LYMPHOCYTE-MEDIATED INFLAMM-AGING IN THE HORSE

Melissa Hope Siard  
*University of Kentucky*, melissa.siard@uky.edu  
Author ORCID Identifier:  
[https://orcid.org/0000-0003-4056-0611](https://orcid.org/0000-0003-4056-0611)  
Digital Object Identifier: [https://doi.org/10.13023/ETD.2017.281](https://doi.org/10.13023/ETD.2017.281)

**Recommended Citation**  
[https://uknowledge.uky.edu/gluck_etds/32](https://uknowledge.uky.edu/gluck_etds/32)
STUDENT AGREEMENT:

I represent that my thesis or dissertation and abstract are my original work. Proper attribution has been given to all outside sources. I understand that I am solely responsible for obtaining any needed copyright permissions. I have obtained needed written permission statement(s) from the owner(s) of each third-party copyrighted matter to be included in my work, allowing electronic distribution (if such use is not permitted by the fair use doctrine) which will be submitted to UKnowledge as Additional File.

I hereby grant to The University of Kentucky and its agents the irrevocable, non-exclusive, and royalty-free license to archive and make accessible my work in whole or in part in all forms of media, now or hereafter known. I agree that the document mentioned above may be made available immediately for worldwide access unless an embargo applies.

I retain all other ownership rights to the copyright of my work. I also retain the right to use in future works (such as articles or books) all or part of my work. I understand that I am free to register the copyright to my work.

REVIEW, APPROVAL AND ACCEPTANCE

The document mentioned above has been reviewed and accepted by the student’s advisor, on behalf of the advisory committee, and by the Director of Graduate Studies (DGS), on behalf of the program; we verify that this is the final, approved version of the student’s thesis including all changes required by the advisory committee. The undersigned agree to abide by the statements above.

Melissa Hope Siard, Student
Dr. Amanda A. Adams, Major Professor
Dr. Daniel Howe, Director of Graduate Studies
LYMHPOCYTE-MEDIATED INFLAMM-AGING IN THE HORSE

Dissertation Submitted in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment
at the University of Kentucky

By

Melissa Hope Siard

Lexington, Kentucky

Co-Directors: Dr. Amanda A. Adams, Assistant Professor of
Equine Immunology
and
Dr. David W. Horohov, Professor of Immunology,
Schlaikjer Chair in Equine Immunology,
Director of Gluck Equine Research Center

2017

Copyright © Melissa Hope Siard 2017
LYMPHOCYTE-MEDIATED INFLAMM-AGING IN THE HORSE

Senior horses (≥20 years) exhibit inflamm-aging, or chronic, low-grade inflammation that occurs systemically with aging, similarly to humans. Inflamm-aging has previously been characterized in the horse in circulation as well as specifically being mediated by lymphocytes and monocytes. In humans, inflamm-aging has been associated with increased morbidity and mortality. However, in the horse, relatively little about inflamm-aging is known regarding clinical effects or factors influencing severity. The contribution of lymphocytes to inflamm-aging of senior horses was examined, specifically through determining the relationships of inflamm-aging with various other health parameters, effects of seasonality, and the extent to which inflamm-aging can be modulated by anti-inflammatory phytonutrient curcumin. The overall hypothesis of this research is that lymphocyte-mediated inflamm-aging of the senior horse is associated with various factors including season, endocrine function, body composition, and nutritional status, and may be modulated by polyphenol curcumin. The effect of season on lymphocyte-mediated inflamm-aging was examined, and senior horses exhibited elevated inflammation compared to adult horse as expected, while also exhibiting changes in inflammatory cytokine production and gene expression throughout the year. In addition to season, pituitary pars intermedia dysfunction (PPID), a common endocrinopathy in senior horses that is associated with immunosuppression, was examined in a group of senior horses to determine any effects on degree of inflamm-aging. Results indicated no significant differences between age-matched PPID and non-PPID horses for lymphocyte-mediated inflammatory cytokine production or gene expression. The immunosuppressive aspect of PPID does not appear to be associated with the degree of lymphocyte-mediated inflammation of the aged horse. Additionally, an expansive correlative study was undertaken to determine relationships between inflamm-aging and basal nutritional status, body composition, age, and PPID within a similarly-managed senior horse population. Results showed various relationships between inflammatory markers and nutritional status, particularly yielding positive associations with serum folate and with serum fatty acids C22:2n6c and C22:5n3c. Inflammation was also associated with age itself but was not associated with body composition parameters and showed mild association with PPID (and serum inflammatory C-reactive protein). As a whole, this study demonstrates that nutritional status can be associated with inflammatory markers. Similarly, many phytonutrients have exhibited anti-inflammatory properties, which may be beneficial to the senior horse exhibiting inflamm-aging. Specifically, the effects of polyphenols including curcuminoids, resveratrol, quercetin, pterostilbene, and hydroxypterostilbene on lymphocyte production of inflammatory cytokines by senior horses were examined in vitro and found to significantly reduce inflammation similarly to common non-steroidal anti-inflammatory drugs. This study led to the in vivo investigation of the effectiveness of curcumin in modulating chronic inflammation of the senior horse. No significant differences were seen between groups receiving curcumin and placebo for the various
inflammatory parameters, which may be due to the dose or low bioavailability of curcumin. As a whole, this research provides further understanding of factors associated with inflamm-aging of the senior horse.

KEYWORDS: Horse, Inflamm-aging, Lymphocyte, Aging, Curcumin, PPID

Melissa Hope Siard

May 26, 2017
Date
LYMPHOCYTE-MEDIATED INFLAMM-AGING
IN THE HORSE

By
Melissa Hope Siard

Dr. Amanda A. Adams
Director of Dissertation

Dr. David W. Horohov
Co-Director of Dissertation

Dr. Daniel Howe
Director of Graduate Studies

May 26, 2017
ACKNOWLEDGMENTS

Many people have contributed to my PhD journey over the years, and I am very thankful. First of all, I would like to thank my advisor, Dr. Amanda Adams for all her help, encouragement, and support throughout my research endeavors. When I started as an undergrad in the lab the summer before my senior year, I certainly did not know I was beginning what would become a PhD journey under your guidance. Your enthusiasm for equine research, vast knowledge of immunology, and ability to collaborate well with many others, all while keeping in mind the big picture, truly make you an outstanding researcher that I am privileged to have worked under. Beyond the academic aspect, you care deeply for your students both as people and as professionals. I will never forget all the bonding experiences we have had over the years and am truly thankful to count you as a role model, particularly as a young woman in academia.

I am also thankful for my committee members including my co-advisor, Dr. David Horohov, Dr. Thomas Chambers, Dr. Elizabeth Head, Dr. Kristine Urschel, and my outside examiner, Dr. Mary Rossano. I am particularly grateful to Dr. Head for connecting me with contacts, eventually leading to funding of the curcumin supplementation project. Each member of my committee contributed different aspects, and the committee worked well together to better my research. I remember coming out of my qualifying exam, counting myself privileged to have five committee members each reading my grant proposal and bringing questions and ways to improve it.
I would also like to thank the members of the Adams and Horohov lab, as so many people have contributed to my research in various ways. I would particularly like to thank Day Barker, our lab technician who has helped extensively with countless projects. Thank you for reading my mind in preparation for collection days, always being conscientious, keeping everything organized, training countless undergrads who helped with our research, and for always being there to help with whatever needs done. I don’t know what we would do without you! In the earlier days of my PhD program, I also appreciated the contributions of Craig Stewart and Alex Betancourt who helped me out numerous times, especially when I had classes while trying to do research studies. I will always appreciate you two, including your help training me during my undergraduate research. And last but certainly not least, Sarah Elzinga, I will always be grateful you joined our lab as a PhD student shortly after I did. You showed me the ropes regarding the role of a graduate student and were my opposite in nearly every way, and yet somehow we almost always ended up at the same bottom line. I have appreciated your friendship and miss you already (as well as your little stress-relieving pooch Arie)! Working with you has shown me just how much opposites can complement and balance each other, and hopefully our friendship of balancing each other out will continue throughout our careers, as you have headed back to the northern lands!

Additionally, I would like to thank the numerous undergraduates who have helped with projects throughout the years. In particular, I would like to thank Kellie McMurry who worked with us for over a year. Kellie, you certainly helped kick-start the Adams lab and inadvertently helped generate my first PhD project, as the quick stilbene project became the stilbene saga of my first year of grad school. Thank you for your tireless
labeling skills and for always going with the flow (sometimes quite literally) in helping with the latest project! Brooke Hadley (now Brooke Barber), I appreciated you spending your summer helping me thaw cells from the liquid nitrogen (a year’s worth of monthly collections) for the seasonal study—it was certainly a much needed help! And Vivian Lages Cotta Martins, I also appreciated your constant good attitude and hard work as well as your stallion whispering skills! I’m truly grateful for the caliber of people I have had the opportunity to work with, certainly including our undergrads.

