and NOP10) that result in critically short telomeres and clinically manifest themselves as fatal genetic diseases such as idiopathic pulmonary fibrosis and dyskeratosis congenita (Nelson and Bertuch, 2012; Alder et al., 2008; Wegman-Ostroksy and Savage, 2017). Adult-onset disease is created by a combination of factors, but the general concept is that after prolonged exposure to oxidative stress and inflammation, cells will develop short telomeres that trigger senescence and ultimately alter the cytokine profile of the cell to produce more pro-inflammatory cytokines (Aviv and Shay, 2018). This process can be hastened by the presence of immune related disease. Patients with auto-immune disease such as rheumatoid arthritis (RA) and diabetes mellitus have been found to have

shortened TL in leukocytes compared to healthy controls (Koetz et al., 2000; Testa et al., 2011). Rheumatoid arthritis is a chronic inflammatory disease often used in TL studies due to the increased proliferation of immune cells at the sites of inflammation. A study of hematopoietic progenitor cells (HPC) in rheumatoid arthritis patients found that CD34+ HPC were shortened by 1,600 bp compared to controls (Colmegna et al., 2008). This may be explained by a study conducted by Fujii et al. (2009) that observed naïve CD4 T cells of RA were defective in up-regulating telomerase activity. Reduced TL in rheumatoid arthritis patients is speculated to be the result of this telomerase regulation defect.

Another causal agent for telomere attrition is acquired disease; either viral or bacterial. When the immune system encounters an antigen, it undergoes clonal expansion or a rapid proliferation in preparation to clear the system of the offending foreign substance. This has been most studied in T lymphocytes as they have the ability to respond to a broad range of antigens either directly or through antigen presenting cells. Many studies have observed T cell telomere dynamics in-vitro and in-vivo. Chronic viral infections such as Human Immunodeficiency Virus, Epstein-Barr Virus, and cytomegalovirus have been used to observe reduced TL and diminished replicative capacity in antigen specific T cells (Van et al., 2008). In animal models it has been observed that repeated exposure to bacteria was associated with rapid telomere attrition in mice and that chronic infection of malaria was also linked to reduced TL in avian species (Ilmonen et al., 2008; Asghar et al., 2015a).

Mood, Behavior, and Mental Illness

Mental illness has also been correlated with telomere attenuation. Individuals with mood disorders have been found to have shortened TL (Simon et al., 2006) as well as an increase in morbidity and mortality due to age-related disease (Kupfer, 2005). Medical illnesses associated with mood disorders include cardiovascular disease, stroke, dementia, cancer, obesity, type II diabetes mellitus, and osteoporosis (Evens et al., 2005). Reduced TL has been observed in a variety of other mental health disorders including bipolar disorder and schizophrenia (Price et al, 2013). Simon et al. (2006) speculates that reduced TL from chronic mental illness may be an explanation for the increase in age-related disease in patients with diagnosed mood disorders. Progression of age and acquisition of disease are the biotic elements of telomere plasticity of abiotic elements which have a well-documented impact on telomere dynamics.

Perceived and Environmental Stress

Chronic perceived stress has been linked to poor health and reduced TL but the dynamics behind stress response can be described as "dose-dependent" and variable when compounded with lifestyle choices. Examples of perceived stress resulting in reducing TL include individuals who recently experienced interpersonal violence, rape, lower socioeconomic status, and stress of caregiving (Epel et al., 2006; Price et al., 2013; Mathur et al., 2016; Lin et al., 2012). Results from a meta-analysis of 22 current perceived stress studies found that there was a statistically significant reduction of TL in subjects involved in perceived stress such as post-traumatic stress disorder, cancer survival, caregiving, abuse, cardiovascular disease, childhood adversity, and chronic

pain (Mathur et al., 2016). Perceived stress has been modeled in avian species and reduced telomere length was found after a perceived stress challenge as well as chronic glucocorticoid exposure (Haussmann and Heidinger, 2015). *In-vitro* work has also shown that high concentrations of glucocorticoids dampen telomerase activity in T lymphocytes and *in-vivo* work in humans shows that shorter telomeres are associated with greater cortisol reactivity (Choi et al., 2008; Gotlib et al., 2015). This was best demonstrated by Epel et al., (2004) in a study of caregiving mothers of healthy and ill children, that found mothers of ill children had higher urinary cortisol concentrations, lower telomerase reactivity, and shorter TL compared to mothers of healthy children. Shalev et al., (2013) theorizes that overall stress induced secretion of glucocorticoids such as cortisol may down-regulate telomerase and increase oxidative stress which will indirectly and directly lead to erosion of telomeres. However, in times of acute perceived stress, increased cortisol has been shown to up-regulate telomerase activity in elderly women (Epel et al., 2010). Dynamics of acute and chronic stress are best observed in patients with major depressive disorder and other psychiatric disorders. In a review of 10 major depressive disorder studies, significantly shortened leukocyte TL was found in patients with chronic, severe depression compared to shorter periods of depression (Shalev et al., 2013). However, compounding adverse life events seems to also impact the degree of TL attrition. In studies of anxiety disorders, individuals suffering from post-traumatic stress disorder showed significantly shorter TL than controls, but this result seemed to be largely related to the presence of adverse childhood events (Kim et al., 2017). This demonstrates the dose-dependent manner of perceived stress on

telomerase and TL. It is clear that environmental stressors and life events ultimately aid in the degree of biological aging an individual undergoes through life.

Reproduction

Female reproduction is one of nature's most complex physiological processes and is tightly linked to telomere dynamics through glucocorticoid signaling and the psychological stress of parental effort. Cross talk between the hypothalamic-pituitary-adrenal axis, HPA axis, and fetal/placental signals during pregnancy form a very intricate system for maintaining homeostasis of not only the mother but the fetus as well. It is important to note that pregnancy is a period of hypercortisolism in the mother and that placenta-derived corticotropin releasing hormone (CRH) increases through pregnancy driving the release of cortisol via the maternal HPA axis (Valsamakis et al., 2018; Magiakou et al., 1996). The altered glucocorticoid profile of pregnancy is most likely the cause of telomere shortening with parity by means of reduced telomerase activity. Interestingly, estrogen is known to up-regulate telomerase activity by binding to the promoter region of hTERT (Kyo et al., 1999). However, during gestation estrogen is suppressed to allow for the dominance of progesterone for the maintenance of pregnancy. This may also be a factor in telomere homeostasis during reproduction. Parity has been associated with reduced telomere length in women and that parous women had 4.2% less telomere sequences (116 fewer base pairs) than nulliparous women (Pollack et al., 2018). However, other studies have found that women with more surviving children were associated with longer telomeres (Barha et al., 2016). This is theorized to be the result of increased lifetime estradiol exposure and improved social

23

factors associated with increased reproductive effort (Barha et al., 2016). Interesting, it is very well established by Epel et al. (2004, 2010) that caregiving stress results in reduced TL. A variety of animal models have found telomere length to be negatively associated with reproductive effort (Kotrschal et al., 2007; Plot et al., 2012, Bauch et al., 2013; Reichert et al., 2014; Sudyka et al., 2014). Within these studies, it was also observed that reproductive effort was positively associated with elevated glucocorticoid concentrations (Bonier et al., 2011; Love et al., 2014; Sudyka et al., 2014; Reichert et al., 2014). As previously stated, parental effort is perceived stress and necessary for the survival of offspring in most species. Here, glucocorticoid signaling plays another important role in the degree of parental effort. An intense glucocorticoid stress response has been associated with a reduction in parental effort while a weak stress response has been associated with increased parental effort (Wingfield and Sapolsky, 2003; Lendvai et al., 2007; Krause et al., 2016). As previously stated, reduced TL is associated with increased stress reactivity and this suggests that TL may be linked to parental fitness. A variety of stressors have the potential to reduce an individual's health and longevity; however, there is minimal research to explore this concept in production animals such as beef cattle.

Telomeres in Cattle

The desire for a biological marker of longevity, specifically productive longevity, is growing in the livestock industry as well as a marker for health and animal welfare. While TL has been studied in humans and murine species since the early 1990's, the use of TL as a potential marker for livestock welfare, health, and longevity has recently

become a topic of interest in the last seven years (Bateson, 2015; Brown et al., 2012; Fairlie et al., 2016, Laubenthal et al., 2016, Yamada et al., 2015). Selection of livestock that stay productive longer is a logical method for reducing the number of animals in production by reducing the need for replacement females, minimizing waste, decreasing greenhouse gas emissions and the cost of farming (Garnsworthy, 2004). Considering the world population is predicted to reach at least 9.9 billion people by the year 2050 (Population Reference Bureau, 2018), the need to increase the efficiency by which animal protein is produced is critical.

Telomere sequences were first discovered on bovine chromosomes within blastocyte embryos in 1999 during the rise of popularity in the cloning of livestock (Betts and King, 1999). The presence of telomerase was also detected in bovine morula and blastocytes and gave the livestock cloning movement a greater understanding of the implications of aged telomeres in somatic cell nuclear transfer and the possible mitigation by telomerase during embryogenesis (Betts and King, 1999; Xu and Yang, 2003). Currently, TL research in cattle has been centered around survival and productivity in dairy breeds. The initial use of TL measurements in Holstein cattle was to quantify the effects of management practices and stress on cow physiology due to the confounding factors associated with cortisol measurements in other stress/physiology studies. Specifically, Brown et al. (2012) found that cows with above average quantity of leukocyte telomere (qT) were less likely to be culled than cows with below average qT and found a negative association between qT and age. This suggests that cattle with longer TL may be productive longer and live to older ages. Investigation of metabolic

tissue TL within the liver, subcutaneous adipose tissue, mammary gland, and leukocytes

found that mammary tissue and peripheral leukocytes had decreased TL between early

and late lactation (Laubenthal et al., 2016). These results would be in line with the high

proliferation rate of mammary tissue during mammogenesis and leukocytes post-partum

reducing TL due to replicative loss but could also be related to telomerase inhibitors

such as cortisol. This suggests that the telomere dynamics of cattle are similar to that of

previously studied mammalian vertebrates such as humans. Reproductive effort has been

linked to telomere attrition due to elevated levels of glucocorticoids during pregnancy

and parturition (Angelier et al., 2017) Since cows are expected to raise a calf every year,

reproductive and parental effort could be a large contributor in cattle telomere dynamics.

A recent longitudinal study of TL in dairy cattle found heritability estimates of 0.36-0.47

(SE=0.05-0.08) and that individual differences of TL were not correlated with

productive lifespan (Seeker et al., 2018a). This is in disagreement with the results found

by Brown et al. (2012) that individual TL determined culling status and survivability.

The inclusion of geriatric cattle, or cattle greater than 6 years of age as defined by Seeker

at el., (2018), could explain why a linear relationship between TL and culling

status/survivability was seen. There is now strong evidence that TL and telomere

dynamics are tightly linked to fitness, longevity, and survival in multiple vertebrate

models (Angelier et al., 2017). Heritability of TL makes it an appealing selection

criterion for dairy cattle but the inconclusive findings of TL as a measurement of

productive lifespan leaves room for further studies. Currently, there has been no

investigation on the reproducibility of these studies in beef breeds of cattle. The

mechanisms behind bovine telomere dynamics between birth and culling (or survival)

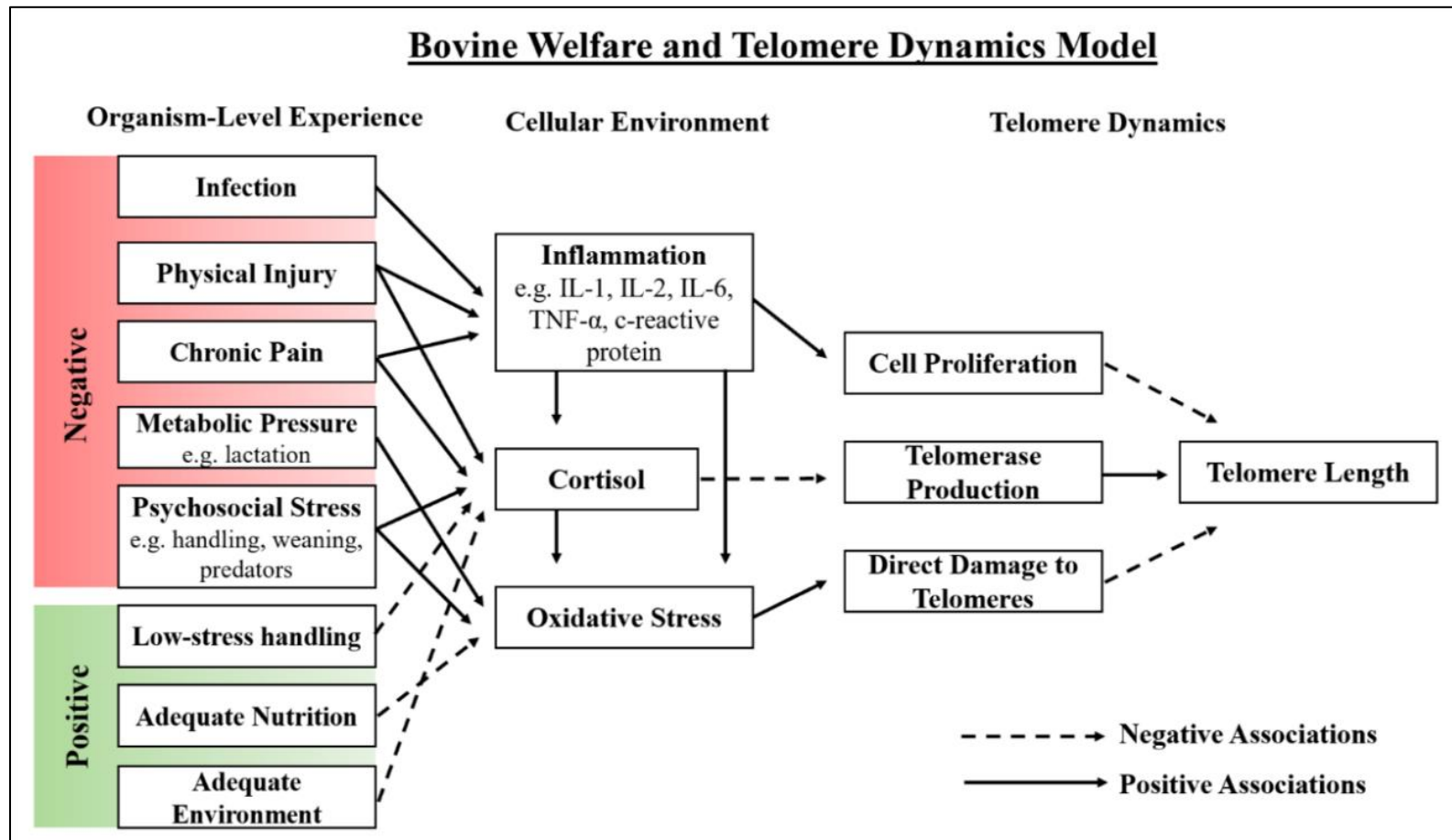remain a topic of exploration.

Figure 3. A schematic representation of the cascade of organismal experiences and their modes of action on telomere dynamics (Modified from Bateson, 2015).

CHAPTER III

EVALUATION OF LIPOPOLYSACCHARIDE INDUCED ACUTE

INFLAMMATORY STRESS ON TELOMERE LENGTH IN BRAHMAN HEIFERS

Introduction

Telomeres (5' – TTAGGG – 3') are tandem, non-coding DNA sequences oriented toward chromosome termini (Blackburn and Gall, 1978). In many vertebrates, these sequences play a crucial role in maintaining chromosome stability during DNA replication (Blackburn, 2005). Telomeres are lost slowly over time due to the "end replication problem"; however, this is mitigated by the ribonucleoprotein enzyme telomerase (Greider and Blackburn, 1989). Despite lengthening mechanisms, the gradual shortening of telomeres has been associated with increased mortality and the occurrence of multiple age-related disease in humans (Cawthon, 2003; Landsorp, 2009). These results lead to the theory that TL would be a reliable proxy for age or "mitotic clock", however, the relationship between aging and TL has proven to be more complex (Haussmann and Vleck, 2002; Angelier et al., 2017). This complexity can be largely attributed to the high plasticity of TL and telomerase behavior when exposed to environmental stressors and the resulting cascade of glucocorticoid signaling (Angelier et al., 2017). There is now strong evidence that telomere length and dynamics are tightly linked to fitness, longevity, and survival in captive and wild vertebrates (Haussmann and Marchetto, 2010; Haussmann and Heidinger, 2015; Asghar et al., 2015; Fairlie et al., 2016; Brown et al., 2012; Seeker et al., 2018a; Seeker et al., 2018b). Telomeres are

29

considered to be not only a molecular marker of biological age but also representative of the "wear and tear" of organismal experiences and may be an important tool to assess the influence of life-history events and environmental conditions in wild and domestic vertebrates (Anglier et al., 2017; Bateson, 2015).

Animal welfare is assumed to be influenced by the cumulative effects of the positive and negative events experienced by an individual (Mendl et al., 2010). Currently, leukocyte TL is the gold standard for assessing disease stress resulting in chronic inflammation due to rapid leukocyte turn-over (Bateson, 2015). Many human and non-human vertebrate studies have found significant telomere shortening in circulating leukocytes with chronic inflammation (Koetz et al., 2000; Testa et al., 2011; Colmegna et al., 2008; Fujii et al., 2009; Hau et al., 2015). However, little research has been done to asses TL following acute inflammatory events.

Materials and Methods

Animal procedures for this project were approved by the Texas A&M AgriLife Research Agricultural Animal Care and Use Committee (2017-037A). Animal procedures were carried out at the Texas A&M AgriLife Research and Extension Center located in Overton, TX. Twelve 20-21 mo old Brahman heifers were utilized for this project.

Experimental Design

Brahman (*Bos indicus*) heifers were grouped into either lipopolysaccharide (LPS) (n = 6) or control (n = 5) groups based on birthdate, temperament score, and sire. All heifers were fitted with a self-contained, indwelling vaginal temperature probe 7 d

before experimental treatments began (Burdick et al., 2011). At the onset of the trial heifers were approximately 640.6 ± 4.46 days of age. From February 16th to March 9th of 2018, heifers were kept on pastures consisting of 'Coastal' bermudagrass (*Cynodon dactylon*) overseeded with 'Maton' rye (*Secale cereale L.*) and 'Nelson' ryegrass (*Lolium multiflorum* Lam.). In addition to pasture and free choice Coastal bermudagrass hay, heifers were fed approximately 3.6 kg of a 3:1 corn:corn gluten grain mix per head per day.

Table 1 Heifer BW (kg) and BCS for each treatment and treatment day (MEAN ± SE).

Treatment Day					
	Treatment	**d 0**	**d 7**	**d 14**	**d 21**
BW (kg)	**LPS**	327 ± 13	305 ± 9	331 ± 16	329 ± 15
	Control	324 ± 8	299 ± 10	319 ± 9	327 ± 10
BCS	**LPS**	5.92 ± 0.26	5.92 ± 0.22	5.92 ± 0.17	5.92 ± 0.22
	Control	5.5 ± 0.14	5.58 ± 0.09	5.67 ± 0.12	6 ± 0.20

On d 0 (February 16th) LPS treatment heifers were administered 0.25 µg/kg of lipopolysaccharide (LPS; Sigma Chem etc; O111:B4), from *Escherichia coli*, subcutaneously in the neck after having approximately 20 ml of blood drawn by jugular venipuncture. Increasing doses of LPS were utilized on d 7 (0.5 µg/kg; February 23rd) and d 14 (0.75 µg/kg; March 2nd). Control heifers were bled to an equal amount and administered physiological saline at the same dosage and injection method on d 0, d 7,

and d 14. Heifers were observed for sickness behavior scores after injection every 30 min for 2 hrs and every hr for an additional 10 hrs. On d 21 (March 9th), no treatments were administered, and blood was drawn. On all treatment and sampling days, d 0 - d 21, heifers were weighed and had BCS recorded and an additional 5 ml of blood was collected and sent to the Texas A&M Veterinary Medical Diagnostic Laboratory for complete blood counts prior to treatments. Evaluation of BCS (1 – 9 scale) was done by a trained professional with over 40 years of experience. Ambient temperature was recorded continuously for the duration of the trial.

White Blood Cell Isolation

Whole blood was centrifuged at 2,675 RCF (relative centrifugal force) for 30 minutes at 5°C. After centrifuging, the white blood cell (WBC) layer located between the red blood cell (RBC) and serum interface, was aspirated with a 1,000 µl pipette and transferred into a 1.5 ml storage tube. The WBC were then cleaned of any remaining RBC and serum utilizing an RBC lysis buffer containing EDTA and water. The WBC were mixed with the RBC lysis buffer for 5 minutes and microfuged for 5 minutes at 5,000 RCF. The remaining RBC, serum, and RBC lysis buffer was aspirated from the top of the WBC pellet. The WBC pellet was then stored at -80°C.

DNA Extraction

Extraction of DNA was performed utilizing spin column GeneJET Genomic DNA Purification Kits (Thermo Scientific; Waltham, MA, USA). However, prior to extraction, frozen WBC were crushed with liquid nitrogen utilizing a mortar and pestle and weighed to approximately 20 mg to accommodate the purification kits. Remaining

crushed WBC were returned to -80°C storage. Quality control of extracted DNA was

conducted at the Texas A&M University Institute for Genome Sciences and Society

Genomics Core lab and criteria were as follows: yield > 20 ng/µl, integrity score < 3,

260/280 > 1.7 and 260/230 > 1.8 (as outlined in Seeker et al., 2016). DNA that passed

quality control was aliquoted and diluted to 10 ng/µl utilizing nuclease free water into

low-binding tubes and stored at -80°C.

Quantitative PCR

Quantity of telomere (qT) sequences was determined utilizing real-time

quantitative PCR methods utilizing the ratio of telomere to β-2-globulin (B2G) genes.

Custom primers for amplifying bovine telomere sequences (telg = 5'-aca cta agg ttt ggg

ttt ggg ttt ggg ttt ggg tta gtgt - 3', and telc = 5'- tgt tag gta tcc cta tcc cta tcc cta tcc cta

tcc cta aca - 3') and the (B2G) gene (β-1 = 5'- cgg cgg cgg gcg gcg cgg gct ggg cgg gaa

ggc cca tgg caa gaa gg - 3'), and β-2 = 5'- gcc ggc ccg ccg cgc ccg tcc cgc cgc tca ctc

agc gca gca aagg - 3') (defined by Brown et al., 2012 and Cawthon et al., 2009) were

designed and purchased from Integrative DNA Technologies (IDT; Coralville, IA,

USA). The bovine β-2-globulin gene was selected as a relatively constant housekeeping

gene that operates as a control gene for determining relative qT (Brown et al., 2012).

The master mix for the telomere and B2G reaction contained 10 µl 2X PowerUp SYBR

Green Master Mix (Applied Biosystems; Foster City, CA, USA), 1 µl of bovine telomere

primers (forward and reverse, each to 500 nmol final concentration), 0.6 µl of B2G

primers (forward and reverse, each 300 nmol final concentration), and 4.8 µl nuclease

free water. Master mix was loaded prior to 20 ng/µl of DNA. Standard double-stranded

DNA oligonucleotides of bovine telomere (sense 5'-tta ggg tta ggg tta ggg tta ggg tta ggg tta ggg tta ggg tta ggg tta ggg tta ggg tta ggg tta ggg tta ggg tta ggg - 3'; antisense 5'- ccc taa ccc taa ccc taa ccc taa ccc taa ccc taa ccc taa ccc taa ccc taa ccc taa ccc taa ccc taa - 3') and B2G (sense 5'- ggt gaa ggc cca tgg caa gaa ggt gct aga ttc ctt tag taa tgg cat gaa gca tct cga tga cct caa ggg cac ctt tgc gct gag tga gct g - 3'; antisense 5'-cag ctc act cag cgc agc aaa ggt gcc ctt gag gtc atc gag atg ctt cat gcc att act aaa gga atc tag cac ctt ctt gcc atg ggc ctt cac c - 3') were designed and purchased from IDT. A six-step, 1:10 serial dilutions of bovine telomere oligo (10^{12} - 10^7) and bovine B2G oligo (10^9 - 10^4) were used to create standard curves and were generated immediately prior to loading. Samples were randomly allocated on a 384-well qPCR plate and amplified in triplicate, 20 µL reactions. A negative control containing water in place of DNA was included to monitor for contamination of the master mix. A real-time system thermocyler (BioRad; Hercules, CA, USA) was programmed with multiplex settings defined by Brown et al. (2012). Cycle threshold (C_q) value triplicates were checked for consistency. Triplicates were included if the cycle difference was less than 0.3 cycles. Single replicates with differences larger than 0.3 cycles were removed if an obvious outlier could be identified, and the remaining wells were included. Samples with remaining wells with differences larger than 0.3 cycles were excluded from analysis. Starting quantities (SQ) were averaged for samples with adequate C_q replicate differences. Copy number of telomere sequence (5' – TTAGGG - 3') and B2G gene was represented by SQ and a ratio of telomere SQ and B2G SQ was utilized to determine T/S ratio.

Statistical Analyses

Statistical Analyses were conducted using mixed linear models with the MIX procedure of SAS 9.4 (SAS Inst., Inc., Cary, NC, USA). All T/S ratio measurements were $\log_{10}$ transformed in an attempt to normalize distribution. Treatment (LPS challenged or control) was the fixed effect of interest. Other investigated fixed effects included treatment day, birthdate and their interactions as well as BW and BCS. Random effects included sire and day of record as a repeated measurement on heifers. Pearson correlation coefficients were calculated using the CORR procedures of SAS and tested for T/S ratio with these measured traits for these heifers: neutrophil and lymphocyte counts and their ratio, monocyte counts, and total white blood cell counts.

Results

Vaginal temperature data and measurements of clinical symptoms were analyzed independently of T/S ratio. Three criteria were utilized to evaluate vaginal temperature: maximum temperature (MaxTemp, °C), time to maximum temperature (TmaxTemp, min), and change in temperature (ΔTemp, °C). Treatment was a significant predictor of an increase in MaxTemp (P = 0.04), decrease in TmaxTemp (P < 0.0001), and increase in ΔTemp (P < 0.0001) (Fig. 7,8, and 9). Treatment day was significant for MaxTemp (P = 0.04) and TmaxTemp (P = 0.04) but not ΔTemp (P = 0.74). Differences for MaxTemp were seen between 0.25 µg/kg BW and 0.50 µg/kg BW (P = 0.01) as well as 0.50 µg/kg BW and 0.75 µg/kg BW for TmaxTemp (P = 0.01). No treatment-dose interactions were detected for MaxTemp (P = 0.51), TMaxTemp (P = 0.18), and ΔTemp (P = 0.38). No SBS were observed. No differences in BW (P = 0.37) and BCS (P = 0.18) score were

35

detected by day. No differences in CBC were detected for treatment or treatment dose

interactions. However, treatment day was a significant predictor of neutrophil count (P =

0.007) and neutrophil to lymphocyte ratio (N:L ratio) (P = 0.01). However, when

analyzed using CORR procedures, the differences in neutrophil count and N:L ratio were

not significant (Table 1). Treatment day did not affect CBC measurements (Table 2).
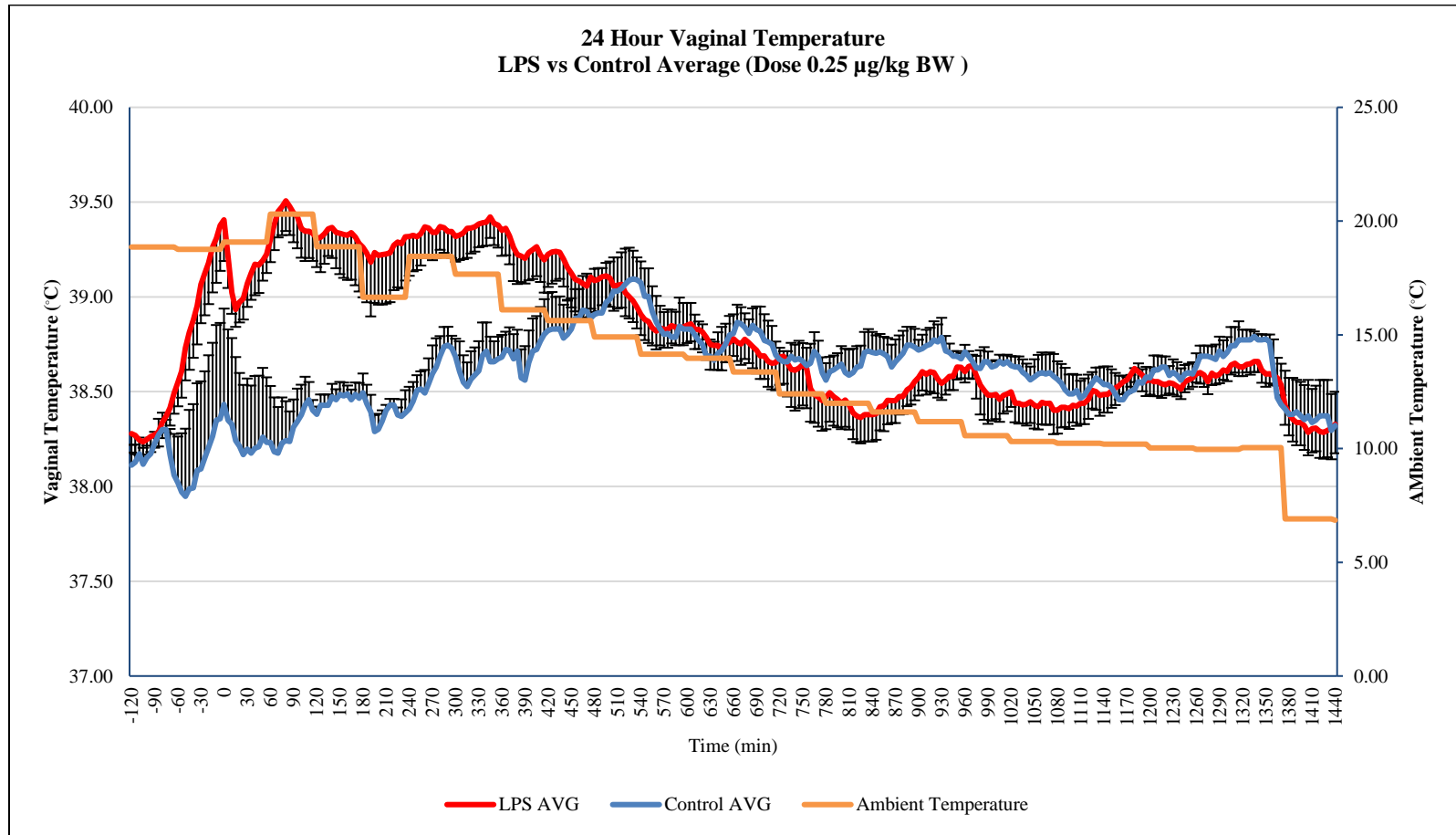
Figure 4. Average vaginal temperature for LPS and control groups spanning 24 hr with average hourly ambient temperature for d0.
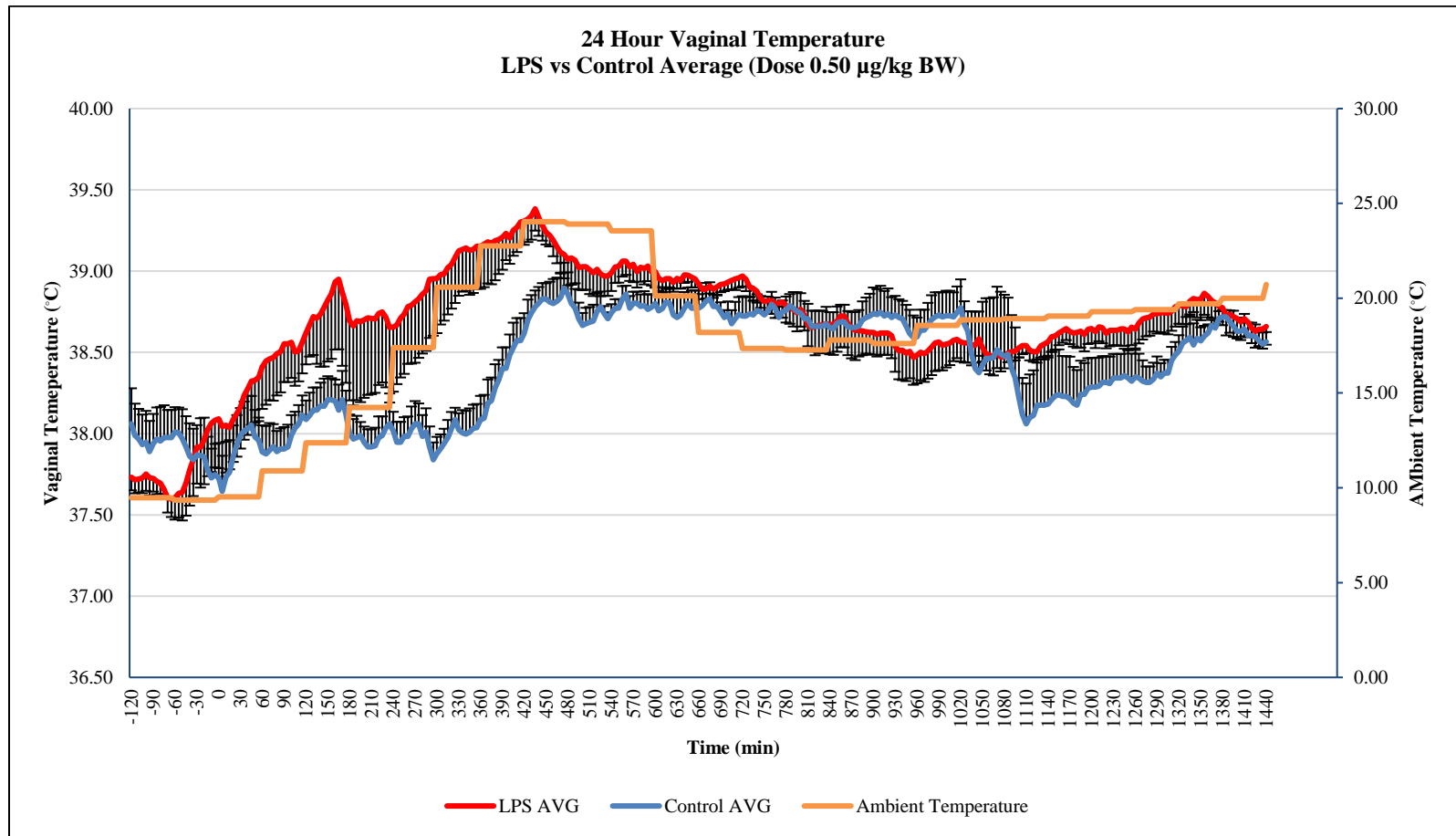
Figure 5. Average vaginal temperature for LPS and control groups spanning 24 hr with average hourly ambient temperature for d7.
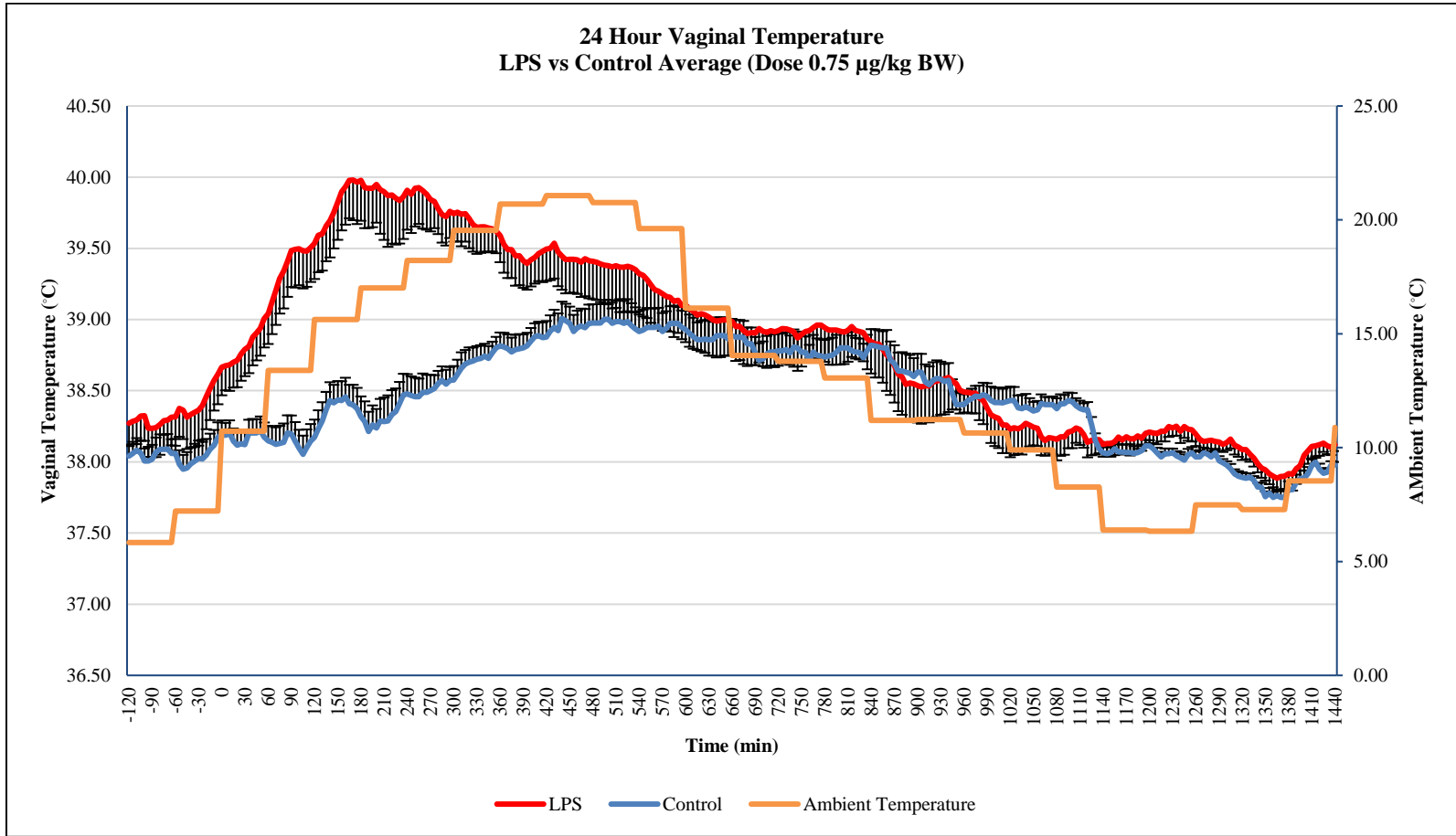
Figure 6. Average vaginal temperature for LPS and control groups spanning 24 hr with average hourly ambient temperature for d14.