Furthermore, I would like to thank the UK farm crew, particularly Mason Mulholland, John Allen, Chip Stamper, Seth Hatfield, and Chad Tucker. Additionally, I would like to thank Courtney Lawson especially for helping with our big collection days for multiple studies and always having a cheerful attitude. And, although Stephanie Reedy is not part of the farm crew, she frequently helped at the farm during our big collections days as well, for which we were certainly grateful. I also appreciate Lynn Ennis and Kevin Gallagher for their work as farm managers. And of course, none of this research would be possible without the UK horses, many of which have become near and dear to me (Clover, Mr. Friendly, Angel). I’m thankful to have gotten to work with so many good horses, particularly our oldies.

I’m also incredibly thankful to my family and friends who have supported me throughout this PhD journey. I will always be grateful for both the comradery of my fellow graduate students and the many friends outside of UK who have been there for me during various parts of this PhD journey. My family including my grandparents who traveled to my defense have always been supportive of me. And my parents have supported me as a person, in my academic pursuits, in my equine pursuits, and as a Christ follower my entire
life, and I could never say enough to thank them for who they are and what they have done for me throughout my life. Thanks so much Mom and Dad!

And Alex, you sure came into my life at an interesting time. Who knew that the cattle nutrition PhD student who came in to learn immunology techniques last spring would become my fiancé by this summer! I am so incredibly thankful for you—who you are and how much you have been there for me in my crazy last year of grad school! It has been a wild ride to be sure, but I wouldn’t trade it for the world! I will always treasure how much you have taken care of me particularly in the last months of writing my dissertation—cooking for me, stopping by to check on me every day, and making sure I took little breaks. I love you, Alex Altman, and look forward to being your wife!

Lastly, I want to thank God for charting my course and seeing me through this crazy journey so far, and for all the good things I’m sure He has in store for the future. I certainly never would have imagined I would find my twenty-six year old self with a PhD, headed to a Christian university I love to teach equine science courses. It would have seemed too good to be true—but here I am by the grace of God. I am so thankful for all the people God has placed in my life over the years who have taught me so much, given me many opportunities, and helped mold me into the person I am today. But most of all I am thankful for His salvation and also for grace as I attempt to tackle each new season of life in His strength. To God be the glory!
# TABLE OF CONTENTS

Acknowledgments....................................................................................................................iii

List of Tables........................................................................................................................................ix

List of Figures.................................................................................................................................x

Chapter 1  Literature Review...........................................................................................................1
Hypothesis and Specific Aims...........................................................................................................16

Chapter 2  Effect of Season on Lymphocyte-Mediated Inflamm-aging and
Immunosenescence in the Horse.........................................................................................................17
  Introduction...................................................................................................................................19
  Methods.........................................................................................................................................21
  Results...........................................................................................................................................27
  Discussion.....................................................................................................................................31

Chapter 3  Relationships of Inflamm-aging with Nutritional Status, Body Composition,
Age, and Pituitary Pars Intermedia Dysfunction in a Senior Horse Population..................45
  Introduction...................................................................................................................................46
  Methods.........................................................................................................................................50
  Results...........................................................................................................................................61
  Discussion.....................................................................................................................................68

Chapter 4  Peripheral Blood Mononuclear Cell-Mediated Inflamm-aging and
Immunosenescence of Aged Horses with vs. without Pituitary Pars Intermedia Dysfunction
(PPID)..............................................................................................................................................84
  Introduction...................................................................................................................................85
  Methods.........................................................................................................................................87
  Results...........................................................................................................................................92
  Discussion.....................................................................................................................................93

Chapter 5  Effects of Polyphenols including Curcuminoids, Resveratrol, Quercetin,
Pterostilbene, and Hydroxypterostilbene on Lymphocyte Pro-inflammatory Cytokine
Production of Senior Horses in Vitro............................................................................................101
  Introduction...................................................................................................................................102
  Methods.........................................................................................................................................106
  Results..........................................................................................................................................110
  Discussion....................................................................................................................................115
Chapter 6 Effectiveness of Curcumin in Modulating Chronic Inflammation of the Senior Horse

- Introduction ................................................................. 128
- Methods ................................................................. 133
- Results ................................................................. 142
- Discussion .............................................................. 145

Chapter 7 Conclusions and Future Research Directions ......................................... 164

References ........................................................................ 168

Vita ............................................................................. 186
LIST OF TABLES

Table 2.1. Ambient temperatures and length of daylight by representative month from each season………………………………………………………………………………………………….38

Table 3.1. Nutrient composition (DM basis) of feed and hay……………………………………76

Table 3.2. Comparisons of various inflammatory measures to serum levels of vitamins, minerals, and fatty acids………………………………………………………………………………….77

Table 3.3. Comparisons of various inflammatory measures to complete blood count and chemistry panel measures…………………………………………………………………..79

Table 3.4. Comparisons of measures of PPID to various inflammatory, vitamin, mineral, fatty acid, complete blood count, chemistry panel, and body composition measures………………………………………………………………………………….81

Table 3.5. Comparisons of various inflammatory, vitamin, mineral, fatty acid, complete blood count, chemistry panel, body composition, and PPID measures to age………………………………………………………………………………………..82

Table 3.6. Comparison of deuterium oxide methods of body composition determination with body composition determinations via ultrasound and muscle scoring………………….83

Table 6.1. RT-PCR gene expression of horses supplemented with curcumin or placebo for 90 days………………………………………………………………………………………………….154
LIST OF FIGURES

Figure 2.1. Lymphocyte production of pro-inflammatory cytokine interferon(IFN)-γ after stimulation with PMA/ionomycin for n=8 senior (≥20 years) and n=8 adult (9-12 years) horses for each season……………………………………………………………………………………………………39

Figure 2.2. Lymphocyte production of pro-inflammatory cytokine tumor necrosis factor(TNF)-α after stimulation with PMA/ionomycin for n=8 senior (≥20 years) and n=8 adult (9-12 years) horses for each season……………………………………………………………………………………………………40

Figure 2.3. Lymphocyte proliferation of n=8 senior (≥20 years) and n=8 adult (9-12 years) horses for each season……………………………………………………………………………………………………41

Figure 2.4. Peripheral blood mononuclear cell-mediated gene expression of 8 senior (≥20 years) and 8 adult horses………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………….
Figure 5.3. Effect of compound concentration on cell viability and lymphocyte production of IFN-γ and TNF-α for the common NSAIDs flunixin meglumine (FM) and phenylbutazone (Bute).………………………………………………………………………………124

Figure 5.4. Comparison of the positive control of DMSO/PMA to optimal concentrations for each compound in examining inflammation……………………………………………………………………………125

Figure 5.5. Effect of optimal compound concentrations on % viability of PBMC following incubation with PMA/ionomycin…………………………………………………………………………126

Figure 6.1. Peripheral blood mononuclear cell (PBMC)-mediated production of interferon(IFN)-γ and tumor necrosis factor(TNF)-α from n=15 curcumin and n=15 placebo-receiving senior horses (≥20 years)………………………………………………………………………………156

Figure 6.2. Serum Prostaglandin E2 (PGE2) production in curcumin and placebo supplemented senior horses…………………………………………………………………………………..157

Figure 6.3. PBMC-mediated inflammatory gene expression of curcumin (n=15) and placebo (n=15)-receiving senior horses (≥20 years)……………………………………………………158

Figure 6.4. Thyrotropin releasing hormone (TRH) stimulation testing in senior horses (≥20 years) pre and post 90-day supplementation with curcumin (n=15) or placebo (n=15)…………………………………………………………………………………………159

Figure 6.5. Basal insulin pre vs. post study for curcumin (n=15) vs. placebo-receiving (n=15) horses…………………………………………………………………………………………160

Figure 6.6. Plasma Aβ pre vs. post study for curcumin (n=15) and placebo-receiving (n=15) senior horses (≥ 20 yrs)……………………………………………………………………161

Figure 6.7. Blood chemistry panel parameters with significant (p<0.05) or trending (p<0.10) interactions between time point and treatment group (n=15 curcumin and n=15 placebo-treated horses)………………………………………………………………………………162

Figure 6.8. Body weights and body condition scores (BCS) for senior (≥20 yrs) horses supplemented with curcumin (n=15) or placebo (n=15) for 90 days………………………………………………………………………………163
CHAPTER 1

LITERATURE REVIEW

Senior horses generally refer to those ≥ 20 years old, which similar to the senior human population, is a demographic of increasing importance as numbers continue to rise. Senior horses (≥20 years) comprise approximately 11.4% of the equine population in the United States (USDA, 2016), while those ≥15 years compose approximately 29% of the horses in the United Kingdom (≥15 years) (Ireland et al., 2011a). This USDA estimate of senior horses (≥20 years) comprising 11.4% of the US equine population (USDA, 2016) is increased from the 7.6% estimated only ten years prior (USDA, 2006), indicating the growth of the senior demographic among equids. Furthermore, among equine facilities in the United States, 44.6% possessed one or more senior horse (≥20 years) (USDA, 2016). These senior horses are frequently ridden for pleasure or kept as companion animals, while some continue engaging in athletic competitions and breeding into their late teens and twenties (Ireland et al., 2011a). Causes of mortality among horses aged 20 or older in the United States include: other (26.6%, with old age as the most common specified cause), colic (13.4%), cancer (13.2%), neurologic problems (12.1%), and chronic weight loss (11.7%) (USDA, 2016).