Table 2. Pearson correlation for investigated fixed effects and treatment day.

Treatment Day

Covariate	Correlation Coefficient	Prob > |r|	Number of Observations
Neutrophil Count	0.13479	0.383	44
Lymphocyte Count	0.00361	0.9815	44
N:L Ratio	0.14141	0.3599	44
Monocyte Count	-0.12278	0.4328	43
Total WBC	0.04021	0.7955	44

Eleven heifers were included in T/S ratio analysis. One heifer was excluded due to injury during data collection. Two samples were excluded due to failure to meet C_q replicate criteria. No differences were seen in T/S ratio by treatment (Fig. 4) (P = 0.35), day (P = 0.73), or the interaction between treatment and day (Fig. 4 and 5) (P = 0.94). A trend was observed for heifer T/S ratio by birthdate (Fig. 6) (P = 0.07) where older heifers tended to have approximately 36.81 ± 17.66 more telomere sequences. No differences in BW (P = 0.30) and BCS (P = 0.32) score were detected by T/S ratio. No relationship was detected for T/S ratio or treatment day with any other investigated fixed effects of these heifers (Tables 1 and 2).
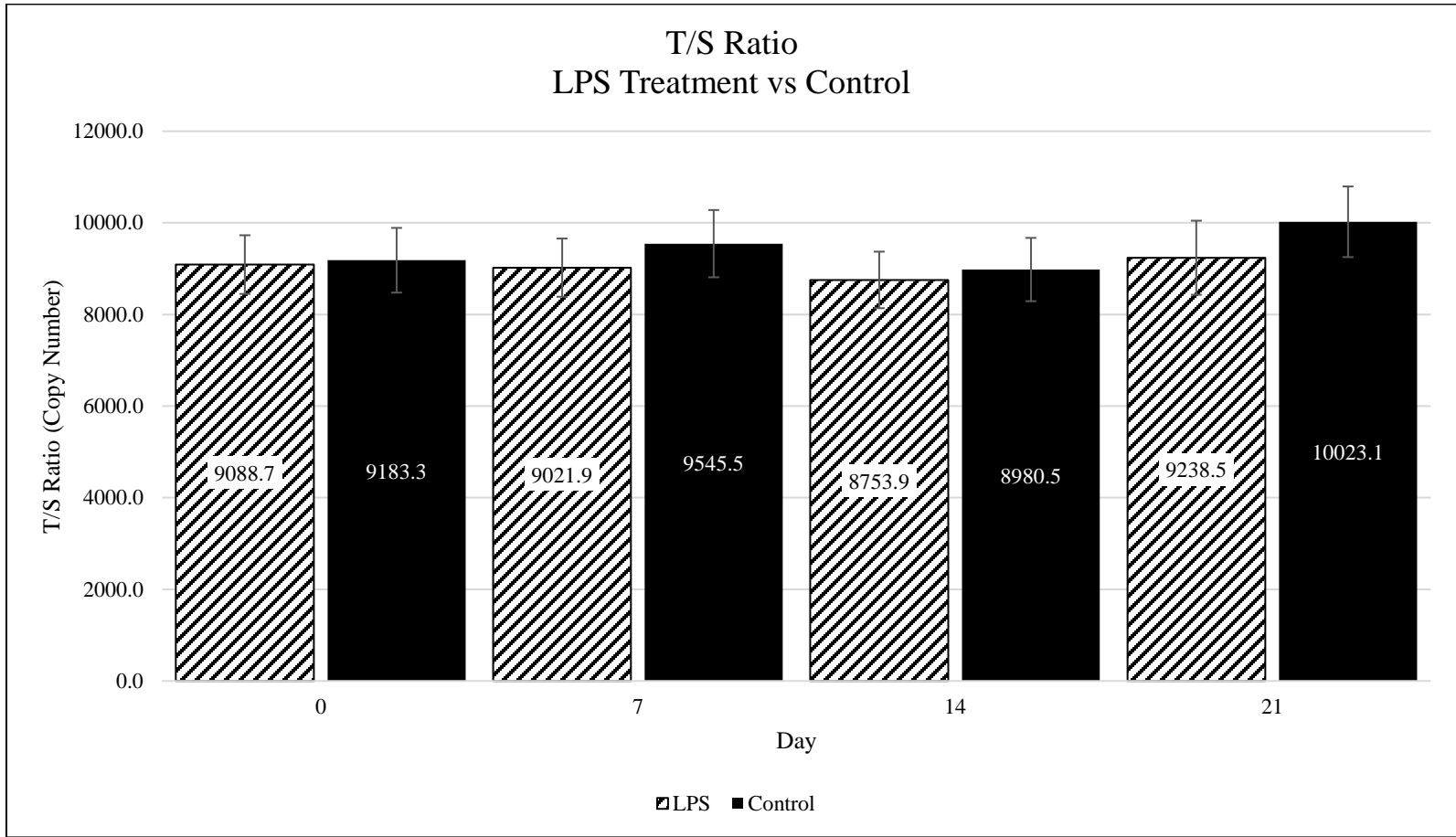
Figure 7. Average T/S ratio for LPS and Control groups by sample day.
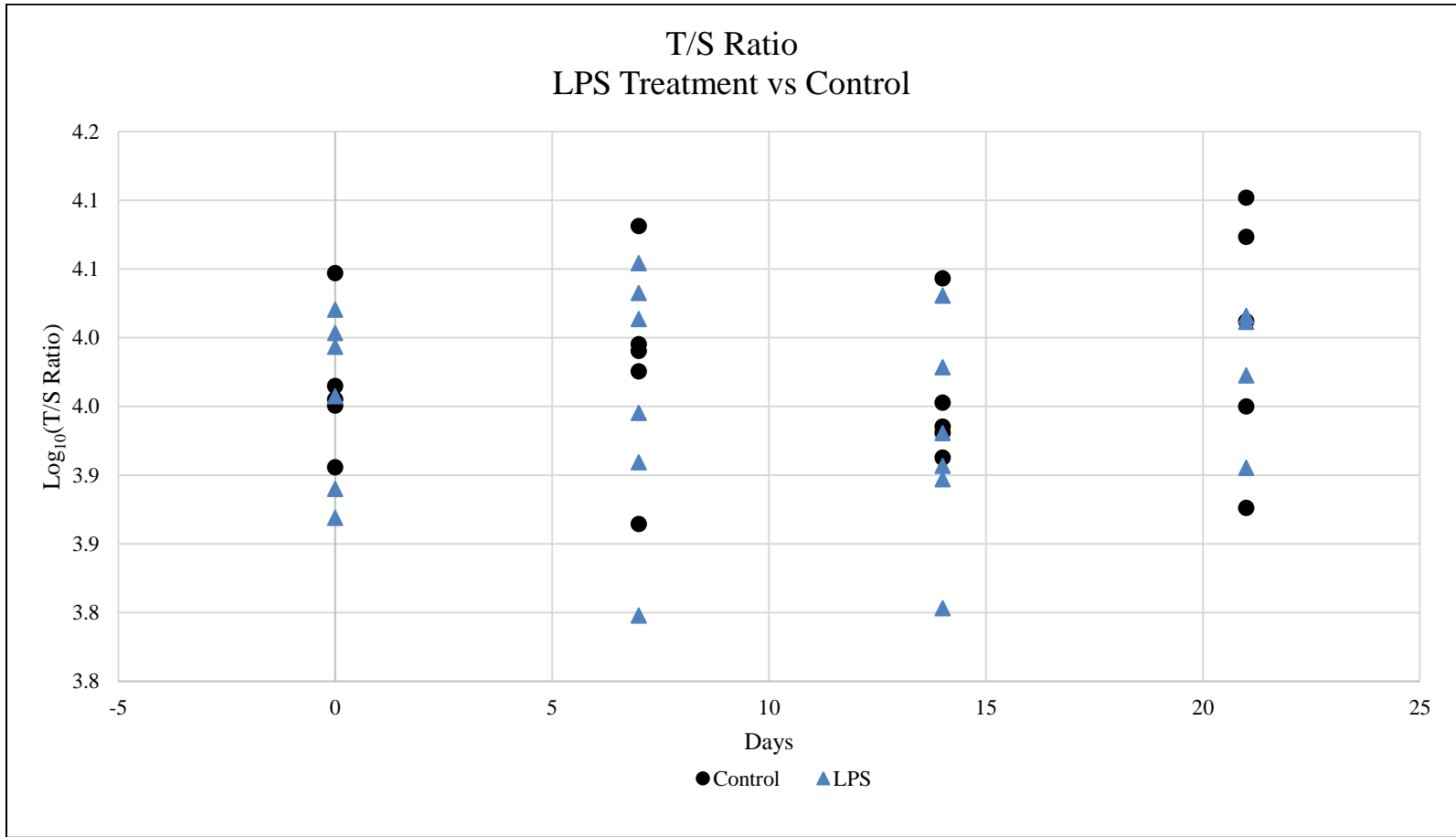
Figure 8. Graphical representation of the log₁₀ transformed distribution of individuals by treatment and day.

Table 3. Pearson correlations of investigated fixed effects and T/S ratio.

T/S Ratio			
Covariate	Correlation Coefficient	Prob > |r|	Number of Observations
d0 Temperament Score	0.08032	0.6131	42
Neutrophil Count	-0.01708	0.9145	42
Lymphocyte Count	0.20159	0.2005	42
N:L Ratio	-0.0437	0.7835	42
Monocyte Count	-0.00033	0.9984	41
Total WBC	0.03387	0.8314	42
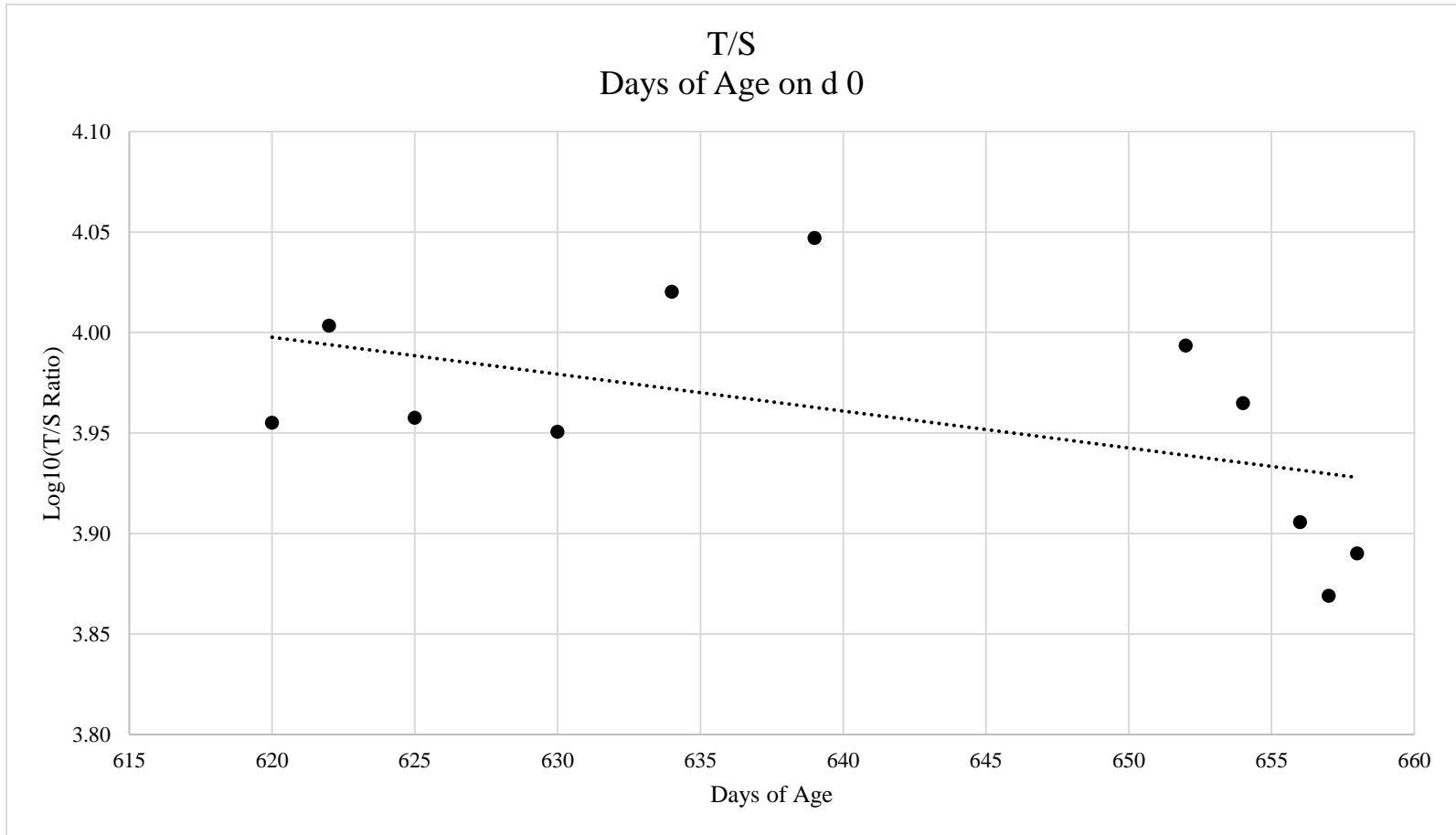
Figure 9. Individual T/S ratio of all heifers before onset of treatments (d0).

Discussion

Telomeres are highly influenced by chronic inflammation and have been found to shorten in the presence of chronic inflammatory disease. The lack of a relationship between treatments and T/S ratio was unexpected but upon further investigation into LPS responses in cattle, it is explicable. It is well established that susceptibility to LPS administration shows significantly interindividual variation for all clinical, hematological, and blood biochemical responses in intravenously injected cattle (Jacobsen et al., 2005). However, in this experiment LPS was injected subcutaneously to elicit a low-level inflammatory event. The expected increased proliferation of WBC following LPS administration may not have occurred in low LPS response individuals. This was supported by the finding of Jacobsen et al. (2005) that more than half of the statistical variation in WBC counts could be attributed to the individual. However, it is impossible to know the dynamics of WBC proliferation in this experiment due to blood sampling occurring 7 days post LPS administration, which missed the innate immune response window. Vaginal temperature did show dramatic differences in LPS response and heifers could be graphically grouped into low (maximum temperature remains $\leq$ 39.5$°$C, Fig. 10), medium (maximum temperature is $> 39.5°$C but $< 40°$C, Fig. 11), and high responders (maximum temperature is $\geq 40°$C, Fig. 12). For the purpose of this experiment, hyperthermia or "fever" was considered $\geq 39.5°$C and normal body temperature around 38.6$°$C. These visual inter-individual differences in response tended to stay constant with increasing doses with the exception of one high responding heifer not responding to the 0.50 µg/kg BW dose and one low responding heifer responding

46

dramatically to the 0.75 µg/kg BW dose. This class switching was observed in Jacobsen et al., (2005) and that not all responses were equally dose responsive. This may be attributed to individual changes in numerous inflammatory mediators that cause the clinical, hematological and blood biochemical changes in response to LPS (Eades, 1993; Plaza et al., 1997). Pyrogenic variability may also be sourced to genetic differences in LPS receptors found on macrophages (Michaels and Banks, 1988; Semrad and Dubielzig, 1993). The presence of low responding heifers may have decreased the power within the experiment, masking any significant changes in TL by treatment. T/S ratio was not analyzed by response group due to small sample size within response groups. It is possible that telomerase activity mitigated any depletion in TL, but the role of telomerase was beyond the scope of this study.
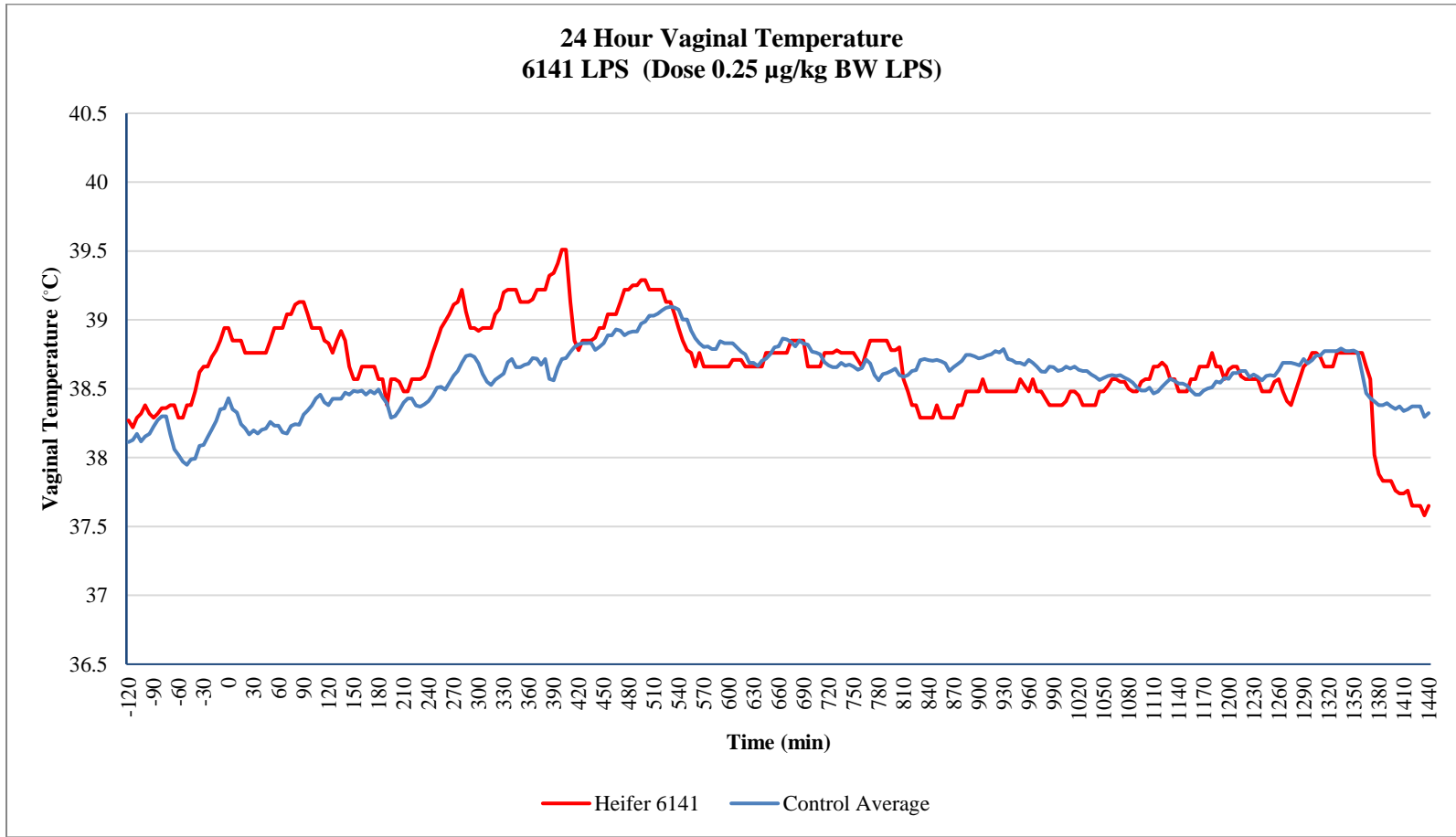
Figure 10. An example of a low LPS responding individual. The maximum temperature did not exceed 39.5°C.

24 Hour Vaginal Temperature
6160 LPS (Dose 0.25 µg/kg BW LPS)

Legend: Heifer 6160 — Control Average
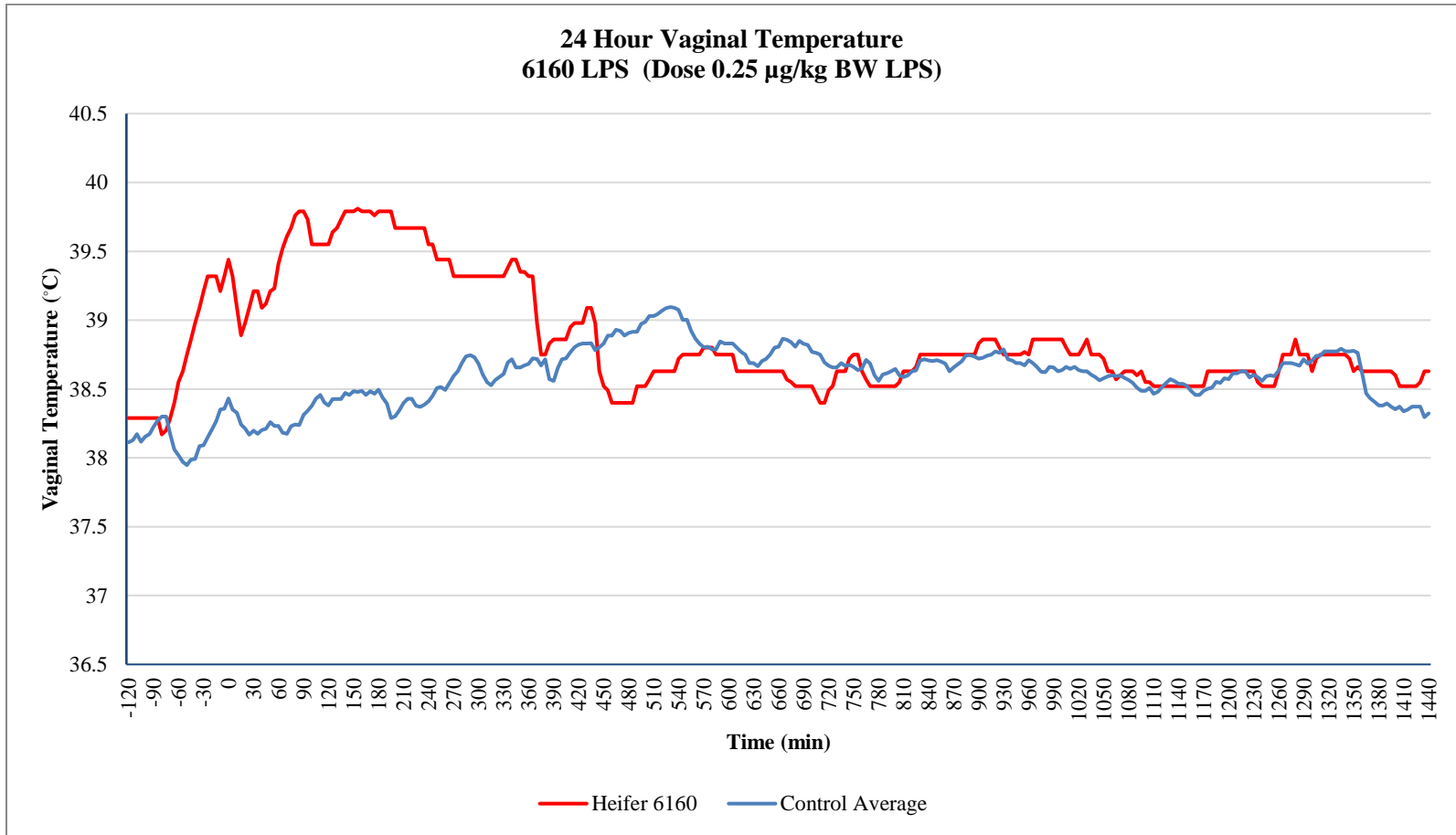
Figure 11. An example of a medium LPS response heifer. The maximum temperature exceeds 39.5°C but remains below 40°C.

24 Hour Vaginal Temperature
6195 LPS (Dose 0.25 µg/kg BW LPS)

Legend: heifer 6195 — Control
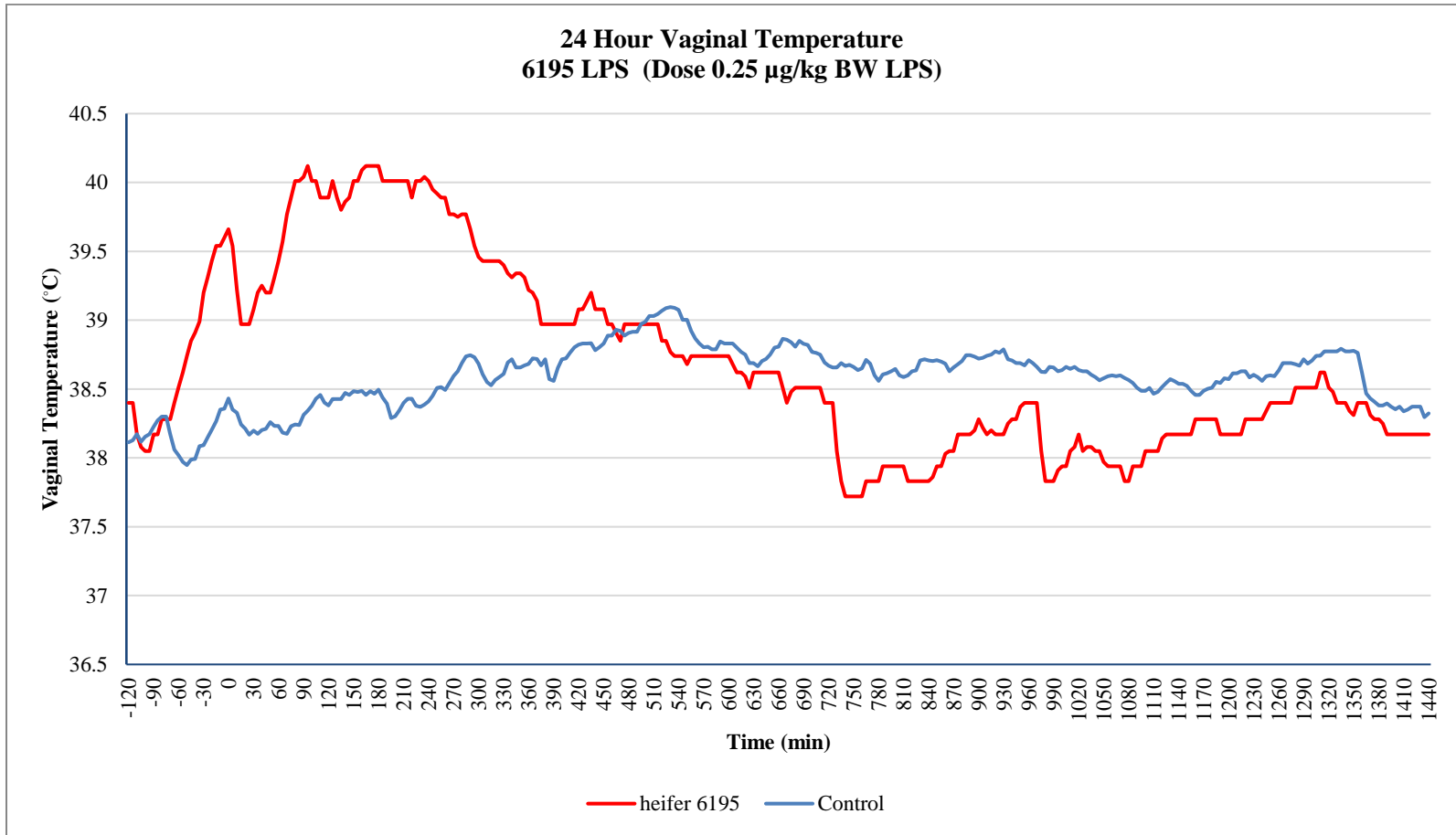
Figure 12. An example of a high LPS response heifer. The maximum temperature exceeds 40°C.

Telomere shortening has also been related to aging and lifespan. The trend in d 0 TL was expected. Multiple studies utilizing cattle and Soay sheep report high individual variability in telomere length (Seeker et al., 2018a, 2018b; Fairlie et al., 2016; Brown et al., 2012; Laubenthal et al., 2016). Statistically, it appears that older heifers have a tendency to have shorter telomeres, however, the difference between the oldest and youngest heifer was approximately 38 days. There was little evidence the in literature to support changes in TL over that short of a period that can be attributed to age in cattle over the age of one month (Seeker et al., 2018a; Fairlie et al., 2016). It is well established that vertebrate TL shortens most rapidly shortly after birth followed by a steady decline through adulthood (Anglier et al., 2017; Seeker et al., 2018a; Fairlie et al., 2016). However, it is more likely that those differences can be attributed to inter-individual differences in TL at birth. Alternately, all previous bovine research has been conducted in dairy breeds of cattle that are known to be earlier maturing. The telomere dynamics in later maturing Brahman cattle may be different. In this study the average age of these heifers was 640 ± 4.56 days of age and were less mature than their dairy counterparts at the same chronological ages. It is possible that the window of rapid telomere attrition may be shifted to an older age to accommodate the continual growth and development at older ages. However, this is not likely at 640 days of age as Seeker et al., (2018a) found the rapid attrition to be within 1 month of birth in Holstein-Friesian dairy cattle and Fairlie et al. (2016) found rapid attrition to be within 4 months of birth in Soay sheep.

Summary

Subcutaneously injected LPS between the doses of 0.25 µg/kg BW and 0.75 µg/kg BW produced a febrile response in Brahman heifers but did not seem to commence a large enough immune response to impact TL in peripheral leukocytes. Brahman heifers seemed to have varying degrees of sensitivity to LPS and consistently react to increasing doses. However, the redistribution of immune cells was not known during the window of innate immune response to further quantify differences in LPS sensitivity. Increased days of age seemed to show a tendency to decrease TL; however, TL is individually diverse based on heritability and the summation of life experiences and may be more related to the latter. LPS administration shows promising potential in studying the effects of acute illness on cattle TL with modifications to dose level and/or route of injection to mount a greater immune response

CHAPTER IV

COMPARISON OF TELOMERE LENGTH IN AGE-MATCHED PRIMIPAROUS

AND MULTIPAROUS BRAHMAN COWS

Introduction

Telomeres are the short G-rich sequences oriented toward the termini of linear

chromosomes responsible for protecting genetic integrity (Teasley and Stewart, 2016).

Telomere sequences (5' – TTAGGG – 3') are supported by a complex of Sheltrin

proteins and maintained by the reverse transcriptase telomerase (de Lang, 2005; Diede

and Gottschling, 1999). Shortening of telomeres has been observed with cellular aging

due to the "end replication problem" after every replication cycle *in-vitro* (Morrison et

al., 1996). However, this is variable by cell type as well as biotic and abiotic factors

influencing replication and behavior of telomerase (Epel et al., 2004). In most cases,

telomere shortening is the hallmark of cellular aging which has led to the idea that TL

may be associated with organismal aging and lifespan (Aviv and Shay, 2018).

Shortening of average leukocyte TL measured in mammalian and non-

mammalian vertebrates seems to be related to lower survival rate and potentially is a link

to longevity (Seeker et al., 2018b; Angelier et al., 2017). This association with longevity

has led to the study of cumulative stress on TL as a biomarker of health and animal

welfare in the livestock industry (Bateson, 2015). Productive longevity or "functional

longevity" (as defined by Seeker et al., 2018b), has remained an elusive trait to quantify

in the dairy cattle industry. Heritability of functional longevity was estimated to be low,

approximately 0.01 - 0.06, until a longitudinal study of 308 Holstein-Friesian dairy cows found heritability estimates of relative leukocyte telomere length (RLTL) to be 0.38 ± 0.03 and 0.32 ± 0.08 among cow and calf data sets (Pritchard et al., 2013; Seeker et al., 2018b). Within this study, it was concluded that relative leukocyte telomere length (RLTL) measured between one and five years of age was positively correlated with productive lifespan (p < 0.05) (Seeker et al., 2018b). This had previously been suggested in a study that found dairy cattle with short telomeres were more likely to be culled within one year of measurement for poor health or fertility (Brown et al., 2012). This is very strong evidence that leukocyte TL is linked to survivability in dairy cattle and associated with "functional longevity" as well as a potential selection criterion for breeding programs.

However, there is no literature investigating this association in alternate cattle breeds with varying differences in production and longevity. A biomarker for productive longevity in commercial cow-calf operations remains elusive; however, telomere dynamics may be different than what is found in commercial dairy operations. It is common practice in the modern dairy industry for the calf to be removed shortly after birth to allow for maximum milk yield from the cow. This eliminates the very well-established stress of caregiving that is known to result in shortened telomeres in humans, avian species, and mice (Epel et al., 2004, 2010; Sudyka et al., 2014; Reichert et al., 2014; Kotrschal et al., 2007) Further investigation is needed to elucidate the impact of parity and successfully raising a calf on bovine telomere dynamics.

Materials and Methods

Animal procedures for this project were approved by the Texas A&M AgriLife Research Agricultural Animal Care and Use Committee (2017-035A). Animal procedures were carried out at the Texas A&M AgriLife Research and Extension Center located in Overton, TX. Nineteen primiparous and multiparous 4-yr-old Brahman cows were utilized for this project.

Experimental Design

Brahman (Bos indicus) multiparous cows (n = 11) and primiparous cows (n = 8) that had been determined to be pregnant and of 4 years of age were kept on pastures consisting of Coastal bermudagrass (*Cynodon dactylon*) overseeded with Maton rye (*Secale cereal* L.) and Nelson ryegrass (*Lolium multiflorum* Lam.) from March 1st to May 26th of 2018. All multiparous cows were undergoing the second gestation of their lifetime while primiparous cows were undergoing their first gestation. In addition to pasture and free choice Coastal bermudagrass hay, cows and heifers were fed approximately 3.6 kg of a 3:1 corn:corn gluten grain mix per head per day prior to calving. Cows were bled by jugular venipuncture, weighed, and BCS recorded d – 28 prior to calving and d + 7 and d + 29 post calving respectively. Evaluation of BCS (1 – 9 scale) was done by a trained professional with 40 years of experience.

Table 4. Cow BW (kg) and BCS for parity and sample day (MEAN ± SE).

		Sample Day		
	Parity	d - 28	d + 7	d + 28
BW (kg)	Parity 1	499 ± 15	507 ± 17	513 ± 9
	Parity 2	526 ± 14	521 ± 15	547 ± 10
BCS	Parity 1	7.00 ± 0.17	6.75 ± 0.21	6.63 ± 0.16
	Parity 2	6.9 ± 0.22	6.73 ± 0.22	6.68 ± 0.23

At the time of parturition, cows and heifers were monitored for duration of labor, calving ease, and retained placenta. Cows were monitored at the onset of observable signs of calving such as relaxation of the pelvic ligaments, swollen vulva, and fluid discharge. However, timing of duration of labor began at signs of hard labor such as the cow laying down with observable contractions to expulsion of the calf. Calf birth weight and gender were recorded within 24 hours of parturition.

White Blood Cell Isolation

Based on individual calving dates, cows and heifers were blood sampled by the jugular vein (20 ml total per animal) 28 d prior to calving as well as on d 7 and 28 after calving utilizing sterile 10 ml EDTA vacutainer tubes. Approximately 20 µl of whole blood was utilized to make blood slides for CBC analysis. Whole blood was centrifuged at 2,675 RCF for 30 minutes at 5°C. After centrifuging, the WBC layer located between the RBC and serum interface, was aspirated with a pipette and transferred into a 1.5 ml storage tube. The WBC were then cleaned of any remaining RBC and serum utilizing a

RBC lysis buffer containing EDTA and water. The WBC were mixed with the RBC lysis buffer for 5 minutes and microfuged for 5 minutes at 5,000 RCF. The remaining RBC, serum, and RBC lysis buffer was aspirated from the top of the WBC pellet. The WBC pellet was then stored at -80°C.

DNA Extraction

Extraction of DNA was performed utilizing spin column GeneJET Genomic DNA Purification Kits (Thermo Scientific; Waltham, MA, USA). However, prior to extraction, frozen WBC were crushed with liquid nitrogen utilizing a mortar and pestle and weighed to approximately 20 mg to accommodate the purification kits. Remaining crushed WBC were returned to -80°C storage. Quality control of extracted DNA was conducted at the Texas A&M University Institute for Genome Sciences and Society Genomics Core lab and criteria were as follows: yield > 20 ng/ul, integrity score < 3, 260/280 > 1.7 and 260/230 > 1.8 (as outlined in Seeker et al., 2016). The DNA that passed quality control was aliquoted and diluted in low-binding tubes to 10 ng/µl, utilizing nuclease free water and stored at -80°C.

Quantitative PCR

Quantity of telomere sequences was determined utilizing real-time quantitative PCR methods utilizing the ratio of telomere to B2G genes. Custom primers for amplifying bovine telomere sequences (telg = 5' - aca cta agg ttt ggg ttt ggg ttt ggg ttt ggg tta gtgt - 3', and telc = 5' - tgt tag gta tcc cta tcc cta tcc cta tcc cta tcc cta aca - 3') and the B2G gene (β-1 = 5' - cgg cgg cgg gcg gcg cgg gct ggg cgg gaa ggc cca tgg caa gaa gg - 3', and β-2 = 5' - gcc ggc ccg ccg cgc ccg tcc cgc cgc tca ctc agc gca gca aagg -

3') (defined by Brown et al., 2012 and Cawthon et al., 2009) were designed and

purchased from Integrative DNA Technologies (IDT; Coralville, IA, USA). The bovine

beta-2-globulin gene was selected as a relatively constant housekeeping gene that

operates as a control gene for determining relative qT (Brown et al., 2012). The master

mix for the telomere and B2G reaction contained 10 µl 2X PowerUp SYBR Green

Master Mix (Applied Biosystems; Foster City, CA, USA), 1 µl of bovine telomere

primers (forward and reverse, each to 500 nmol final concentration), and 0.6 µl of B2G

primers (forward and reverse, each to 300 nmol final concentration), and 4.8 µl nuclease

free water. Master mix was loaded prior to 20 ng/µl of DNA. Standard double-stranded

DNA oligonucleotides (oligo) of bovine telomere (sense 5' - tta ggg tta ggg tta ggg tta

ggg tta ggg tta ggg tta ggg tta ggg tta ggg tta ggg tta ggg tta ggg tta ggg tta ggg - 3';

antisense 5' - ccc taa ccc taa ccc taa ccc taa ccc taa ccc taa ccc taa ccc taa ccc taa ccc taa

ccc taa ccc taa ccc taa - 3') and B2G (sense 5'- ggt gaa ggc cca tgg caa gaa ggt gct aga

ttc ctt tag taa tgg cat gaa gca tct cga tga cct caa ggg cac ctt tgc gct gag tga gct g - 3';

antisense 5'- cag ctc act cag cgc agc aaa ggt gcc ctt gag gtc atc gag atg ctt cat gcc att act

aaa gga atc tag cac ctt ctt gcc atg ggc ctt cac c - 3') were designed and purchased from

IDT. Six-step, 1:10 serial dilutions of both bovine telomere standard (10^{12}-10^7) and

bovine B2G (10^9-10^4) to create standard curves were generated immediately prior to

loading. Samples were randomly allocated on a 384-well qPCR plate and amplified in

triplicate, 20 µl reactions. A negative control containing water in place of DNA was

included to monitor for contamination of master mix. A real-time system thermocyler

(BioRad; Hercules, CA, USA) was programed with multiplex settings defined by Brown

58

et al., 2012. Cycle threshold (C_q) value triplicates were checked for consistency. Triplicates were included if the cycle difference was less than 0.25 cycles. Single replicates with differences larger than 0.25 cycles were removed if an obvious outlier could be identified, and the remaining wells were included. Samples with remaining wells with differences larger than 0.25 cycles were excluded from analysis. Starting quantities were averaged for samples with adequate C_q replicate differences. Copy number of telomere sequence (5' – TTAGGG - 3') and B2G gene is represented by SQ and a ratio of telomere SQ and B2G SQ was utilized to determine T/S ratio.