An Overview of Inflamm-aging

The onset of old age is associated with multiple physiological changes, particularly those associated with the immune system. Horses, like humans, exhibit the phenomenon of inflamm-aging (chronic, systemic, low-grade inflammation that is associated with advanced age), in addition to exhibiting immunosenescence (decreased immune response
associated with old age). Initially the occurrence of inflamm-aging appears somewhat paradoxical in the face of the existence of immunosenescence; however, this phenomenon of inflamm-aging has traditionally been thought to occur as an overload response of the immune system being subjected to countless mitogens over the course of a lifetime. (Adams et al., 2008; Franceschi et al., 2000; Vasto et al., 2007). More recently, inflamm-aging has specifically been thought to derive from at least some of the following factors: 1) increased cell debris due to potential accumulation time and/or deficient elimination, 2) senescent cells and their secretory pro-inflammatory cytokines, 3) immunosenescence, which is likely exacerbated by persistent infections, 4) endogenous microbiota of the digestive tract potentially leaking into the surrounding tissues with age, causing chronic inflammation, or a shift toward more pro-inflammatory types of microbiota with age, and 5) increased coagulation with aging (Franceschi and Campisi, 2014).

Given the variety of stimuli thought to trigger inflamm-aging, it is not surprising that quite a few mechanisms may be involved in this phenomenon. The classic inflammatory cascade is regulated by nuclear factor(NF)-κβ and is thought to be the primary pathway by which inflamm-aging is mediated (Salminen et al., 2008; Xia et al., 2016). However, the target of rapamycin (TOR) pathway has also been researched in regards to inflamm-aging, as it regulates longevity through a variety of means (Stanfel et al., 2009) and also is associated with NF-κB activation, suggesting a regulatory role in inflamm-aging (Temiz-Resitoglu et al., 2017; Xia et al., 2016). Other pathways that may be involved in inflamm-aging either due to their effects on aging as a whole or due to their effects specifically on inflammation (whether pro- or anti-inflammatory) include: Retinoic-
acid-inducible gene-I (RIG-I), Notch, Sirtuin, Transforming growth factor(TGF)-β, and Ras pathways (Xia et al., 2016).

Inflamm-aging manifests itself by increased gene expression and protein production of various inflammatory cytokines, both systemically and specifically mediated by peripheral blood mononuclear cells (PBMC) (Adams et al., 2008; Adams et al., 2009; Fagiolo et al., 1993). In humans, old age has been linked to increases in pro-inflammatory interleukin(IL)-1, IL-6, tumor necrosis factor-alpha (TNF-α), C-reactive protein (CRP), and serum amyloid A (SAA) (Franceschi et al., 2000; Ostan et al., 2015). Similarly, in the horse, inflamm-aging has been characterized by increased production of IFN-γ and TNF-α mediated by lymphocytes and monocytes as well as increased serum TNF-α protein levels and increased whole blood gene expression of IL-1β, IL-15, IL-18, and TNF-α (Adams et al., 2008; Adams et al., 2009). More recently, inflamm-aging in the horse has also been characterized by increased PBMC-mediated IFN-γ gene expression (Adams et al., 2015).

In humans, inflamm-aging is associated with morbidity and mortality (Franceschi and Campisi, 2014). Furthermore, inflamm-aging underlies various maladies including Alzheimer’s disease, Parkinson’s disease, macular degeneration, osteoporosis, cardiovascular diseases such as atherosclerosis, and various others (Xia et al., 2016). Chronic inflammation even appears to be associated with cancer and diabetes (Freund et al., 2010; Vasto et al., 2009; Xia et al., 2016), two maladies particularly rampant in the United States currently. Given the association of inflamm-aging with numerous diseases in aged humans, it is anticipated that inflamm-aging has negative implications on equine
health; however, specific clinical conditions associated with inflamm-aging in the horse have not yet been identified.

**Inflamm-aging and Season**

It is unknown whether external conditions, such as season, exacerbate inflamm-aging. Seasonal immune changes in other species have been demonstrated (Bilbo et al., 2002; Bowden et al., 2007; McFarlane et al., 2012). Seasonality in cytokine levels both circulating in serum and mediated by PBMC have been demonstrated in baboons (McFarlane et al., 2012). Particularly, pro-inflammatory serum C-reactive protein and mitogen-stimulated PBMC production of TNF-α and IL-6 have exhibited lowest levels in December, while serum IL-6 levels for baboons housed outdoors were actually highest in December, indicating that different types of cells may be producing cytokines quite differentially at a given time of year (McFarlane et al., 2012). Siberian hamsters subjected to lipopolysaccharide injection also exhibited decreased inflammatory response (IL-6 and IL-1β) in addition to decreased fever and anorexia duration, when exposed to photoperiods reflecting winter in comparison to summer (Bilbo et al., 2002).

In the horse specifically, a few studies have been performed regarding seasonal changes in immune parameters; however, several of these studies primarily examined horses in diseased states known to have seasonal elements. For example, in a study examining summer pasture-associated obstructive pulmonary disease (SPAOPD), PBMC-mediated gene expression of IFN-γ was higher in summer compared to winter in both diseased (p<0.05) and control (p<0.10) horses (Beadle et al., 2002). In a large-scale retrospective study examining lower airway inflammation of racehorses, bronchoalveolar lavage (BAL) revealed influences of season on cell profiles; specifically, lower airway
inflammation was associated with an increase in mast cell percentage, which occurred most commonly in spring (Secombe et al., 2015). Another study examining stable air quality seasonally found that BAL samples from trotting horses in training had elevated levels of IL-6 expression in winter compared to summer, however the horses were stabled all but 4-7 hours of the day during winter, while only around 12 hours during summer which may have also affected the immune system aside from season alone (Riihimaki et al., 2008). Additionally, serum IL-4 has been shown to be elevated in winter compared to late spring in ponies (Wray et al., 2013). Thus, the horse has demonstrated some seasonal variations in immune parameters, but specifically examining inflamm-aging seasonally has not yet been investigated.

Several inflammatory diseases in humans have demonstrated seasonal fluctuations (Araki et al., 2017; Feldthusen et al., 2016; Vinnik et al., 2016). Inflamm-aging itself has not been examined seasonally to determine whether it appears exacerbated at certain times of year. However, given the prevalence of seasonality in inflammatory human diseases, seasonal fluctuations associated with inflamm-aging are likely.

Additionally, although little is known regarding seasonal changes in inflammation in the horse, seasonal rhythms in various hormones are known to occur in horses. In healthy horses, α-melanocyte stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH) both display rather marked seasonal patterns, with levels of both these hormones peaking in fall as horses prepare for winter (McFarlane et al., 2011). These seasonal hormonal patterns appear to derive from both the hypothalamus and the pituitary, as demonstrated by a sheep model in which the neural tissue connecting the two organs was surgically removed (Lincoln and Richardson, 1998). Although ACTH does not have a
clearly defined relationship with either pro- or anti-inflammatory cytokines, α-MSH has demonstrated numerous anti-inflammatory, antioxidant effects (Bhardwaj et al., 1996; Catania et al., 1999; Luger and Brzoska, 2007; Oktar et al., 2004). Specifically, α-MSH increases anti-inflammatory IL-10 production by monocytes (Bhardwaj et al., 1996) and also downregulates LPS-activated NF-κB (Luger et al., 2000). Thus, the seasonal rhythm of anti-inflammatory α-MSH in the horse may influence inflammatory cytokine production over the course of the year.