Statistical Analyses

Statistical analyses were conducted using mixed linear models with the MIXED procedure of SAS 9.4 (SAS Inst., Inc., Cary, NC). All T/S ratio measurements were $\log_{10}$ transformed in an attempt to normalize their distribution. Parity (first or second) was the fixed effect of interest. Other investigated fixed effects included calf birth weight, duration of labor, and calving ease and their interactions as well as BW and BCS. Random effects included sire and day of record as a repeated measurement on heifers. Pearson correlation coefficients were calculated using CORR procedures of SAS and tested for T/S ratio with these measured traits of these cows: neutrophil and lymphocyte counts and their ratio, monocyte counts, and total white blood cell counts.

Results

Nineteen cows were included in the analysis. Two calves died within 28 days post calving. Blood samples collected after the death of the calves were excluded from analysis. Two samples were excluded from analysis that did not meet PCR C_q replicate

criteria. Significant differences were seen between parity 1 (123196.9 ± 4426) and parity 2 (113972.0 ± 3973) (P = 0.02) with Parity 2 cows having approximately 9224.5 less copies of telomere sequences (Fig. 13). No differences were seen in day (P = 0.23) and the interaction of parity and day (P = 0.76) (Fig. 14 and 15). A trend was observed between duration of labor (Tlabor) and T/S ratio (P = 0.06). No differences were detected for BW and BCS for sample day (P = 0.18; P = 0.30) or T/S ratio (P = 0.57; P = 0.38). Pearson correlations show a positive correlation (coefficient 0.46) between duration of labor and T/S ratio (prob > |r| 0.0008). However, no differences in T/S ratio were observed for calving ease (P = 0.19) or calf birth weight (P = 0.79). Pearson correlations did not indicate differences in other investigated fixed effects relating to T/S ratio or sampling day (Tables 3 and 4).
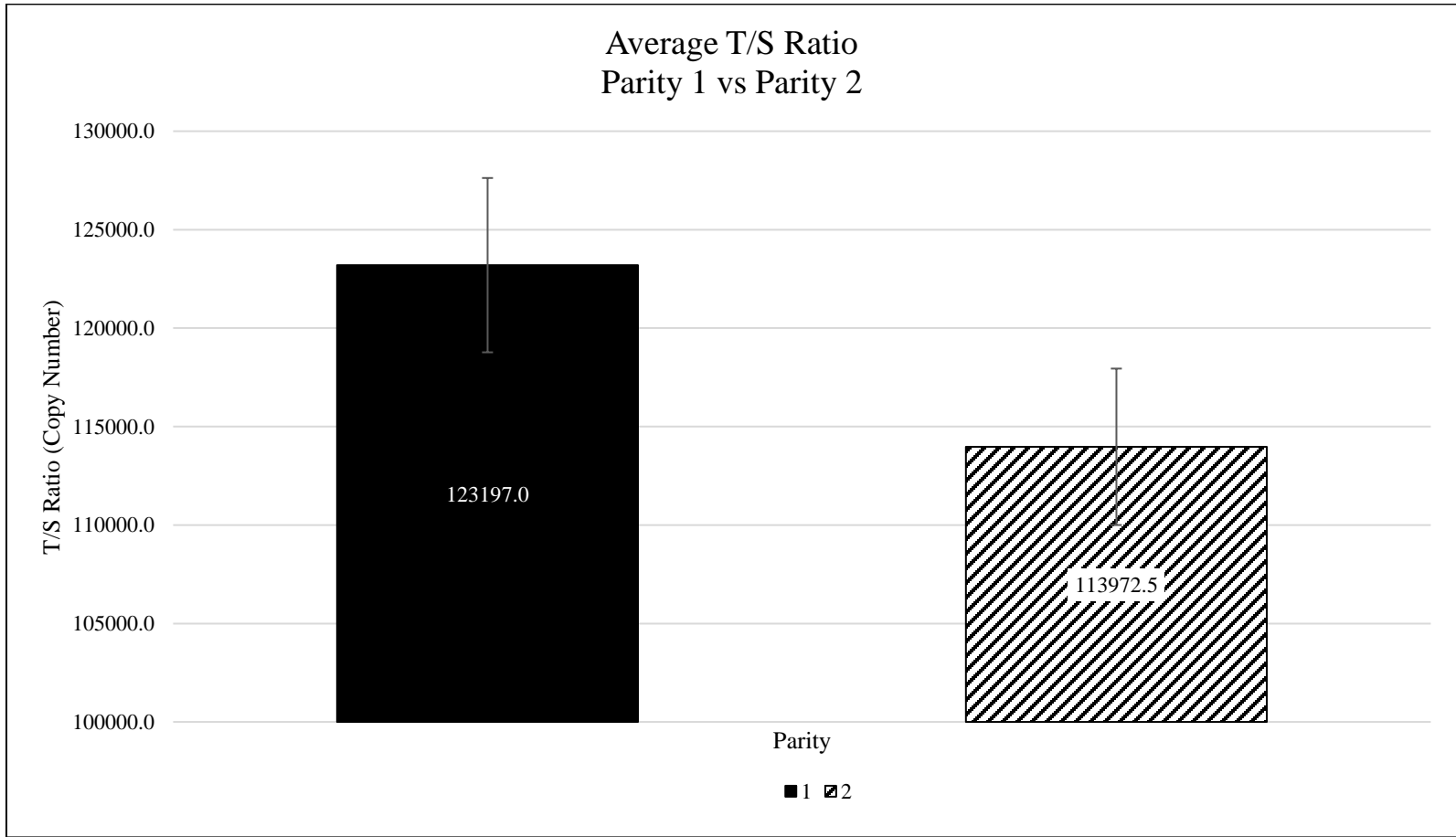
Figure 13. Graphical representation of average T/S ratio between parity 1 and parity 2.
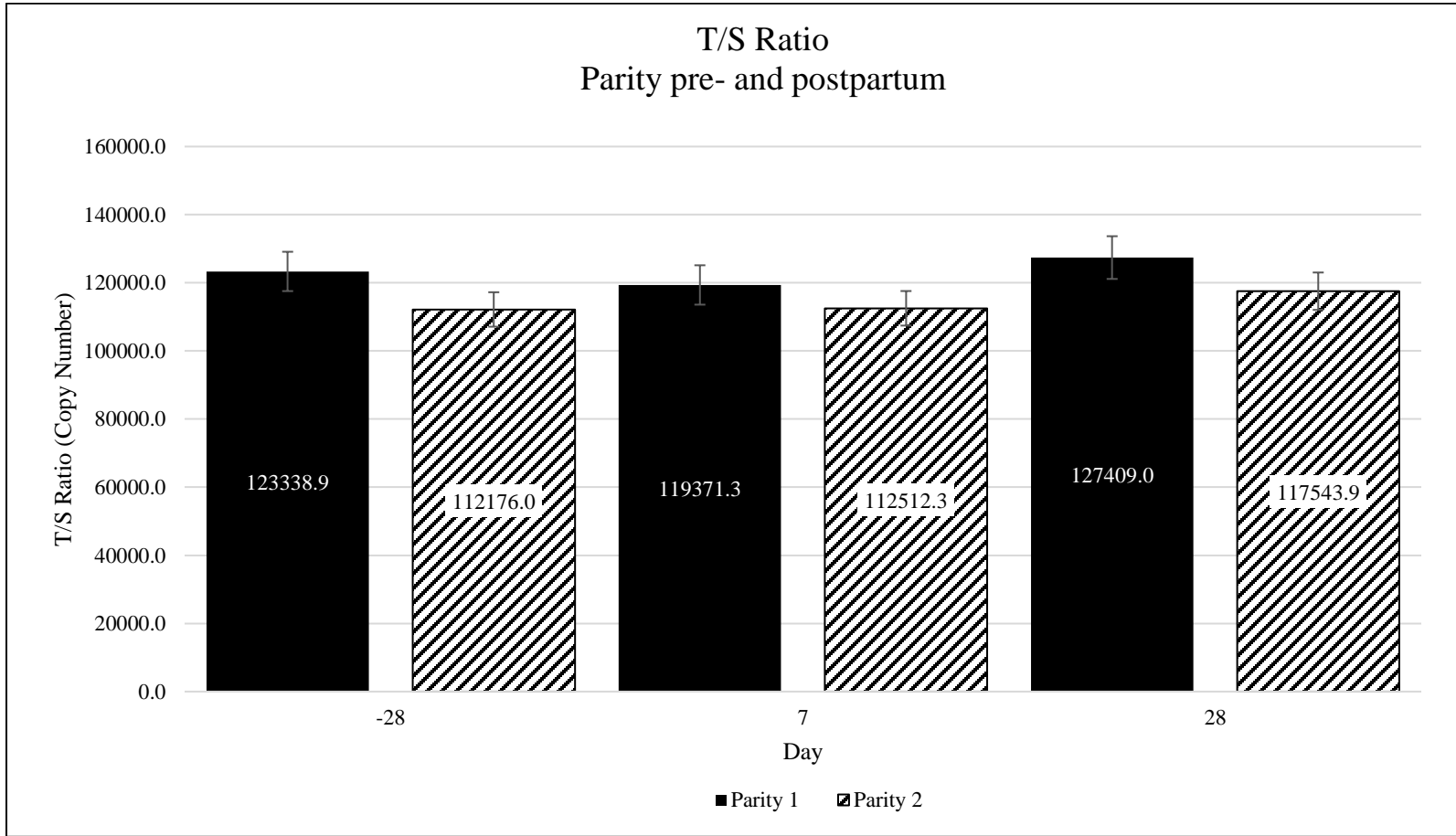
Figure 14. Graphical representation of T/S ratio for parity 1 and parity 2 pre-calving and post-calving.
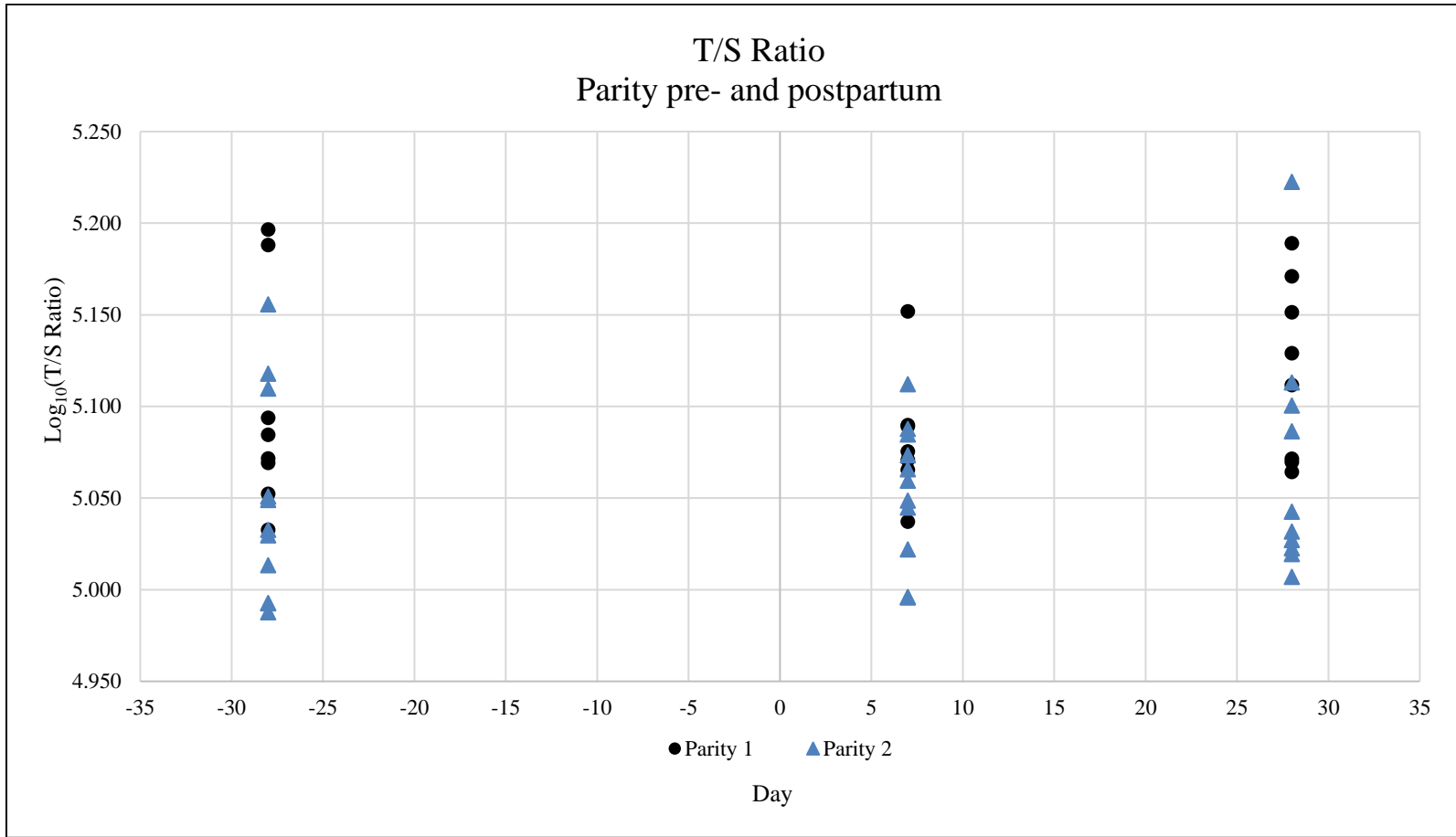
Figure 15. Graphical representation of the log$_{10}$ transformed distribution of individuals by parity and day.

Table 5. Pearson correlations of investigated fixed effects and T/S ratio.

T/S Ratio

Covariate	Correlation Coefficient	Prob > |r|	Number of Observations
Total WBC	0.10885	0.4379	53
Neutrophil Count	0.02256	0.8726	53
Lymphocyte Count	0.13568	0.3327	53
N:L Ratio	-0.18446	0.1861	53
Monocyte Count	0.10125	0.4707	53

Table 6. Pearson correlations of investigated fixed effects and sample day.

Sample Day

Covariate	Correlation Coefficient	Prob > |r|	Number of Observations
Total WBC	-0.00544	0.968	57
Neutrophil Count	0.06582	0.6267	57
Lymphocyte Count	-0.0434	0.7485	57
N:L Ratio	0.11864	0.3794	57
Monocyte Count	0.16639	0.2161	57

Discussion

Multiparous and primiparous cows showed differences in TL overall despite the interaction of parity and day not being significant. It was not surprising that parity had a significant effect on telomere length. Literature in humans, avian species, and cattle suggest that parity, lactation, and parental effort have a negative effect on TL (Seeker et al., 2018a, Brown et al., 2012; Laubenthal et al., 2016; Pollack et al, 2018; Kotrschal et al., 2007; Plot et al., 2012, Bauch et al., 2013; Reichert et al., 2014; Sudyka et al., 2014; Epel et al., 2004, 2010). The difference between nulliparous and multiparous TL was observed in women in that multiparous women had 4.2% less telomeric sequences than nulliparous women (Pollack et al., 2018). In the present study, multiparous cows had approximately a 7.5% reduction in T/S ratio compared to primiparous heifers. The lack of differences in the interaction of day and parity was unexpected. Parturition is a complex cascade of glucocorticoid and signaling and changes in hormone profiles that are suggested in the literature to impact the activity of telomerase and direct damage to telomeres (Effros et al., 2005; Kyo et al., 1999; Choi et al., 2008; Barnes et al., 2018). However, the lack of differences in TL prepartum and postpartum may be attributed to the protective effects of estrogen. The postpartum resumption of ovarian function and return to estrus means that estrogen concentrations will return normal estrous cyclic levels (Murphy et al., 1990). Estrogen is known to bind to the hTERT promoter region of telomerase, upregulating activity (Kyo et al., 1999). It is possible that this is a

mechanism in place to protect telomeres after the physiological stress of gestation and parturition.

The overall difference in TL between parity 1 and parity 2 may be related to lactation and the psychological stress of caring for a calf. Laubenthal et al., (2016) found a strong positive correlation between initial leukocyte qT in early lactation and the extent of qT attrition in late lactation. However, this was limited to cows with initially high qT values than those with low qT values (Laubenthal et al., 2016). This indicates that the protective mechanisms of telomeres act preferentially on lower qT values or "shorter" telomeres in dairy cattle. However, Seeker et al., (2018a) did not detect a difference in RLTL between two genetic lines for high milk yield and control cows but did find that milk yield significantly affected lifespan ($P > 0.001$). While Brown et al., (2012) did not evaluate milk yield, they did report a linear relationship between age and qT as well as a relationship between qT and culling status in cows. This conflicts with Seeker et al., (2018a) that did not report an age-related decline to 6 years of age in cows but did acknowledge that geriatric (13 - 14 years of age) cows were included in the Brown et al., (2012) study. These conflicting studies suggest that parity and lactation in dairy cattle may or may not be influencing telomere dynamics. It is important to note that all previous research conducted in cattle has been with dairy cattle, and in the dairy industry cows are required to produce a calf and off-set their maintenance costs with milk yield. In all of the previously mentioned studies, cows were not responsible for raising their calf. However, in the beef cow-calf industry cows are responsible for raising their calves to weaning. This suggests that the reduction in T/S ratio in this study was most likely

67

driven by the physiological stress of successfully raising a calf. This is in line with many human studies that find a strong relationship between caregiving stress and TL (Epel et al., 2004, 2010).