Inflamm-aging and Pituitary Pars Intermedia Dysfunction (PPID)

ACTH and α-MSH hormones are elevated in horses with the endocrinopathy known as pituitary pars intermedia dysfunction (PPID) or equine Cushing’s disease (McFarlane et al., 2011). PPID is prevalent among aged horses, affecting 15-30% of this demographic, and is characterized by the presence of hypertrichosis (McFarlane et al., 2011). Other clinical signs of PPID include polydipsia, polyuria, hyperhidrosis, muscle atrophy, laminitis, regionalized adiposity, immunosuppression and increased susceptibility to disease, behavioral abnormalities (such as lethargy), reproductive infertility, and neurologic impairment (McFarlane, 2011). PPID is thought to be caused by dopaminergic neurodegeneration of the pituitary pars intermedia, compromising the natural negative feedback loop such that the PPID horse produces elevated levels of pro-opiomelanocortin (POMC)-derived hormones ACTH and α-MSH (McFarlane, 2011). These elevated levels of ACTH and/or α-MSH are likely the mediators of the clinical signs that characterize PPID. Although exact mechanisms are not clear (Hart et al., 2016), these POMC-derived hormones have demonstrated preliminary effects on hair pigmentation in humans (Bohm et al., 2006; Tobin, 2008), and may affect hair growth in the horse, leading to the
characteristic hypertrichosis. As previously discussed, α-MSH also has many anti-inflammatory properties (Bhardwaj et al., 1996; Catania et al., 1999; Luger and Brzoska, 2007; Oktar et al., 2004), which when produced at the elevated levels seen in PPID, may lead to the clinical symptoms of immunosuppression and increased susceptibility to disease characterizing PPID. Specifically, PPID horses have been reported to exhibit increased bacterial infections causing dermatophilosis, pneumonia, and abscesses (McFarlane, 2011). This decreased ability of the PPID horse to respond to pathogens may be exacerbated by the fact that the senior horse is already immune-compromised, exhibiting the age-related phenomena of inflamm-aging and immunosenescence.

A few studies have been performed to determine immune changes associated with PPID. PPID horses have demonstrated decreased neutrophil function, evidence by decreased oxidative burst activity as well as decreased adhesion (McFarlane et al., 2015). In examining oxidative burst activity, a positive association was shown for the ratio of insulin:α-MSH, indicating a potential role for hormones in immune function of the PPID horse (McFarlane et al., 2015). Another study reported that PPID horses exhibit total leukocyte cytokine dysregulation, in which cytokines varied as to whether they reflected age-matched non-PPID (IL-8) or adult non-PPID horses (IFN-γ, IL-6) depending on the cytokine, although the spread of much of the data for each cytokine was quite large (McFarlane and Holbrook, 2008). Still, this differential expression of cytokines in the PPID horse suggests that the aged, PPID horse may exhibit inflamm-aging differently from the non-PPID, aged horse.

Although some research regarding PPID has been performed, studies to better understand the pathogenesis of this endocrine disease, particularly as it relates to
immunosuppression and susceptibility to opportunistic infections, need to be undertaken. Furthermore, inflamm-aging of the senior horse has been established both in whole blood as well as being mediated by PBMC. Thus, it would be of interest to determine if PPID specifically affects PBMC function of the senior horse exhibiting inflamm-aging.

*Inflamm-aging and General Health*

In addition to the effects of PPID on the immune system of the senior horse, little is known regarding the implications of inflamm-aging of the horse in relation to overall health, specifically regarding nutritional status and muscle composition. Given the prevalence of senior horses, with nearly half of the equine operations in the US having at least one senior horse (USDA, 2016), promoting healthspan in addition to lifespan is key. And, given that inflamm-aging appears to underlie most age-associated diseases in humans (Freund et al., 2010; Vasto et al., 2009; Xia et al., 2016), understanding physiological relationships of other health parameters with inflamm-aging will likely help promote better quality of life through improved management of the senior horse.

Loss of muscle mass is associated not only with the occurrence of PPID, as previously mentioned, but is also associated with advanced aging. This old age-associated loss of muscle mass is termed sarcopenia. In humans, sarcopenia occurs at a rate of approximately 6% per decade starting mid-life (Janssen, 2010; Janssen and Ross, 2005). Although longitudinal studies examining rates of sarcopenia have not been performed in the horse, the occurrence of sarcopenia in the horse is a known phenomenon (Lehnhard et al., 2004; Reed et al., 2015). In fact, an owner-reported survey of over 900 horses in the United Kingdom found that 23.9% of horses aged 15 years or older have exhibited loss of muscle tone (Ireland et al., 2011b).
Some studies have found an inverse relationship between inflammation and mass and strength of skeletal muscle. Various human studies have found increased pro-inflammatory cytokines (TNF-α, IL-6, and CRP) associated with decreased muscle mass and strength, including in the elderly (Schaap et al., 2009; Schaap et al., 2006; Visser et al., 2002). Additionally, sarcopenia in cattle appears to be associated with lymphocyte (primarily CD8+) inflammation (Costagliola et al., 2016).

In the horse, specific muscle changes associated with age include: shifts in muscle fiber types (increased percentage of myosin heavy chain types I and IIA) (Li et al., 2016), decreased density of mitochondria (Li et al., 2016), and decreased satellite cell activity in response to exercise (Reed et al., 2015). This decreased satellite cell activity in response to exercise is thought to be associated with the increased inflammatory state of the senior horse experiencing inflamm-aging. It is thought that inflamm-aging may diminish the ability of the senior horse to experience hypertrophy and acute exercise-associated inflammation similarly to their younger counterparts during strenuous exercise (Reed et al., 2015), making the maintenance of physical fitness more difficult in the senior horse. As exercise is key for prevention and treatment of sarcopenia and considering regular exercise in humans and mice tends to be anti-inflammatory, including in regards to inflamm-aging (Lesniewski et al., 2011; Montero-Fernandez and Serra-Rexach, 2013; Sallam and Laher, 2016; Woods et al., 2012), further research should be performed to better understand any connections between sarcopenia and inflamm-aging in the horse in order to better manage the senior horse.

In relation to nutritional status of the senior horse and potential relationships with inflamm-aging, various vitamins, minerals, and fatty acids have demonstrated biological
effects on immunity and specifically inflammation. Vitamin D has well-established anti-inflammatory properties (Abbas, 2016; Capri et al., 2006; Moore et al., 2005). Vitamin C has been shown to downregulate IL-6 in bone marrow mononuclear cells of adults aged 67 ± 6.8 years, another population of cells where inflamm-aging has been demonstrated (Pangrazzi et al., 2017). Vitamin E has also been shown to mitigate inflamm-aging, downregulating the elevated levels of pro-inflammatory prostaglandin E2 (PGE2) in aged mice (Wu et al., 2001). In the aged horse, supplementation with Vitamin E has improved bacterial killing capacity of monocytes and neutrophils (Petersson et al., 2010). The immunity of the horse also appears to be affected by some minerals, such as deficiencies in selenium (Brummer et al., 2013).

Poly-unsaturated fatty acids (PUFA) have been associated with anti-inflammatory activity as well. Independent of age, PUFA, especially n-3 fatty acids, exhibited positive correlations with anti-inflammatory cytokines (IL-10, transforming growth factor[TGF]-β) and negative correlations with pro-inflammatory cytokines (IL-6, TNF-α) in a large-scale human population study primarily focused on the aged population (78.9% ≥65 yrs; age range = 20-98 yrs) (Ferrucci et al., 2006). This suggests that increased levels of dietary PUFA may decrease the degree of inflamm-aging (Ferrucci et al., 2006). Another short-term study found that an infusion of fish oil-based n-3 fatty acids decreased endotoxin-stimulated production of TNF-α, IL-1, IL-6, and IL-8 by monocytes, again indicating an anti-inflammatory capacity of n-3 fatty acids (Mayer et al., 2003). Furthermore, in horses with chronic lower airway inflammatory disease, supplementation with n-3 fatty acids has also demonstrated clinical benefits (Nogradi et al., 2015). Clearly nutrition has demonstrated clinical effects on inflammation and specifically inflamm-aging (Dasilva et
Inflamm-aging and Mitigation by Polyphenols, Particularly Curcumin

In addition to vitamins, minerals, and fatty acids, various plant-derived bioactive polyphenols have anti-inflammatory, antioxidant effects that may benefit the senior horse exhibiting inflam-aging. Some polyphenols of interest include resveratrol, quercetin, pterostilbene, hydroxypterostilbene, and curcumin.