The positive trend observed in duration of labor in relation to T/S ratio was unexpected. Parturition is a physiologically strenuous event and parity has been found to negatively impact TL as stated above. However, endocrine signaling related to the onset of labor may play a role in telomere dynamics. During parturition in ruminants, the onset of labor is dependent upon the activation of the fetal HPA axis (Wood, 1999). This increases the plasma concentration of cortisol to induce activity of 17-hydroxylase and 17,20 lyase in the placenta, which results in the biosynthesis of estrogen relative to progesterone (Wood, 1999). While cortisol is known to have an inhibitory effect on telomerase, it is possible that the biosynthesis of estrogen during labor may be impacting telomerase activity. Differences in change of ($\log_{10}$(T/S ratio)) show that while there were minor differences in duration of labor between d7 and d-28 (Figure 16), there are two individuals that had telomere lengthening, in relation to longer duration of labor between d28 and d-28 (Figure 17). Those individuals may be driving the trend. It is also observed that parity 2 cows had a shorter duration of labor and less variability in change of ($\log_{10}$(T/S ratio)). However, it is important to note that duration of labor was measured by observation and signs of labor are subjective. The true time cows' spent in labor may be variable from what was recorded which may confound this analysis.
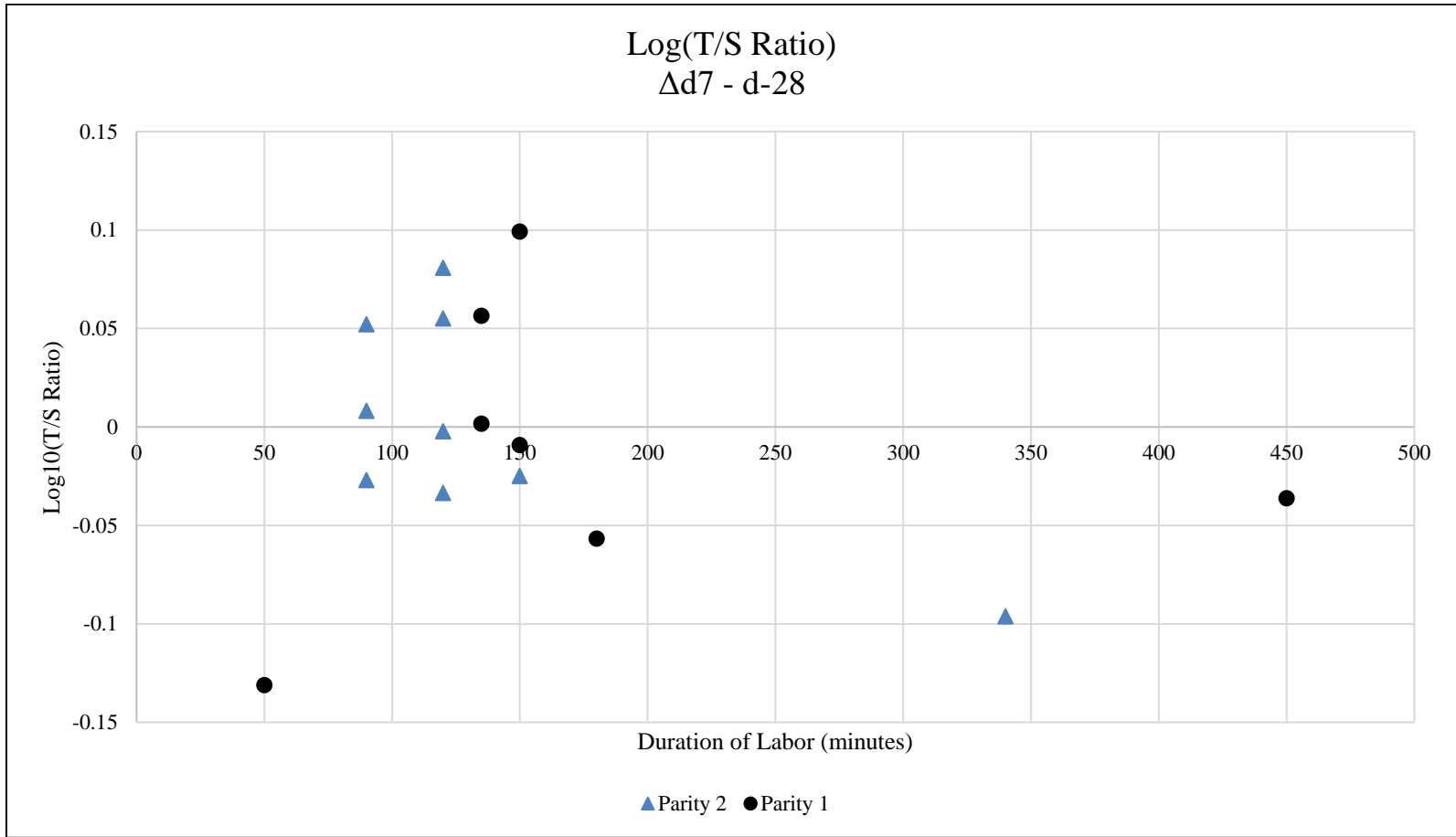
Figure 16. Change in (log10(T/S ratio)) between d7 to d-28 by duration of labor and parity.
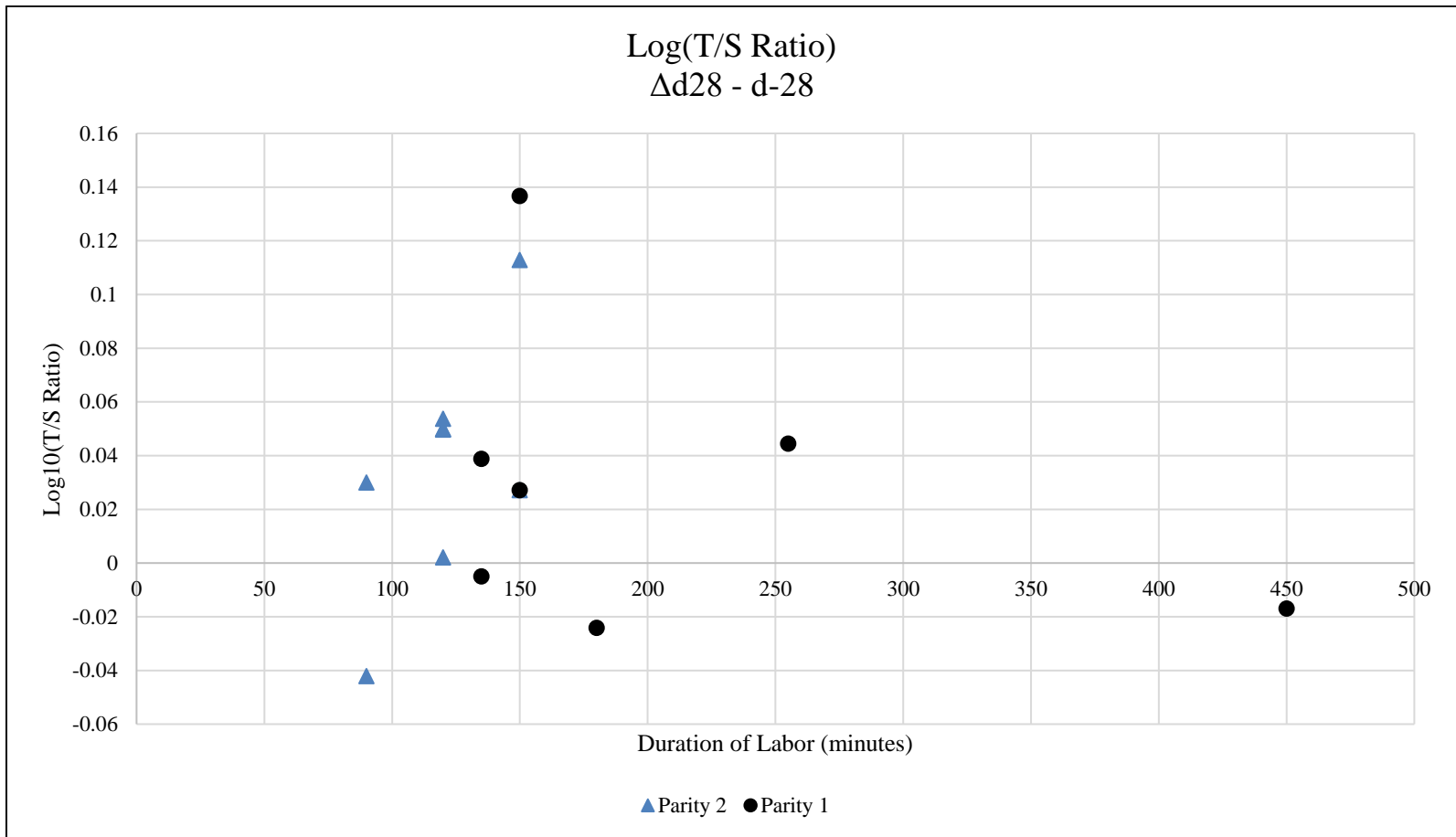
Figure 17. Change in (log10(T/S Ratio)) between d28 to d-28 by duration of labor and parity.

Summary

Parity was a significant predictor of telomere attrition when Brahman cows are controlled for age. While no changes in TL were detected between d -28 and d 28 for parity 1 and 2, the difference was most likely attributed to parental effort of caring for a calf. Telomere length shows a tendency to extend with an increase in time spent in hard labor. Changes in hormone signaling during parturition, particularly estrogen, may act as protective mechanisms in a time of increased physiological stress. However, this may be a unique artifact of studying primiparous Brahman cows that are undergoing the stress of birth and motherhood for the first time. Studying age-matched cows of varying parities beyond two calves is necessary to fully understand the relationship between parturition and telomere attenuation.

CHAPTER V

CONCLUSIONS

Chapter III Evaluation of Lipopolysaccharide Induced Acute Inflammatory Stress on Telomere Length in Brahman Heifers

Telomere dynamics are ultimately dependent on an individual's ability to maintain allostasis through glucocorticoid signaling and behavior of telomerase. Chronic inflammation has been shown in several publications to affect TL and the subsequent activation or de-activation of telomerase. However, little research exists to evaluate the effects of repeated, acute inflammatory events and none exist in cattle on telomere dynamics. In this experiment, repeated, increasing low-doses of subcutaneously injected LPS did not shorten TL or impact other physiological measures of stress such as WBC, BW, or BCS. However, vaginal temperature did confirm the presence of a sub-acute inflammation in LPS treated heifers, though no clinical signs were observed. Days of age at d0 did indicate the presence of an age effect on TL or the presence of inter-individual variation in TL prior to this experiment. As there was only a subclinical response to any dose of LPS it must be concluded that a greater concentration of endotoxin is needed to mount a greater immune response to result in shortening of telomeres in Brahman cattle. Further research regarding the effect of acute inflammation on TL in Brahman cattle is warranted.

Chapter IV Comparison of Telomere Length in Age-Matched Primiparous and Multiparous Brahman Cows

Parity and caregiving have been shown by recent research publications to be lifelong influencers of telomere dynamics in humans, mice, and avian species. During parturition, cows undergo rapid endocrine changes in response to signals generated by the fetal HPA axis. Increased concentrations of cortisol are known to inhibit telomerase resulting in telomere attrition; however, short term stimulation can increase telomerase activity and result in telomere lengthening. The resumption of ovarian function post-partum allows for steroidogenesis to continue and the production of estrogen which may also mitigate telomere attrition through telomerase. In this experiment parity was a significant indicator of telomere attrition, most likely related to the long-term stress of raising a calf to weaning. More time spent in labor showed a positive trend in telomere length suggesting that endocrine changes during parturition have possible positive impact on telomerase activity. Extrapolating TL differences in multiparous cows beyond 1 or 2 calves may give insight into productive longevity and lifespan as primiparous cows may not be representative. Blood sampling at weaning of the current calf and d -28 of calving for the following calf could be done to further understand when TL attrition is occurring. The role of telomerase is also needed to fully understand telomere dynamics during parturition and the mechanisms of lengthening or shortening of TL. Creating a large sample size, multigenerational longitudinal study of multiparous Brahman cows involving the role of telomerase is a promising next step in understanding cow longevity in a beef cow-calf system scenario.

REFERENCES

Aikata, H. H. Takaishi, Y. Kawakami, S. Takahashi, M. Kitamoto. T. Nakanishi, Y. Nakamura, F. Shimamoto, G. Kajiyama, and T. Ide. 2000. Telomere reduction in human liver tissues with age and chronic inflammation. Exp. Cell. Res. 256:578-582.

Akbar, A.N. and M. Vukmanovic-Stejic. 2007. Telomerase in T lymphocytes: us it or lose it? J. Immunol. 178:6689-6694.

Alder, J.K., J.J. Chen, L. Lancaster, S. Danoff, S.C. Su, J.D. Cogan, I. Vulto, M. Xie, X. Qi, R.M. Tuder, J.A. Phillips, P.M. Lansdorp, J.E. Loyd, and M.Y. Armanios. 2008. Short telomeres are a risk factor in idiopathic pulmonary fibrosis. Proc. Natl. Acad. Sci. USA. 105:13051-13056.

Angelier, F., D. Costantini, P. Blevin, and O. Chastel. 2017. Do glucocorticoids mediate the link between environmental conditions and telomere dynamics in wild vertebrates? A review. Gen. Comp. Endocrinol. 10:1016.

Armanios, M. and E.H. Blackburn. 2012. The telomere syndromes. Nat. Rev. Genet. 13:693-704.

Asghar, M., D. Hasselquist, B. Hasson, P. Zehtindjiev, H. Westerdahl, and S. Bensch, 2015a. Hidden costs of infection: chronic malaria accelerates telomere degradation and senescence in wild birds. Science. 347:436-438.

Asghar, M., S. Bensch, M. Tarka, B. Hansson, and D. Hasselquist. 2015b. Maternal and genetic factors determine early life telomere length. Proc. R. Soc. Edinb. Biol. Sci. 282:20142263.

Aviv, A. 2012. Genetics of leukocyte telomere length and its role in atherosclerosis. Mutat. Res. 730:68-74.

Aviv, A., and J.W. Shay. 2018. Reflection on telomere dynamics and aging-related disease in humans. Phil. Trans. R. Soc. B. 373:20160436.

Barha, C.K., C.W. Hanna, K.G. Salvante, S.L. Wilson, W.P. Robinson, R.M. Altman. 2016. Number of children and telomere length in women: A prospective, longitudinal evaluation. PLoS ONE. 11:e0146424.

Barnes, R.P., E. Fouquerel, and P.L. Opresko. 2018. The impact of oxidative DNA damage and stress on telomere homeostasis. Mech. Age. Dev. 177:37-45.

Bateson, M. 2015. Cumulative stress in research animals: Telomere attrition as a biomarker in a welfare context? Bioessays. 38:201-212.

Bauch, C., P.H. Becker, and S. Verhulst. 2013. Telomere length reflects phenotypic quality and costs of reproduction in a long-lived seabird. Proc. R. Soc. London B: Biol. Sci. 280: 20122540.

Betts, D.H. and W.A. King. 1999. Telomerase activity and telomere detection during early bovine development. Dev. Genet. 24:397-403.

Blackburn, E.H. and J.G. Gall. 1978. A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in Tetrahymena. J. Mol. Biol. 120:33-55.

Blackburn, E.H. 2001. Switching and signaling at the telomere. Cell 106:661-673.

Blackburn, E.H., E.S. Epel, and J. Lin. 2015. Human telomere biology: a contributory and interactive factor in aging, disease risks, and protection. Aging 350:1193-1198.

Blackburn, E.H. 2005. Telomeres and telomerase: their mechanisms of action and the effects of altering their functions. FEBS Lett. 579:859-862.

Bonier, F., I.T. Moore, and R.J. Robertson. 2011. The stress of parenthood? Increased glucocorticoids in birds with experimentally enlarged broods. Biol. Lett. 7:944-946.

Broccoli, D., J.W. Young, T. de Lang. 1995. Telomerase activity in normal and malignant hematopoietic cell. Proc. Natl. Acad. USA. 92:9082-9086.

Brown, D.E., C.D. Dechow, W.S. Liu, K.J. and Harvatine, T.L. 2012. Hot Topic: Association of telomere length with age, herd, and culling in lactating Holsteins. J. Dairy Sci. 95:6384-6387.

Burdick, N.C., J.A. Carroll, J.W. Dailey, R.D. Randel, S.M. Falkenberg, and T.B. Schmidt. 2011. Development of a self-contained, indwelling vaginal temperature probe for use in cattle research. J. Therm. Biol. 37:339-343

Campisi, J. 2011. Cellular senescence: putting the paradoxes in perspective. Curr. Opin. Genet. Dev. 21:107-112.

Cawthon, R.M. 2009. Telomere length measurement by a novel monochrome multiplex quantitative PCR method. Nucleic Acids Res. 37: e21.

Cawthon, R.M., K.R. Smith, E. O'Brien, A. Sivatchenko, and R.A. Kerber. 2003. Association between telomere length in blood and mortality in people aged 60 years or older. Lancet. 361:360-361.

Cesare, A.J. and R.R. Reddel. 2010. Alternative lengthening of telomeres: models, mechanisms, and implications. Nat. Rev. Genet. 11:319-330.

Cesare, A.J. and J. Kalseder. 2012.A three-state model of telomere control over human proliferative boundaries. Curr. Opin. Cell. Biol. 24:731-738.

Choi, J., S.R. Fauce, and R.B. Effros. 2008. Reduced telomerase activity in human T lymphocytes exposed to cortisol. Brain Behav. Immun. 22:600-605.

Colmegna, I., A. Diaz-Borjon, H. Fujii, L. Schaefer, J.J. Goronzy, and C.M. Weyand. 2008. Defective proliferative capacity and accelerated telomeric loss of hematopoietic progenitor cells in rheumatoid arthritis. Arthritis and Rheum. 58:990-1000.

Damjanovic, A.K., Y. Yang, R. Glaser, J.K. Kiecolt-Glaser, H. Nguyen, B. Laskowski, Y. Zou, D.Q. Beversdorf, and N.P. Weng. 2007. Accelerated telomere erosion is associated with declining immune function of caregivers of Alzheimer's disease patients. J. Immunol. 179:4249-4254.

Deng, Z., Z. Wang, N. Strong, R. Plasschaert, A. Moczan, H.S. Chen, S. Hu, P. Wilramasinghe, R.V. Davuluri, M.S. Bartolomei, H. Riethman, and P.M. Lieberman. 2012. A role for CTCF and cohesion in subtelomere chromatin organization, TERRA transcription, and telomere end protection. EMBO J. 31:4165-4178.

Deng, Y., S.S. Chan, and S. Chang. 2008. Telomere dysfunction and tumor suppression: the senescence connection. Nat. Rev. Cancer. 8:450-458.

De Lang, T. 2004. T-loops and the origin of telomeres. Nat. Reviews: Mol. Cell Bio. 5:323-329.

De Lang, T. 2005. Sheltrin: the protein complex that shapes and safeguards human telomeres. Genes and Dev. 19:2100-1250.

De Meyer, T. 2011. Telomere length integrates physiological factors in the successful aging story, but what about the biology? Psychosom. Med. 73:524-527.

Dhabhar, F. and B. McEwen. 1997. Acute stress enhances while chronic stress suppresses immune function in vivo. A role for leukocyte trafficking. Brain Behav. Immun. 11:286-306.

Diede, S.J. and D.E. Gottschling. 1999. Telomerase-mediated telomere addition *in-vivo* requires DNA primase and DNA polymerase a and gamma. Cell. 99:723-733.