Resveratrol, a phytoalexin and sirtuin-1 activator found in red wine, has demonstrated anti-inflammatory effects both in vitro and in vivo (Kelly, 2010; Knutson and Leeuwenburgh, 2008). Resveratrol mediates anti-inflammatory effects through both the NF-κB and MAPK pathways, decreasing expression of TNF-α and IL-1β while also decreasing activity of myeloperoxidase, a marker of oxidative stress (Zhang et al., 2017). Furthermore, resveratrol has exhibited various anti-inflammatory and anti-oxidant effects in an aged mouse model, including decreasing age-related inflammatory profiles (Gines et al., 2017; Wong et al., 2011), indicating that resveratrol is able to modulate the degree of inflam-aging. In the horse, in vitro work has demonstrated the ability of resveratrol to decrease neutrophil-mediated oxidative stress (Kohnen et al., 2007). Additionally, oral supplementation of horses with resveratrol has reportedly decreased some measures of lameness (Watts et al., 2016) and also increased antioxidant capacity of older horses (Ememe et al., 2015), indicating that resveratrol is biologically active in the horse and may be able to decrease inflam-aging.
Quercetin, a flavonoid commonly found in various fruits, vegetables and tea, has exhibited anti-inflammatory and antioxidant effects both in vitro and in vivo, as well. Quercetin has particularly been successful in mitigating inflammation induced by high-fat and high-fructose diets in rodents (Das et al., 2013; Vazquez Prieto et al., 2015). Quercetin has been shown to exert anti-inflammatory effects by downregulating the NF-κβ and p38 MAPK signaling pathways (Wang et al., 2017). In a model of age-associated macular degeneration, which has an underlying inflammatory component, quercetin was shown to decrease inflammation (IL-6, IL-8) (Hytti et al., 2015), showing potential for the capability of quercetin to decrease age-associated inflammation.

Pterostilbene, an analog of resveratrol found in blueberries, and hydroxypterostilbene, an analog of pterostilbene found in the plant Sphaerophysa salsula, have likewise demonstrated anti-inflammatory and antioxidant effects. Pterostilbene acts as a nuclear factor erythroid 2-related factor-2 (Nrf2) activator, decreasing inflammatory cytokines in a mouse model of diabetes (Sireesh et al., 2017). Pterostilbene as well as resveratrol and quercetin have been shown to decrease pro-inflammatory expression of TNF-α, IL-1β, IL-6, and iNOS, while also decreasing NF-κB activation in macrophage and kidney cell lines, respectively. Hydroxypterostilbene has demonstrated anti-inflammatory activity as well by decreasing pro-inflammatory COX-2 protein levels (Cheng et al., 2014; Takemoto et al., 2015). Hydroxypterostilbene mediated these effects through the PI3K/Akt, MAPK, and mTOR signaling pathways (Cheng et al., 2014).

Curcumin, the primary anti-inflammatory component of the turmeric spice derived from the rhizomes of Curcuma longa (Kumar et al., 2010), is of particular interest regarding anti-inflammatory capacity. In addition to the extensive evidence of its various
anti-inflammatory, anti-oxidant, anti-tumor, and various other health-promoting effects (Franck et al., 2008; Schaaf et al., 2009; Venkatesan and Chandrakasan, 1995), curcumin has specifically demonstrated effects in the horse. Several equine studies in vivo and ex vivo have shown the potential of curcumin as an anti-inflammatory, antioxidant agent (Clutterbuck et al., 2009; Derochette et al., 2013; Franck et al., 2006; Franck et al., 2008; Siard et al., 2016). In a preliminary study, horses supplemented orally with curcumin demonstrated anti-inflammatory effects, showing decreased levels of whole blood IL-1β and IL-1RN expression (Farinacci et al., 2009b). Taken together, curcumin in particular appears a prime candidate to mitigate inflam-aging of the senior horse.

Curcumin is a pleiotropic agent, exerting various biological effects by a vast number of mechanisms, with even anti-inflammatory effects being mediated through quite a variety of different ways (Di Pierro et al., 2013). Primary signaling pathways through which curcumin suppresses inflammation include NF-κB and COX. Specifically, curcumin inhibits IκB kinase (IKK) activity, decreasing phosphorylation of IκBα, thereby sequestering more NF-κB outside of the nucleus and keeping NF-κB from acting as a transcription factor, leading to decreased production of inflammatory cytokines, such as IL-6, IL-12, and TNF-α (Bharti et al., 2003; Nanji et al., 2003). Curcumin modulates the COX pathway, which may actually be a caveat of the NF-κB pathway (Kim et al., 2006), by selectively inhibiting COX-2 activity (Goel et al., 2001), such that it cannot convert arachidonic acid into pro-inflammatory PGE2 (Chizzolini and Brembilla, 2009; Park and Conteas, 2010), thereby suppressing inflammation.

Curcumin has mediated these anti-inflammatory effects extensively in vitro (Derochette et al., 2013; Lin et al., 2014) while also having numerous biological effects in
vivo, including decreased inflammation (Venkatesan and Chandrakasan, 1995). In addition to the previously mentioned small-scale study in horses examining curcumin supplementation on inflammation (Farinacci et al., 2009b), several other studies in vivo and ex vivo have demonstrated the potential of curcumin as an anti-inflammatory, antioxidant agent in the horse (Clutterbuck et al., 2009; Derochette et al., 2013; Franck et al., 2006; Franck et al., 2008; Siard et al., 2016).

Although free curcumin is readily metabolized following oral administration (Kumar et al., 2010) and therefore not biologically active, many formulations have been engineered to enable curcumin to remain bioavailable and bioactive. Some of the most researched formulations that have been shown to increase bioavailability include solid lipid curcumin particle™ technology (Longvida®) (Gota et al., 2010) and phosphatidylcholine curcumin phytosomes (Meriva®) (Cuomo et al., 2011). In older humans, Longvida® administered orally has demonstrated biological effects by improving mood and working memory (Cox et al., 2015) as well as improving vascular endothelial function (Santos-Parker et al., 2017). Additionally, Longivida® has decreased pathology associated with Alzheimer’s disease in an aged mouse model (Ma et al., 2013). Furthermore, Longvida has recently demonstrated anti-inflammatory effects, reducing serum TNF-α and IL-8, as well as decreasing circulating marker of muscle damage creatine kinase following exercise (McFarlin et al., 2016). Oral administration of Meriva® has also resulted in various anti-inflammatory effects, ranging from improvements in inflammatory ocular conditions (Mazzolani and Togni, 2013) to decreased levels of pain, similarly to acetaminophen (Di Pierro et al., 2013). In a study of osteoarthritic patients, all markers of inflammation [interleukin(IL)-1β, IL-6, soluble CD40 ligand, soluble vascular adhesion molecule-1
(sVCAM-1), and erythrocyte sedimentation rate (ESR)] were significantly reduced in the treatment group (n=50) but not the control group (n=50) over the course of the 8 month study (Belcaro et al., 2010a). A previous study from the same group had also shown a decrease in elevated levels of CRP when treated with curcumin phytosomes over the course of 3 months (Belcaro et al., 2010b). Thus, these studies show that curcumin can certainly mediate biological effects, including decreasing inflammation, when administered orally using an enhanced bioavailability formulation.

Given the bioavailability and bioactivity of various curcumin formulations and the fact that polyphenols including curcumin have been shown to decrease PBMC- and specifically lymphocyte-mediated inflammation (Afman et al., 2014; Ford et al., 2016; Literat et al., 2001; Siard et al., 2016), it is therefore likely that curcumin may be able to modulate lymphocyte-mediated inflamm-aging of the senior horse.

Furthermore, inflamm-aging of the senior horse would provide a good model in which to test the anti-inflammatory nature of these phytonutrients, like curcumin, not only for potential benefits to the senior horse experiencing inflamm-aging but also for the many chronic inflammatory conditions common in the horse. Chronic inflammation is frequently treated with nonsteroidal anti-inflammatory drugs (NSAIDs), despite the fact that many NSAIDs have adverse side effects and/or are not indicated for chronic treatment of inflammation (Bessone, 2010; Ingrasciotta et al., 2015; Martinez Aranzales et al., 2015; McConnico et al., 2008; Monreal et al., 2004; Reed et al., 2006). Thus, the potential of polyphenols, such as curcumin, to attenuate inflammation in the senior horse may provide an alternative to the use of NSAIDs in treating chronic inflammation, while also benefiting the health of the senior horse as a whole by decreasing the degree of inflamm-aging.
Hypothesis and Specific Aims

The overall hypothesis of this research is that lymphocyte-mediated inflamm-aging of the senior horse is associated with various factors including season, endocrine function, body composition, and nutritional status, and may be modulated by curcumin, a plant-derived polyphenol.

Specific aims are as follows:

1. To determine the effects of season on lymphocyte-mediated inflamm-aging of the senior horse.
2. To determine the relationship between lymphocyte-mediated inflamm-aging of the senior horse and nutrient status, body composition, hematology/biochemistry, and pituitary pars intermedia dysfunction (PPID).
3. To determine the relationship between lymphocyte-mediated inflamm-aging and the age-associated endocrinopathy PPID in the senior horse.
4. To determine effects of polyphenols curcumin, resveratrol, pterostilbene, hydroxypterostilbene, and quercetin on lymphocyte-mediated inflamm-aging of the senior horse in vitro.
5. To determine the effectiveness of curcumin as an anti-inflammatory supplement to modulate inflamm-aging of the senior horse.
CHAPTER 2

EFFECT OF SEASON ON LYMPHOCYTE-MEDIATED INFLAMM-AGING
AND IMMUNOSENESCENCE IN THE HORSE

Abstract

Senior horses (≥20 years) exhibit changes in the immune system associated with advanced age, similarly to those evidenced in humans. Specifically, senior horses experience inflamm-aging or systemic, chronic, low-grade inflammation and immunosenescence or decreased immune response, particularly evidenced by decreased response to vaccination and decreased lymphocytes proliferation. While age is a factor known to impact immune responses, season has also been shown to influence immune responses of many species including fish, rodents, and primates. Although a few studies examining diseased horses have explored seasonal relationships with immune function, healthy horses have not yet been examined to determine if they display seasonal variations. Furthermore, the senior horse is of interest to gain a better understanding of when these immunosuppressed horses may be more prone to disease.

In this study, peripheral blood mononuclear cells (PBMC) were collected from n=8 senior (mean age = 23.38±1.66 yr) and n=8 adult horses (mean age = 10.75 ± 0.83 yr) monthly for one year. PBMC to be used for lymphocyte proliferation assay using carboxyfluorescein succinimidyl ester (CFSE) staining were stimulated with concanavalin A for 96 hours prior to flow cytometry analysis. PBMC for all other assays were stimulated with phorbol 12-myristate 13-acetate (PMA) for 4 hours. PBMC were then intracellularly stained for inflammatory cytokines interferon(IFN)-γ and tumor necrosis factor(TNF)-α
and analyzed using flow cytometry to determine the percent of lymphocytes producing each cytokine (%) as well as the mean fluorescence intensity (MFI). PBMC were also analyzed seasonally (January, April, July, and October) using RNA isolation, reverse transcription, and real-time polymerase chain reaction (RT-PCR) to determine gene expression of IFN-γ, TNF-α, interleukin(IL)-4, IL-6, and IL-10. Cortisol, adrenocorticotropic hormone (ACTH), and body weights were analyzed seasonally (January, April, July, and October) as well. Statistical analyses were performed using SAS 9.4 software mixed procedures with repeated measures, and significance was set at p<0.05.

Results showed significant differences between senior and adult horses for all flow cytometry measures of inflammation and for lymphocyte proliferation (p<0.05), as expected. Seasonal differences were identified for IFN-γ MFI, %TNF-α, and TNF-α MFI (p<0.05). Gene expression of IL-6 varied seasonally, being significantly decreased in July (p<0.05), while TNF-α gene expression was highest in July compared to January and April (p<0.05). Gene expression of IFN-γ and TNF-α was also significantly greater for senior horses than adult horses (p<0.05). Body weight appeared to have some effects on immune parameters, but these seasonal changes are likely due to a wide and complex variety of mechanisms. In general, inflammation was decreased during winter and elevated in spring and summer. Furthermore, senior horses exhibited overall increased inflammation and decreased lymphocyte proliferation in comparison to adult horses, demonstrating the immune-compromising effects of aging. Thus, the senior horse exhibiting inflam-aging and immunosenescence appears to be affected by seasonal immune changes, which may have management implications to best promote health.
Introduction

In multiple species, old age is associated with various alterations in immune function, which can have negative implications on health. The horse is no exception, experiencing both inflamm-aging (systemic, chronic low-grade inflammation) and immunosenescence (decreased immune response) similar to elderly humans (Adams et al., 2008; Franceschi et al., 2000). Inflamm-aging in the horse has been characterized as increased circulating pro-inflammatory cytokines, whole blood gene expression of inflammatory cytokines, and peripheral blood mononuclear cell (PBMC)-mediated production of inflammatory cytokines (Adams et al., 2008; Adams et al., 2009). Immunosenescence has also been characterized in the horse, encompassing both decreased response to vaccination and decreased lymphocyte proliferation of old horses in relation to young horses (Adams et al., 2008; Goto et al., 1993; Horohov et al., 1999). The occurrence of these age-associated changes in immune function have been well-established; however, the potential effect of season on the aged immune system merits investigation in the horse.

Many species are known to exhibit seasonal changes in various parameters including immune response, body weight, adipose tissue content, voluntary food intake, metabolic rate, heat production, reproductive activity, length and color of hair coat, and hormone levels (McFarlane et al., 2012). Some of the species exhibiting seasonal changes, particularly in immune response, include various types of fish, rodents, and primates (Bilbo et al., 2002; Bowden et al., 2007; Mann et al., 2000; McFarlane et al., 2012). In a baboon study specifically examining seasonal changes in inflammation in the northern hemisphere, serum C-reactive protein (CRP), an acute phase protein, was decreased in December compared to June (p<0.05), and serum interleukin(IL)-6 also showed an effect of season
(p<0.001) (McFarlane et al., 2012). In another study, Siberian hamsters injected with lipopolysaccharide and subjected to short vs. long day length (to mimic winter and summer, respectively) experienced decreased inflammatory cytokine production (IL-6 and IL-1β) as well as decreased duration of fever and anorexia during short day length, indicating that immune response may be diminished during winter (Bilbo et al., 2002).

A few studies have also demonstrated effects of season on immune parameters in the horse (Riihimaki et al., 2008; Secombe et al., 2015; Wray et al., 2013). Peripheral blood mononuclear cells have been examined in equids to determine the effects on seasonal allergy-related changes in cytokine production (Beadle et al., 2002; Hamza et al., 2007). For example, a study examining equine insect bite hypersensitivity in Icelandic horses showed increased PBMC-mediated interferon(IFN)-γ and decreased IL-4 production and mRNA expression in winter compared to summer (Hamza et al., 2007). A study examining summer pasture-associated obstructive pulmonary disease (SPAOPD), another malady thought to have an allergic component, showed seasonal changes in PBMC-mediated IFN-γ expression as well; however, in this study, IFN-γ expression was lower in the winter compared to summer, for both control and diseased horses (Beadle et al., 2002).

The present study was performed to determine seasonal changes in lymphocyte-mediated inflammatory cytokine production of senior horses (which are known to experience inflamm-aging and immunosenescence) compared to adult horses. We hypothesized that season will affect immune function in both senior and adult horses, with senior horses experiencing elevated inflammation and diminished lymphocyte proliferation relative to adult horses overall. Determining seasonal effects on immunity will provide a better understanding of when senior horses, which are already immunocompromised, may
be more prone to disease. Furthermore, determining whether season may be a potential confounding factor may aid in improving experimental design of future studies.

**Methods and Materials**

2.1. *Animals*

Eight senior horses (mean age ± SD = 23.38 ± 1.77 yr) and eight adult horses (mean age ± SD = 10.75 ± 0.89) were housed at the University of Kentucky, Department of Veterinary Science Maine Chance Farm. Horses were maintained on pasture throughout the year, receiving mixed grass hay ad libitum during winter months. The horses also had access to salt and mineral blocks. All horses were free of clinical signs of infectious disease and did not exhibit signs of lameness throughout the study, and all sixteen horses enrolled completed the study. All procedures were in accordance with the University of Kentucky Institute of Animal Care and Use Committee.

Due to the fact that the study duration was a year (April 2013 – March 2014), the diets of horses varied, with some horses receiving concentrates during the study. Specifically, all horses received the same isocaloric diet for the first 2 months of the study (50% oats, 50% alfalfa pellets). However, since the adult horses were gaining weight, for management purposes, the adult horses did not receive feed for the following 10 months of the study. The senior horses continued to receive the 50% oats, 50% alfalfa pellets mixture through September; however amounts changed in order to maintain body weight. The senior horses then received a senior feed October through December, then were returned to the maintenance diet of oats and alfalfa pellets, also receiving an additional balancer pellet in February and March. Although diet could not be accounted for in the model as a covariate because it was confounded with month, analysis was performed on
the 2 months at the beginning of the study when all horses received the same diet, as well as during the 10 months for the adult horses in which they received only forage, and similar results were found for these subsets in relation to analyzing all the data. Thus, it is unlikely that differences in feed afforded a significant role in the results of this study (see Section 2.8 & 3.4).