Doksani, Y., J.Y. Wu, T. de Lang, and X. Zhuang. 2013. Super resolution fluorescence imaging of telomeres reveals TRF2-dependant t-loop formation. Cell. 155:345-356.

Drury, S.S., K. Theall, M.M. Gleason. A.T. Smyke, I. De Vivo, J.Y.Y. Wong, N.A. Fox, C.H. Zeanah, and C.A. Nelson. 2011. Telomere length and early severe social deprivation: linking early adversity and cellular aging. Mol. Psychiatry 17:719-727.

Eades, S.C. 1993. Endotoxemia in dairy cattle: role of eicosanoids in reticulorumen stasis. J Dairy Sci. 76:414-420.

Effros, R.B., M. Dagarag, C. Spaulding, and J. Man. 2005. The role of CD8+ T-cell replicative senescence in human aging. Immunol. Rev. 205:147-157.

Elwood, N.J. 2004. Telomere biology of human hematopoietic stem cells. Cancer Control. 11:77-85.

Entringer, S., E.S. Epel, J. Lin, C. Buss, B. Shahbaba, E.H. Blackburn, H.N. Simon, and P.D. Wadhwa. 2013. Maternal psychosocial stress during pregnancy is associated with newborn leukocyte telomere length. Am. J. Obstet. Gynecol. 134:e1-7.

Entringer, S., C. Buss, J.M. Swanson, D.M. Cooper, D.A. Wing, F. Waffarn, and P.D. Wadhwa. 2012a. Fetal programming of body composition, obesity, and metabolic function: The role of intrauterine stress and stress biology. J. Nutr. Metab. 2012:632548.

Entringer, S., C. Buss, and P.D. Wadha. 2012b. Prenatal stress, telomere biology, and fetal programming of health and disease risk. Sci. Signal. 5:pt12.

Entringer, S. E.S. Epel, R. Kumsta, J. Lin, D.H. Hellhammer, E.H. Blackburn, S. Wust, and P.D. Wadhwa. 2011. Stress exposure in intrauterine life is associated with shorter telomere length in young adulthood. Proc. Natl. Acad. Sci. U S A. 108:E513-E518.

Epel, E.S., E.H. Blackburn, J. Lin, F.S. Dhabhae, N.E. Adler, J.D. Morrow, and R.M. Cawthon. 2004. Accelerated telomere shortening in response to life stress. Proc. Natl. Acad. Sci. U S A. 101:17312-17315.

Epel, E.S., J. Lin, F.S. Dhabhar, O.M. Wolkowitz, E. Puterman, L. Karan, and E.H. Blackburn. 2010. Dynamics of telomerase activity in response to acute psychological stress. Brain Behav. Immun. 24:531-539.

Epel, E.S., J. Lin, F.H. Wilhelm, O.M. Wolkowitz, R. Cawthon, N.E. Adler, C. Dolbier, W.B. Mendes, and E.H. Blackburn. 2006. Cell aging in relation to stress arousal and cardiovascular risk factors. Psychoneuroendocrinology. 31:277-287.

Evens, L.E., D.S. Charney, L.L. Lewis, R.N. Golden, J.M. Gorman, K.R. Rama Krishnan, C.B. Nemeroff, J.D. Bremner, R.M. Carney, J.C. Coyne, M.R. Delong, N. Frasure-Smtih, A.H. Glassman, P.W. Gold, I. Grant, L. Gwyther, G. Ironson, R.L. Johnson, A.M. Kanner, W.J. Katon, P.G. Kaufmann, F.J. Keefe, T.P. Laughren, J. Leserman, C.G. Lyketsos, W.M. McDonald, B.S. McEwen, A.H. Miller, D. Musselman, C. O'Connor, J.M. Pititto, B.G. Pollock, R.G. Robinson, S.P. Roose, J. Rowland, Y. Sheline, D.S. Sheps, G. Simon, D. Spiegel, A. Stunkard, T. Sunderland, P. Tibbits Jr., and W.J. Valvo. 2005. Mood disorders in the medically ill:scientific review and recommendations. Biol. Psychiatry. 58:175-189.

Fairlie, J., R. Holland, J.G. Pilkington, J.M. Pemberton, L. Harrington, and D.H. Nussey. 2016. Lifelong leukocyte telomere dynamics and survival in a free-living mammal. Aging Cell. 15:140-148.

Fleming, A.M., C.J. Burrows. 2017. Formation and processing of DNA damage substrates for the hNEIL enzymes. Free Radic. Biol. Med. 107:35-52.

Foote C.G., F. Daunt, J. Gonzalez-Solis, L. Nasir, R.A. Phillips, P. Monaghan. 2010. Individual state and survival prospects: age, sex, and telomere length in long lived seabird. Behav. Ecol. 22:156-161.

Frias, C., J. Pampalona, A. Genesca, and L. Tussell. 2012. Telomere dysfunction and genome instability. Front. Biosci. 17:2181-2196.

Fujii, H., L. Shao, I. Colmegna, J.J. Goronzy, and C.M. Weyand. 2009. Telomerase insufficiency in rheumatoid arthritis. Proc. Natl. Acad. Sci. U S A. 106:4360-4365.

Garnsworthy, P.C. 2004. The environmental impact of fertility in dairy cows: A modelling approach to predict methane and ammonia emissions. Anim. Feed Sci. Technol. 112:211-223.

Gidron, Y., K. Russ, H. Tissarchondou, and J. Warner. 2006. The relation between psychological factors and DNA damage: a critical review. Bio. Psychol. 72:291-304.

Gilson, E. and V. Geli. 2007. How telomeres are replicated. Nat. Rev Mol. Cell. Bio. 8:825-838.

Glass, D., L. Parts, D. Knowles, A. Aviv, and T.D. Spector. 2010. No correlation between childhood maltreatment and telomere length. Biol. Psychiatry. 68:e21-e22.

Gomez, D.E., R.G. Armando, H.G. Farina, P.L. Menna, C.S. Cerrudo, P.D. Ghiringhelli, and D.F. Alonso. 2012. Telomere structure and telomerase in health and disease. Int. J. Oncol. 41:1561-1569.

Goronzy, J.J., H. Fujii, and C.M. Weyand. 2006. Telomeres, immune aging, and autoimmunity. Exp. Gerontol. 41:246-251.

Gotlib, I.H., J. LeMoult, N.L. Colich, L.C. Foland-Ross, J. Hallwayer, J. Joormann, J. Lin, and O.M. Wolkowitz. 2015. Telomere length and cortisol reactivity in children of depressed mothers. Mol. Psychiatry. 20:615-620.

Greider, C.W. and E.H. Blackburn. 1985. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell. 43:405–413.

Greider C.W. and E.H. Blackburn. 1987. The telomere terminal transferase of Tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity. Cell. 51:887–898.

Greider C.W. and E.H. Blackburn. 1989. A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. Nature. 337:331–337.

Hau, M., M.F. Haussmann, T.J. Greives, C. Matlock, D. Costantini, M. Quetting, J.S. Adelman, A.C. Miranda, and J. Partecke. Repeated stressors in adulthood increase the rate of biological ageing. 2015. Front. Zool. 12:4.

Haussman, M.F. and C.M. Vleck. 2002. Telomere length provides a new technique for ageing animals. Oecologia. 130:325-328.

Haussman, M.F., and B.J. Heidinger. 2015. Telomere dynamics may link stress exposure and ageing across generations. Bio. Lett. 11:20150396.

Haussman, M.F. and N.M. Marcheto. 2010. Telomeres: Linking stress and survival, ecology and evolution. Curr. Zool. 56:714-727.

Haussmann, M.F. and B.J. Heidinger. 2015. Telomere dynamics may link stress exposure and aging across generation. Bio. Lett. 11.

Hayflick, L. and P.S. Moorhead. 1961. The serial culture of diploid and transformed human fibroblasts. Exp. Cell. Res. 25:585-621.

Hayflick, L. 1965. The limited in-vitro lifetime of human diploid cell strains. Exp. Cell Res. 37:614-636.

Ilmonen, P., A. Kotrschal, A., and D.J. Penn. 2008. Telomere attrition due to infection. PLoS One. 3:e2143.

Iwama, H., K. Ohyashiki, J.H. Ohyashiki, S. Hayashi, N. Yahata, K. Ando, K. Toyama, A. Hoshika, M. Takasaki, M. Mori, and J.W. Shay. 1998. Telomeric length and

telomerase activity vary with age in peripheral blood cells obtained from normal individuals. Human Genetics. 102:397-402.

Jacobsen, S., T. Toelboell, and P.H. Anderson. 2005. Dose dependency and individual variability in selected clinical, haematological, and blood biochemical responses after systemic lipopolysaccharide challenge in cattle. Vet. Res. 36:167-178.

Kananen, L., I. Surakka, S. Pirkola, J. Suvisaari, J. Lonngvist, L. Peltonen, S. Ripatti, and I. Hovatta. 2010. Childhood adversities are associated with shorter telomere length at adult age both in individuals with anxiety disorder and controls. PloS one. 5:e10826.

Kiecolt-Glaser, J.K., J.P. Gouin. N.P. Weng, W.B. Malarkey, D.Q. Beversdorf, and R. Glaser. 2011. Childhood adversity heightens the impact of later-life caregiving stress on telomere length and inflammation. Psychosom. Med. 73:16-22.

Kim, T.Y., S.J. Kim, J.R. Choi, S.T. Lee, J. Kim, I.S. Hwang, H.G. Chung, J.H. Choi, S.H. Kim, and J.I. Kang. 2017. The effect of trauma and PTSD on telomere length: and exploratory study in people exposed to combat trauma. Nature. 7:4375.

Kliment, C.R. and T.D. Oury. 2010. Oxidative stress, extracellular matrix targets, and idiopathic pulmonary fibrosis. Free Radic. Biol. Med. 49:707-717.

Koetz, K. E. Bryl, K. Spickschen, W.M. O'Fallon, J.J. Goronzy, and C.M. Weyand. 2000. T cell homeostasis in patients with rheumatoid arthritis. Proc. Natl. Acad. Sci. 97:9203-9208.

Kotrschal, A., P. Ilmonen, and D.J. Penn. 2007. Stress impacts telomere dynamics. Biol. Lett. 3:128-130.

Krause, J.S., J.H. Perez, H.E. Chmura, S.L. Meddle, K.E. Hunt, L. Gough, N. Boelman, and J.C. Wingfield. 2016. The stress response is attenuated during inclement weather in parental, but not pre-parental, Lapland longspurs (Calcarius lapponicus). Horm. Behav. 83:68-74.

Kupfer, D.J. 2005. The increasing medical burden in bipolar disorder. JAMA. 293:2528-2530.

Kyo, S. M. Takakura, T. Kanaya. W. Zou, K. Fujimoto, Y. Nishio, A. Orimo, and M. Inoue. 1999. Estrogen activates telomerase. Cancer Res. 59:5917-5921.

Landsorp, P.M. 2005. Major cutbacks at chromosome ends. Trends Biochem. Sci. 30:388-395.

Landsorp, P.M. 2009. Telomeres and disease. EMBO J. 28:2532-2540.

Laubenthal, L., M. Hoelker, J. Frahm, S. Danicke, K. Gerlack, K.H. Sudekum, H. Sauerwein, and S. Haussler. 2016. Short Communication: Telomere lengths in different tissues of dairy cows during early and late lactation. J. Dairy Sci. 99:4881-4885.

Lendvai, A.Z. M. Giraudeau, and O. Chastel. 2007. Reproduction and modulation of the stress response: and experimental test in the house sparrow. Proc. R. Soc. London B: Biol. Sci. 274:391-397.

Lin, J. E. Epel, J. Cheon, C. Kroenke, E. Sinclair, M. Bigos, O. Wolkowitz, S. Mellon, and E. Blackburn. 2010. Analyses and comparisons of telomerase activity and telomere length in human T and B cells: insights for epidemiology for telomere maintenance. J. Immunol. Methods. 352:71-70.

Lin, J. E. Epel. and E. Blackburn. 2012. Telomeres and lifestyle factors: roles in cellular ageing. Mutat. Res.730:85-89.

Longhese, M.P., S. Anbalagan, M. Martina, D. Bonetti. 2012. The role of sheltrin in maintaining telomere integrity. Frontier in Biosci. 17:1715-1728.

Lou, W., J.G. Muller, E.M. Rachlin, and C.J. Burrows. 2001. Characterization of hydantoin products from one-electron oxidation of 8-oxo-7, 8-dihydroguanosine in a nucleoside model. Chem. Res. Toxicol. 14:927-938.

Love, O.P., C.L. Madliger. S. Bourgeon, C.A. Semeniuk, and T.D. Williams. 2014. Evidence of baseline glucocorticoids as mediators of reproductive investiment in a wild bird. Gen. Comp. Endocrinol. 199:65-69.

Luo, W., J.G. Muller, E.M. Rachlin, and C.J. Burrows. 2001. Characterization of hydantoin products from one-electron oxidation of 8-oxo-7,8-dihydroguanosine in a nucleoside model. Chem. Res. Toxicol. 14:927-938.

Magiakou, M.A., G. Mastorako, D. Rabin, B. Dubbert, P.W. Gold, G.P. Chrousos, 1996. Hypothalamic corticotropin-releasing hormone suppression during the postpartum period:implications for the increase of psychiatric manifestations at this time. J. Clin. Endocrinol. Metab. 81:1912-1917.

Mangino, M., S.J. Hwang, T.D. Spector, S.C. Hunt, M. Kimura, A.L. Fitzpatrick, L. Christiansen, I. Peterson, C.C. Elbers, T.H. Wei Chen, S.R. Srinivason, J.D. Kark, A. Benetos, S.E. Shamieh, S. Visvikis-Siest, K. Christensen, G.S. Berenson, A.M. Valdes, A. Vinuela, M. Garcia, D.K. Arnett, U. Broeckel, M.A. Province, J.S. Pankow, C. Kammerer, Y. Liu, M. Nalls, S. Tishkoff, F. Thomas, E. Ziv, B.M. Psaty, J.C. Bis, J.I. Rotter, K.D. Taylor, E. Smith, N.J. Schork, D. Levy, and A. Aviv. 2012. Genome-wide

meta-analysis points to CTC1 and ZNF676 genes regulating telomere homeostasis in humans. Hum. Mol. Genet. 21:5385-5394.

Mantell, L.L. and C.W. Greider. 1994. Telomerase activity in germ line and embryonic stem cells of Xenopus. Embo. J. 13:3211-3217.

Marcand, S., V. Brevet. C. Mann, and E. Gilson. 2000. Cell cycle restriction of telomere elongation. Curr. Biol. 10:487-490.

Mathur, M.B., E. Epel, S. Kind, M. Desai, C.G. Parks, D.P. Sandler, and N. Khazeni. 2016. Perceived stress and telomere length: A systemic review, meta-analyses, and methodologic considerations for advancing the field. Brain Behav. Immun. 54:159-169.

Martin, L.B. 2009. Stress and immunity in wild vertebrates: timing is everything. Gen. Comp. Endocrinol. 163:70-76.

McEachern, M.J., A. Krauskopf, and E.H. Blackburn, 2000. Telomeres and their control. Annu. Rev. Genet. 34:331-358.

McEwen, B.S. 1998. Stress, adaptation, and disease. Ann N Y Acad Sci. 840:33-44.

McEwen, B.S. 2013. The brain on stress: toward an integrative approach to brain, body, and behavior. Perspect Psychol Sci. 8:673-675.

McMullen, S., J.C. Osgerby, L.M. Thurston, T.S. Gadd, P.J. Wood, D.C. Wathes, and A.E. Michael. 2004. Alteration in placental 11β-hydroxysteroid dehydrogenase (11βHSD) activities and fetal cortisol:cortisone ratios induced by nutritional restriction prior to conception and at defined stages of gestation in ewes. Reproduction 127:717-725.

Mendl, M., O.H.P Burman, and E.S. Paul. 2010. An integrative and functional framework for the study of animal emotion and mood. Proc R Soc B Biol Sci. 277:2895-2904.

Metcalf, D. 1988. Molecular control of blood cells. 1st ed. Harvard University Press, Cambridge, MA.

Micheals, F.H. and K.L. Banks. 1988. Contribution of various host factors to resistance to experimentally induced bacterial endotoxemia in calves. Am J Vet Res. 49:557-562.

Morrison, S.J., K.R. Prowse, P. Ho, and I.L. Weissman. 1996. Telomerase activity in hematopoietic cells is associated with self-renewal potential. Immunity 5:207-216.

Mulligan, C.J., N.C. D'Errico, J. Stees, and D.A. Hughes. 2012. Methylation changes at NR3C1 in newborns associate with maternal prenatal stress exposure and newborn birthweight. Epigenetics 7:853-857.

Murphy, B.E., S.J. Clark, I.R. Donald, and D.L. Vedady. 1974. Conversion of maternal cortisol to cortisone during placental transfer to the human fetus. Am. J. Obstet. Gynecol. 118:538-541.

Murphy, M.G., M.P. Boland, and J.F. Roche. 1990. Pattern of follicular growth and resumption of ovarian activity in post-partum beef suckler cows. Repro. 90:523-533.

Nelson, N.D. and A.A. Bertuch. 2012. Dyskeratosis congenita as a disorder of telomere maintenance. Mutat. Res. 730:43-51.

Njajou, O., R.M. Cawthon, C.M. Damcott, S.H. Wu, S. Ott, M.J. Garant, E.H. Blackburn, D.M. Braxton, A.R. Shuldiner, and W.C. Hsueh. 2007. Telomere length is paternally inherited and is associated with parental lifespan. Proc. Natl. Acad. Sci. U S A. 104:12135-12139.

Nzietchueng, R., M. Elfarra, J. Nloga, C. Labat, J.P. Carteaux, P. Maureira, P. Lacolley, J.P. Villemot, and A. Benetos. 2011. Telomere length in vascular tissues from patients with atherosclerotic disease. J. Nutr. Health aging. 15:153-156.

O'Donovan, A., E. Epel, J. Lin, O. Wolkowitz, B. Cohen, S. Maguen, T. Metzler, M. Lenoci, E. Blackburn, and T.C. Neylan. 2011. Childhood trauma associated with short leukocyte telomere length in posttraumatic stress disorder. Biol. Psychiatry. 70:465-471.

Okamoto, K. C. Bartocci, I. Ouzounov, J.K. Diedrich, J.R. Yates 3rd, and E.L. Denchi. 2013. A two-step mechanism for TRF2-mediated chromosome-end protection. Nature. 494:502-505.

Olovnikov, A.M. 1971. A theory of marginotomy: The incomplete copying of template margin in enzymatic synthesis of polynucleotides and biological significance of the phenomenon. J. Theor. Biol. 41:181–190.

Opresko, P.L. and J.W. Shay. 2017. Telomere-associated aging disorders. Ageing. Res. Rev. 33:52-66.

Paeschke, K., K.R. McDonald, and V.A. Zakian. 2011. DNA replication through G-quadruplex motifs is promoted by the *Saccharomyces cerevisae* Pif1 DNA helicase. Cell. 145:678-691.

Pauliny, A., K. Larsson, and D. Blomqvist. 2012. Telomere dynamics in a long-lived bird, the barnacle goose. BMC Evo. Biol. 12:257

Plaza, M.A., J. Fioramonti, and L. Bueno, 1997. Role of central interleukin-iB in gastrointestinal motor disturbances induced by lipopolysaccharide in sheep. Dig Dis Sci. 42:242-250.