2.2. Data and Sample Collection

Over the course of one year, the body weights of the horses were recorded monthly using a weight tape to determine any changes. Air temperatures were also collected using the data reported by the UK Ag Weather Center for Spindletop Farm (adjacent to Maine Chance Farm) at 1000 h on the days of blood collections, and hours of daylight were found on the Weather Underground website for Lexington, Kentucky. Heparinized blood was collected monthly in the morning (at approximately 1000 hours) via jugular venipuncture, from which PBMC were then isolated. Gene expression from PBMC was then examined seasonally (January, April, July, October). Serum and EDTA plasma was collected seasonally (January, April, July, October) to measure basal cortisol and adrenocorticotropic hormone (ACTH), respectively.

2.3. PBMC Isolation and Freezing

PBMC were isolated using a Ficoll-Paque Plus™ (Amersham Biosciences, Piscataway, NJ) density gradient and counted using a VICELL™ Counter-XR (Beckman Coulter, Miami, FL), as previously described (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006; Siard et al., 2016). Each month over the course of the year-long study, PBMCs were frozen in liquid nitrogen at a concentration of 2-5x10^7 in freeze media
(50% RPMI 1640 [Gibco, Grand Island, NY], 40% fetal bovine serum [Sigma-Aldrich, St. Louis, MO], and 10% dimethyl sulfoxide [Sigma]).

2.4. Culture Preparation

At the completion of the study, PBMC were thawed by horse for all months (n=12 samples per horse), washed in cRPMI media (RPMI 1640 [Gibco, Grand Island, NY] supplemented with 2.5% fetal equine serum [FES; BioWest, Nuaillé, France], 55 μM 2-mercaptopethanol [Gibco], and 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/mL streptomycin [HyClone Pen/Strep/Glutamine solution; Thermo Scientific]), spun at 300g x 10 minutes, and resuspended in cRPMI. PBMC were then counted using the VICELL to attain desired plating concentrations.

Prior to intracellular staining and RNA isolation, PBMC were plated at a concentration of 4x10⁶ cells/mL in cRPMI media (in duplicate), stimulated with phorbol 12-myristate 13-acetate (PMA; 25 ng/mL; Sigma) and ionomycin (1 μM; Sigma) (single well per sample), and incubated 4 hours at 37°C, 5% CO₂ (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006; Siard et al., 2016). Brefeldin A (10 μg/mL; Sigma) was also added to all wells (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006; Siard et al., 2016).

Prior to proliferation analysis, PBMC were fluorescently tagged with carboxyfluorescein succinimidyl ester (CFSE), as previously described (Adams et al., 2008), with the exception that the reaction was quenched with FES and washed with 10% FES, (instead of FBS). PBMC were then plated (in duplicate) at a concentration of 2x10⁶ cells/mL in cRPMI media, stimulated with concanavalin A (2.5 μg/mL; Sigma) (single well per sample), and incubated 96 hours at 37°C, 5% CO₂ (Adams et al., 2008).
2.5. IFN-γ and TNF-α Intracellular Staining and Flow Cytometry

Prior to intracellular staining, PBMC were transferred to duplicate 96-well V-bottom plates, fixed with 2% paraformaldehyde (Sigma), and incubated at 4°C overnight. Intracellular staining for IFN-γ and TNF-α was performed as has previously been described (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006; Siard et al., 2016). Briefly, PBMC were washed in saponin buffer [PBS supplemented with 1% fetal bovine serum (FBS), 0.1% saponin (Sigma), and 0.1% sodium azide (Sigma)] and intracellularly stained with IFN-γ FITC mouse anti-bovine antibody (AbD Serotec, Raleigh, NC; 0.1 mg; 1:100 dilution) or TNF-α anti-equine monoclonal antibody (HL801; kindly provided by Dr. Rob MacKay, University of Florida; 1:10 dilution) and secondary antibody FITC-conjugated goat F(ab’)2 anti-mouse IgG (H + L) (Invitrogen; 2 mg/mL; 1:1000 dilution) (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006; Siard et al., 2016). PBMC from both IFN-γ and TNF-α plates were resuspended in FACS Flow (Becton Dickinson, Franklin Lakes, NJ) and transferred to 5-mL round-bottom tubes prior to analysis using a FACS Calibur flow cytometer (Becton Dickinson) and Cell Quest® software (Becton Dickinson) (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006; Siard et al., 2016). Lymphocyte populations were gated on cell size and granularity to determine the percent of lymphocytes producing IFN-γ and TNF-α as well as mean fluorescence intensities (MFI) of lymphocytes for each cytokine (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006).

2.6. RNA Isolation, Reverse Transcription, and Polymerase Chain Reaction

Following the incubation in 24-well plates, PBMC for all horses in each season (January, April, July, October) were stored at -80°C in Trizol® solution, until RNA was
isolated by phenol-chloroform extraction (Breathnach et al., 2006) and quantified using an Epoch microplate spectrophotometer (BioTek, Winooski, VT). RNA was reverse transcribed with master mix reagents (16 μL avian myeloblastosis virus [AMV] buffer 5X, 16 μL MgCl₂, 4 μL dNTP, 1 μL RNasin, 1 μL oligo dT primer, and 0.5 μL AMV reverse transcriptase per sample; Promega, Madison, WI) and a thermocycler (Applied Biosystems, Foster City, CA), incubating samples at 42°C for 15 minutes and 95°C for 5 minutes (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). cDNA samples were then stored at -20°C until real time-polymerase chain reaction (RT-PCR) analysis. To ensure accuracy and precision in aliquoting, an epMotion 5070 (Eppendorf) loaded plates with cDNA samples and 5 equine specific intron-spanning primers and probes including IFN-γ, IL-4, IL-6, IL-10, and TNF-α, (Applied Biosystems) in addition to Beta-glucuronidase (β-GUS), the housekeeping gene (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). The 7900HT Fast RT-PCR System (Applied Biosystems) was used to perform RT-PCR, incubating samples at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15s and 60°C for 60s (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). Linear regression was used to determine cycle threshold (CT) values, with relative changes in cytokine gene expression being quantified using the ΔΔCT method (Livak and Schmittgen, 2001). The calibrator for each cytokine was the average ΔCT of all media alone samples. Data are reported as relative quantity (RQ) values according to the equation RQ = 2^-ΔΔCT (Livak and Schmittgen, 2001).

2.7. Endocrine Analysis.

Basal cortisol and basal ACTH were examined for all horses in each season (January, April, July, October). Serum cortisol and EDTA plasma ACTH were analyzed
by Cornell Animal Health Diagnostic Center, Endocrinology Laboratory using chemiluminescence immunoassay (Immulite® 1000; Siemens, Berlin, Germany)(Place et al., 2010).

2.8. **Statistical Analysis.**

Statistical analysis was performed using SAS 9.4 software (SAS Institute Inc., Cary, NC). Flow cytometry data from each month was grouped into seasons and averaged according to the following: winter (December, January, February); spring (March, April, May); summer (June, July, August); fall (September, October, November). Flow cytometry data was analyzed using a mixed procedure with age category (senior or adult) and season as well as the interaction between age category and season as the fixed effects, with season as the repeated variable. Gene expression, hormone, and body weight data was analyzed similarly but using month (January, April, July, and October) instead of seasonal averages.

Additionally, modeling was then performed with cortisol, ACTH, and body weight data to determine whether these variables may be potential influencing factors on the various immune parameters. Data was blocked by age category and analyzed individually with each of the potential influencing factors (cortisol, ACTH, and body weight), month, and the interaction of month and potential influencing factor as the fixed effects when modeling for each of the immune parameters (%IFN-γ, IFN-γ MFI, %TNF-α, TNF-α MFI, lymphocyte proliferation, IFN-γ RQ, TNF-α RQ, IL-4 RQ, IL-6 RQ, IL-10 RQ). Ambient temperature was modeled over time (January, April, July, October) to determine differences between months.

Due to some changes in diet over the course of the year-long study, additional analysis was performed. Data from the first two months (April & May) in which all horses
received the same isocaloric diet was modeled with age category, month, and age x month to determine whether differences between adult and senior horses were similar to the results of the entire dataset when diets were the same. Additionally, adult horses did not receive feed (forage only) the last 10 months of the study, so those horses were analyzed by month to determine if seasonal effects were similar for this subset as the entire dataset.

All data met skewness and kurtosis requirements, (although ACTH required log-transformation). Analyses with p<0.05 were considered statistically significant.

Results

3.1. Inflammatory cytokine production.

Senior horses exhibited elevated inflammation when compared to adult horses, as expected. Specifically, senior horses exhibited a higher percentage of lymphocytes producing IFN-γ (% IFN-γ) (Fig. 2.1A), as well as higher mean fluorescence intensity (MFI) of lymphocytes intracellularly stained for IFN-γ (Fig. 2.1B) (p<0.001). Senior horses also exhibited higher percentage of lymphocytes producing TNF-α (% TNF-α) (Fig. 2.2A), as well as higher TNF-α MFI (Fig. 2.2B) (p<0.001).