Plot, V. F. Criscuolo, S. Zahn, and J.Y. Georges. 2012. Telomeres, age and reproduction in a long-lived reptile. PLoS One. 7:e40855.

Podlevsky, J.D. and J.J. Chen. 2012. It all comes together at the ends: Telomerase structure, function, and biogenesis. Mutat. Res. 730:3-11.

Pollack, A.Z., K. Rivers, and K.A. Ahrens. 2018. Parity associated with telomere length among U.S. reproductive age women. Hum. Reprod. 33:736-744.

Population Reference Bureau. 2018. 2018 world population data sheet with focus on changing age structures. https://www.prb.org/2018-world-population-data-sheet-with-focus-on-changing-age-structures/ (Accessed 16 May 2018.)

Price, L.H., H.T. Kao, D.E. Burgers, L.L. Carpenter, and A.R. Tyrka. 2013. Telomeres and early life stress: an overview. Biol. Psychiatry. 73:15-23

Pritchard, T., M. Coffey, R. Mrode, and E. Wall. 2013. Understanding the genetics of survival in dairy cows. J. Dairy. Sci. 96:3296-3309.

Rattiste, K., H. Klandorf, J. Urvik, T. Sepp, M. Asghar, D. Hasselquist, C. Cooey, and P. Horak. 2015. Skin pentosidine and telomere length do not covary with age in a long-lived seabird. Biogerontology. 16:435-441.

Redrejo-Rodriguez, M., C. Saint Pierre, S. Couve, A. Mazouzi, A.A. Ishchenko, D. Gasparutto, and M. Saparbaev. 2011. New insights in the removal of the hydantoins, oxidation products of pyrimidines, via base excision and nucleotide incision repair pathways. PLOS One. 6:e21039.

Reichert, S., A. Stier, S. Zahn, M. Arrive, P. Bize, S. Massemin, and F. Criscuolo. 2014. Increased brood size leads to presistant eroded telomeres. Front Ecol. Evol.2:9.

Reichert, S. and A. Stier. 2017. Does oxidative stress shorten telomeres *in vivo*? A review. Biol. Lett. 13:20170463.

Rey, S., C. Quintavalle, K. Burmeister, D. Calabrese, M. Schlageter, L. Quagliata, G. Cathomas, J. Diebold, A. Molinolo, and M.H. Heim. 2017. Liver damage and senescence increases in patients developing hepatocellular carcinoma. J. Gastroenterol. Hepatol. 32:1480-1486.

Seeker, L.A., R. Holland, S. Underwood, J. Fairlie, A. Psifidi, J.J. Ilska, A. Bagnall, B. Whitelaw, M. Coffey, G. Banos, and D.H. Nussey. 2016. Method specific calibration corrections for DNA extraction method effects on relative telomere length measurements by quantitative PCR. PLoS One. 11:e0164046.

Seeker, L.A., J.J. Ilska, A. Psifidi, R.V. Wilbourn, S.L. Underwood, J. Fairlie, R. Holland, H. Froy, A. Bagnall, B. Whitelaw, M. Coffey, D.H. Nussey, and G. Banos. 2018a. Longitudinal changes in telomere length and associated genetic parameter in dairy cattle analyzed using random regression models. PLoS ONE. 13:e0192864.

Seeker, L.A., J.J. Ilska, A. Psifidi, R.V. Wilbourn, S.L. Underwood, J. Fairlie, R. Holland, H. Froy, E. Salvo-Chirnside, A. Bagnall, B. Whitelaw, M.P. Coffey, D.H. Nussey, and G. Banos. 2018b. Bovine telomere dynamics and the association between telomere length and productive lifespan. Sci. Rep. 8:12748.

Semrad, S.D. and R. Dubielzig. 1993. Effect of phenylbutazone and repeated endotoxin administration on hemostasis in neonatal calves. Am J Vet Res. 54:1339-1346.

Sfeir, A.J., S.T. Kosiyatrakul, and D. Hockmeyer. 2009. Mammalian telomeres resemble fragile sites and require TRF1 for efficient replication. Cell. 138:90-103.

Sfeir, A.J. 2012. Telomeres at a glance. J. Cell. Sci.125. 4173-4178.

Shalev, I., S. Entringer, P.D. Wadha, O.M. Wolkowitz, E. Puterman, J. Lin, and E.S. Epel. 2013. Stress and telomere biology: A lifespan perspective. Psychoneuroendochrinology 38:1835-1845.

Shalev, I. 2012. Early life stress and telomere length: Investigating the connection and possible mechanisms. Bioessays 34:943-952.

Simon, N.M., J.W. Smoller, K.L. McNamara, R.S. Maser, A.K. Zalta, M.H. Pollack, A.A. Nierenberg, M. Fava, and K.K. Wong. 2006. Telomere shortening and mood disorders: preliminary support for a chronic stress model of accelerated aging. Biol. Psychiatry. 60:432-435.

Smith, S.M., R.G. Nager, and D. Costantini. 2016. Meta-analysis indicates that oxidative stress is both a constraint on and a cost of growth. Ecol. Evol. 6:2833-2842.

Sobinoff A.P. and H.A. Pickett. 2017. Alternative lengthening of telomeres: DNA repair pathways converge. Trends Genet. 33:921-932.

Sudyka, J., A. Arct. S. Drobniak, A. Dubiec. L. Gustafsson, and M. Cichon. 2014. Experimentally increased reproductive effort alters telomere length in the blue tit (cyanistes caeruleus). J. Evol. Biol. 27:2258-2264.

Surtees, P.G., N.M. Wainwright, K.A. Pooley, R.N. Luben, K.T. Khaw, D.F. Easton, and A.M. Dunning. 2011. Life stress, emotional health, and mean telomere length in the European Prospective Investigation into Cancer (EPIC)-Norfolk population study. J. Gerontol. series A Biol. Sci. Med. Sci. 66:1152-1162.

Szostak J.W. and E.H. Blackburn. 1982. Cloning yeast telomeres on linear plasmid vectors. Cell. 29:245–255.

Teasley, D.C. and S.A. Stewart. 2016. Telomere Biology. In: R. Bradshaw and P. Stahl. Encyclopedia of Cell Biology. Academic Press, Saint Louis, MO. p. 426-434.

Testa, R. F. Olivieri, C. Sirolla, L. Spazzafumo. M.R. Rippo, M. Marra, A.R. Bonfigli, A. Ceriello, R. Antonicelli, C. Franceschi, C. Castellucci, I. Testa, and A.D. Procopio. 2011. Leukocyte telomere length is associated with complications of type 2 diabetes mellitus. Diabet. Med. 28:1388-1394.

Tyrka, A.R., L.H. Price, H.T. Kao, B. Porton, S.A. Marsella, and L.L. Carpenter. 2010. Childhood maltreatment and telomere shortening: preliminary support for an effect of early stress on cellular aging. Biol. Psychiatry. 67:531-534.

Valsamakis, G. G. Chrousos, G. Mastorakos. 2018. Stress, female reproduction, and pregnancy. Psychoneuroendocrinology. 100:48-57.

Van, B.D., N.M. Nanlohy, S. Otto, F.J. Plunkett, J.M. Fletcher, and A.N. Akbar. 2008. Progressive telomere shortening of Epstein-Barr virus-specific memory T cells during HIV infection: contributor to exhaustion? J. Infect. Dis. 198:1353-1357.

von Zglinicki, T. 2002. Oxidative stress shortens telomeres. Trends Biochem. Sci. 27:339-344.

Watson J. 1972. Origin of concatemeric T7 DNA. Nat. N. Biol. 239:197–201.

Wegman-Ostrosky, T. and S.A. Savage. 2017. The genomics of inherited bone marrow failure: from mechanism to the clinic. Br. J. Haematol. 177:526-542.

Weng, N.P., L.D. Palmer, B.L. Levine, H. Clifford Lane, C.H. June, and R.J. Hodes. 1997. Tales of Tails: regulation of telomerase activity during lymphocyte development, differentiation, activation, and aging. Immunol. Rev. 160: 43-54.

Wood, C.E. 1999. Control of parturition in ruminants. J. Reprod. Fertil. Suppl. 54:115-126.

Xu, J. and X. Yang. 2003. Will cloned animals suffer premature aging- The story at the end of clones' chromosomes. Reprod. Biol. Endocrinol. 1:105.

Yamada, T., M. Higuchi, and N. Nakanishi. 2015. Effect of the anatomical site on telomere length and pref-1 gene expression in bovine adipose tissue. Biochemical and biophysical research communications. 463:923-927.

Ye, J., C. Lenain, S. Bauwens, .2010. TRF2 and Apollo cooperate with topoisomerase 2alpha to protect human telomeres from replicative cell damage. Cell. 142:230-242.

Wingfield, J.C. and R.M. Sapolsky. 2003. Reproduction and resistance to stress: when and how. J. Neuroendocrinol. 15:711-724.

Zimmerman. M. 2014. TRF1 negotiates TTAGGG repeat-associated replication problems by recruiting the BLM helicase and the TPP/POT1 repressor of ATR signaling. Genes Dev. 28:2477-2491.

WBC ISOLATION, DNA EXTRACTION, AND PCR PROTOCOLS

White Blood Cell Isolation

Step 1 Collect blood by jugular venipuncture into EDTA vacutainer tubes and store in

refrigerator until WBC isolation steps

- When bleeding animal, make sure vacutainer fills with steady stream of

    blood

- Use 18 ga 1" needles and change needle between every animal

Step 2 Spin whole blood at 2,675 RCF (3,500 RPM) for 30 min at 5°C

Step 3 Carefully aspirate the WBC layer at the serum and RBC interface

- Transfer into a 1.5 ml microfuge tube and place on ice

Step 4 Add RBC lysis buffer to WBC to fill 1.5 ml tube

- Vortex tubes for 20 sec

- Shake tubes for 5 min

Step 5 Spin tubes in microfuge for 5 minutes at 5,000 RCF (14,000 RPM)

- A WBC pellet should form at bottom of tube

Step 6 Aspirate supernatant off the top of WBC pellet

- Repeat steps 4,5, and 6 WBC washing until a clean, white WBC pellet is

    achieved

Step 7 Store WBC pellet in -80°C in 1.5 ml microfuge tube

- Wrap in parafilm for long term storage

DNA Extraction

Utilize a spin column kit (GeneJET Genomic DNA Purification Kits (Thermo Scientific; Waltham, MA, USA.)) that yields high quality, intact DNA

Quality Control – for PCR

Yield > 20 ng/µl, Integrity Score < 3, 260/280 > 1.7, 260/230 >1.8 (as outlined by Seeker et al., 2016)

- Use a Qubit to determine yield, a Nanodrop does not give yield of INTACT genomic DNA

- Nanodrop is used for determining salt content

- Can utilize a Tapestation or Gel for integrity

Aliquot DNA to 10 ng / µl concentration and 10 µl total volume

- Store in low-binding tubes at -80°C

- May need to store at greater volumes for long term storage

- Seal samples with parafilm for long term storage

Real-Time PCR

Materials

Standards and Primers can be ordered through Integrative DNA Technologies

Bovine Telomere Standard Oligonucleotide (IDT Ref #206903826)

Bovine B2G Standard Oligonucleotide (IDT Ref # 206903823)

Bovine Telomere Forward Primer (IDT Ref# 207839206)

Bovine Telomere Reverse Primer (IDT Ref# 207839207)

Bovine B2G Forward Primer (IDT Ref# 207839208)

Bovine B2G Reverse Primer (IDT Ref# 207839209)

Powerup SYBR Master Mix (LTC Catalog # A25776)

Nuclease Free H$_2$0

**Intact Genomic DNA (Integrity checked & Qubit for concentration)

    - Diluted to 10 ng / µl

    - 10 µl aliquots made for all possible plates

Preparatory Steps

Test Primers and Standards prior

S.O. 2019 experiment found 500nM of telomere F/R primer worked best with 300nM of B2G F/R primer

    - Can be altered if another primer ratio is desired

S.O. 2019 experiment found that, for 20-21 mo old heifers and 4-year-old cows, the appropriate standard curve concentrations were between $10^{13} - 10^8$ for telomere and $10^9 - 10^4$ for B2G

    - Older/younger animals may require a different range of standard concentrations

    - Test standard curve on a representative animal prior

Utilize the same thermocycler throughout experiment with plates manufactured by the same company

Step 1 Test Standards

Resuspend standard to 1×10^{16} (that should give a broad enough range for any bovine TL)

See nM given on IDT form

- Multiply given nM by 6.022×10^{17}

- This will give you copy number (#)

- Diluting this will give you # x 10^{18}

- Adjust dilution to give # x 10^{16}

- See S.O. lab notebook for example if confused

Let standards resuspend for 1 hour with finger tapping every 10-15 minutes

- Make aliquots of stock and store in -80*

Do a 1:10 dilution series of the standards utilizing the non-stick (low concentration) tubes

- Test standard for linearity

- Make aliquots of each iteration and store in -80*

Bovine B2G and Telomere standards must be run independently (2 curves on plate)

***Standard works best freshly made, aliquots of frozen dilution may not be linear, so I recommend testing a standard and saving the iteration in addition to making a fresh standard dilution series when running an experiment. That way you have a back-up if your previous standard fails, the freshly diluted standard will work. However, standards vary every time you dilute them so you will not be able to make comparisons between plates. Using the frozen standard gives you the chance to compare between plates if it succeeds but it has not worked in the past.

Step 2 Prepare Master Mix

This is a 20 µL reaction (18 µL MM + 2 µL DNA) meant for a 384 well plate

Master Mix		x # Wells
H20	4.8 µL	X µL
TF	1 µL	X µL
TR	1 µL	X µL
BF	0.6 µL	X µL
BR	0.6 µL	X µL
SYBR	10 µL	X µL
Total Volume:	18 µL	X µL + 5-10% for pipetting error

Tips:

1.) Mix everything well after combining AND before plating
2.) Primers come with instructions to suspend to 100nM from IDT
   - Use a 1:10 dilution to achieve the proper final nM for master mix
3.) Keep covered in foil away from light (anytime Powerup SYBR Master Mix is involved)
4.) Work from cheapest reagent to most expensive
5.) All materials and steps should be done on ice
6.) SYBR is very viscous so pipette slowly otherwise you may run out of master mix when setting up a plate

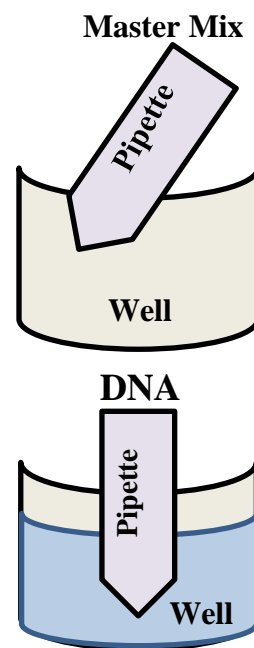*Setting up Plates*

Note: Everything will be done in triplicate

Tip: Set plate on a colored laminated card on ice

   - Dark colors help make the wells visible

   - Plastic keeps moisture off of the plate

Step 1 Pipette master mix in first (18 µL)

   - Mix thoroughly prior

   - Pipette master mix onto well wall to

92

avoid bubbles forming underneath

Step 2 Pipette DNA in second (2 µL)

- Mix thoroughly via finger tapping of the tube

- Pipette DNA into master mix but above

the bottom of the well

- Treat standards like DNA

Tips:

1.) Use recently calibrated pipettes

2.) Go slowly and deliberately

3.) **Rushing will cause your triplicates to be wide and**

your standard curve to fall apart!

Step 3 Setting up thermocycler and finished plate

S.O. et al., (2019) experiment utilized Brown et al., 2012 multiplex thermocycler

settings: "The thermal cycling profile was as follows. Stage 1: 15 min at 95°C; stage 2: 2

cycles of 15 s at 94°C, 15 s at 49°C; stage 3: 40 cycles of 15 s at 94°C, 10 s at 62°C, 15 s

at 73°C with signal acquisition, 10 s at 80°C, and 15 s at 83°C with signal acquisition.

Signal acquisition at 73°C provided the information to calculate cycle threshold (Ct)

values for the telomere product (Ct Tel); signal acquisition at 83°C provided the

information for the β-globin product (Ct βG)"

- This should be tested with primers and standard prior to experiment

Tips: Before imputing plates

1.) Insert plate into plate spinner

- This will prevent any bubbles from staying in the bottom of the well

2.) Seal plate with plastic film

Data Analyses

Download and install CFX Maestro Software

      -if using Bio-Rad thermocycler

Export data from thermocycler

Step 1 Plate set up

Open plate set up in CFX Maestro

- Designate wells as unknown, standards, or negative controls based on plate
  map

- This will have to be done twice on two different saved versions for telomere
  standard and B2G standard set to the appropriate signal acquisition step for
  each

Check linearity of standard curves

- $R > 0.98$ respectively

Step 2 Export data

After the standard curves have been created in plate set up starting quantities of all
unknown samples will be given in addition to C_q values

- Export data into excel

- Make sure the program is in "Target" setting and not "fluorophore"

Check that low C_q values correlate with high starting quantities

- This can be done by sorting data in excel (highest to lowest)

- If this relationship is not correct go back to CFX maestro and double check settings

Step 3 Data Analyses

Correctly label wells with Animal ID

Evaluate C_q differences by subtracting the highest and lowest value within triplicates of an individual sample

- Do this for all samples to see accuracy of plate set up

- Determine a threshold that is acceptable for C_q differences, generally no more than 0.3 differences between highest and lowest value

- Eliminate obvious outliers within replicates to achieve acceptable C_q difference

- Eliminate any samples that exceed 0.3 difference even with outlier removed

- Utilize remaining starting quantities to create an average for a sample

Utilize average starting quantities of telomere and B2G to create T/S ratio for samples

- Divide telomere by B2G starting quantities for all samples

Use T/S ratio for statistical analyses of choice

Instructions for Buffers and Gel Electrophoresis

RBC Buffer

Materials

998 ml millipore filtered H_2O

2 ml of 0.5 M EDTA

Sterile conical vacuum filter

95

Instructions

Add 2 ml of 0.5 M EDTA to 998 ml H_2O and filter through sterile conical vacuum filter

Store at 4°C

Gel Electrophoresis

Instructions for Bio-Rad min sub cell GT tray

Step 1 In a 125 ml flask add 50 ml 1X TAE buffer and 0.5 g agarose

- Cover with plastic wrap and poke to vent

- Microwave for 1.5 min with occasional mixing

Step 2 Let flask with TAE and agarose cool until safe to touch

- Add 2.5 µl of Azura Genomic Smart Stain

- Swirl to mix

Step 3 Pour mixture into tray

- Make sure comb is in place prior to pouring

- Let solidify for 30 minutes

Step 4 Pour TE buffer over gel until it reached the fill line on side of tray

- Carefully remove comb by lifting one side at a time

- Make sure wells stay intact

Step 5 Load wells

- Utilize 100 ng of DNA mixed with Azura Genomics Smart Glow Stain

    o 1 µl of stain for every 5 µl of DNA

    o Prepare all DNA samples prior to loading in PCR strip tubes

- Utilize 6 µl of Thermo Scientific GeneRuler 1 kb DNA ladder

Step 6 Run settings

- Run electrophoresis at 85 V for 1 hr

- Dye should be 2/3 of the way down the gel