Some seasonal changes were observed in lymphocyte-mediated production of IFN-γ. While the percent of lymphocytes producing IFN-γ did not significantly differ seasonally (p>0.05) (Fig. 2.1A), IFN-γ MFI did vary seasonally (p<0.001) (Fig. 2.1B). Specifically, IFN-γ MFI was lower in winter and fall than in spring and summer (p<0.05) (Fig. 2.1B).

Seasonal changes were also observed in lymphocyte-mediated production of TNF-α. The percent of lymphocytes producing TNF-α and TNF-α MFI both significantly varied with season (p<0.01). Specifically, %TNF- α was lower in winter than in spring and summer (p<0.01), and %TNF-α was also lower in fall than spring (Fig. 2.2) (p<0.05).
α MFI was lowest in winter compared to all seasons (p<0.01) (Fig. 2.2B). No significant interactions of season and age category were present for any of the lymphocyte-mediated inflammatory cytokine parameters (p>0.05).

3.2. **Lymphocyte proliferation.**

Lymphocyte proliferation also significantly differed between senior and adult horses (p=0.023), with adult horses having higher proliferation than senior horses (Fig. 2.3). Lymphocyte proliferation did not differ significantly between seasons (p>0.05) (Fig. 2.3). No significant interaction of age category and season was present (p>0.05).

3.3. **Cytokine gene expression.**

Gene expression of various pro- and anti-inflammatory cytokines was examined using PCR to examine any differences seasonally for senior and adult horses. January, April, July, and October were analyzed to determine gene expression of IFN-γ, TNF-α, IL-4, IL-6, and IL-10, as each of these months was considered the middle month for each season (winter, spring, summer, and fall, respectively) (Fig. 2.4). For the cytokines analyzed, significant differences between senior and adult horses were found for IFN-γ and TNF-α (p<0.01) (Fig. 2.4).

A significant overall effect of month was found for IL-6 (p<0.05), while a trend for an effect of month was determined for TNF-α (p=0.055) (Fig. 2.4B&C). Specifically, IL-6 gene expression was lower in July than in all other months analyzed, showing diminished levels in summer (p<0.05). Meanwhile, TNF-α was higher in July than in January or April (p<0.05) but did not significantly differ from October, showing that gene expression of TNF-α is greater in summer than in winter or spring. There were no other significant effects
of month (p>0.05), and there were not significant interactions of age category and month for any of the cytokines analyzed (p>0.05).

3.4. Potential influencing factors.

Serum cortisol, plasma ACTH, body weight, ambient air temperatures, and length of daylight were examined seasonally (in January, April, July, and October) to determine whether they may affect inflammatory and proliferative parameters. Serum cortisol significantly differed between months, with an overall p-value of <0.0001, but did not differ between age groups (Fig. 2.5A). Cortisol was lowest in January and October and highest in April (Fig. 2.5A). Plasma ACTH was statistically significant for differences both between age groups and months (p<0.01) (Fig. 2.5B). Senior horses exhibited elevated plasma ACTH concentrations compared to adult horses (Fig. 2.5B). Overall, ACTH was lowest in January and April and highest in October (Fig. 2.5B). Body weight did not vary by month (p>0.05) and did not differ between senior and adult horses (p>0.05) (Fig. 2.6). Ambient temperatures varied as expected with January (winter) being lowest and July (summer) being highest (compared to all seasons) (p<0.05) (Table 2.1). Likewise, length of daylight followed a similar pattern with January being shortest and July being longest (Table 2.1).

For each of the immune parameters modeling with cortisol, ACTH, and body weight for the months of January, April, July, and October was performed to determine any influence on specific markers of the immune system. In a model incorporating month and month interactions while also blocking for age category, it was found that IFN-γ MFI and ACTH were positively associated (p=0.038). Weight was also individually associated with %IFN-γ (p=0.018; negative), %TNF-α (p=0.003; negative), and lymphocyte
proliferation (p=0.008; positive). No other relationships were found between cortisol, ACTH, or body weight and immune parameters (%IFN-γ, IFN-γ MFI, %TNF-α, TNF-α MFI, lymphocyte proliferation, IFN-γ RQ, TNF-α RQ, IL-4 RQ, IL-6 RQ, IL-10 RQ) (p>0.05) in the aforementioned model.

Although diet was a confounding factor, the adult horses did not receive any feed (only forage) for 10 months and still exhibited various seasonal changes in inflammatory cytokine production. While the forage changed throughout the year, as horses were kept on pasture and fed mixed-grass hay ad libitum during winter months, this is a common management practice of equine facilities and arguably is part of a seasonal effect. In this group of adult horses not receiving feed, seasonal changes were specifically seen for IFN-γ MFI (p<0.0001), %TNF-α (p=0.003), and TNF-α MFI (p<0.001), quite similarly to the results of the complete dataset (Fig. 2.1&2.2). Furthermore, although all horses were only on the same diet for the first two months of the study due to differences in energy needs to keep adult vs. senior horses at maintenance, differences in cytokine production were seen between senior and adult horses. Specifically, differences between senior and adult horses were seen for %IFN-γ (p=0.002), IFN-γ MFI (p=0.051), %TNF-α (p=0.002), and TNF-α MFI (p=0.016), although lymphocyte proliferation did not demonstrate significant effects of age (p=0.283). Additionally, for the full dataset, there were no interactions between age category and month/season. Therefore, seasonal inflammatory changes likely are not due to changes in feed.
Discussion

Many species are known to undergo physiological changes seasonally as an adaptation to varying conditions. These changes in physiology affect everything from hormones to hair coat (McFarlane et al., 2012). Not surprisingly, immune function has also demonstrated seasonal variations in many species as well (Bilbo et al., 2002; Bowden et al., 2007; Mann et al., 2000; McFarlane et al., 2012). Few studies have been conducted in the horse to determine seasonal effects, though some seasonal effects on immune response of allergy-related conditions have been determined (Beadle et al., 2002; Hamza et al., 2007). However, a longitudinal study to determine the effects of season on senior horses in comparison to adult horses has not previously been performed.

Senior horses are known to experience inflamm-aging (chronic, low-grade inflammation occurring systemically during old age) and immunosenescence (aging of the immune system including decreased lymphocyte proliferation and decreased response to vaccination) (Adams et al., 2008; Adams et al., 2009; Adams et al., 2011). The current study determined the effects of season on function of PBMC in these already immune-compromised senior horses in comparison to adult horses.

As hypothesized, season affected lymphocyte-mediated inflammatory cytokine production for multiple parameters examined. IFN-γ MFI, %TNF-α, and TNF-α MFI all exhibited seasonal patterns. Specifically, IFN-γ MFI was lowest in winter and fall and highest in spring and summer. Percent of lymphocytes producing TNF-α was lowest in winter compared to spring and summer, and TNF-α MFI was lowest in winter compared
to all seasons. As a whole, inflammation was diminished in the winter and elevated in the spring and summer.

This general decrease in inflammation during winter is similar to what has been seen in rhesus monkeys and baboons (Mann et al., 2000; McFarlane et al., 2012). Specifically, Mann et al. (2000) showed a decrease in IFN-γ+ PBMC during winter compared to summer (Mann et al., 2000). McFarlane et al. (2012) showed a decrease in pro-inflammatory serum CRP in December compared to June as well as a decrease of inflammatory cytokine production in December following stimulation with both LPS and CpG (deoxynucleotide found in viral, bacterial, fungal and parasitic DNA) (McFarlane et al., 2012). However, the study also showed an increase in serum IL-6 in December compared to June for baboons housed outdoors (McFarlane et al., 2012), showing that various inflammatory cytokines can be expressed differentially from each other dependent on the season.

Senior horses also exhibited elevated levels of inflammatory cytokine protein production relative to adult horses in all measures, as expected. This was likewise seen in the 2 month subset when horses were all receiving the same diet. Inflamm-aging in the horse is a well-characterized phenomenon (Adams et al., 2008; Adams et al., 2009) that was evidenced again in this study.

Senior horses had decreased lymphocyte proliferation compared to adult horses, as expected. Functionally, these results indicate a decreased adaptive response of senior horses to pathogen invasion in comparison to adult horses. Although the two month subset when all horses received the same diet did not exhibit a significant difference between senior and adult horses, previous studies examining lymphocyte proliferation of senior
horses in comparison with adult horses have observed lower levels in senior horses as an aspect of immunosenescence (Adams et al., 2008). It is expected that these differences in lymphocyte proliferation between senior and adult horses would be more pronounced if using a younger population of adult horses for comparison, similarly to previous studies (in which the mean age was 4.5 years as compared to 10.8 years in the current study) (Adams et al., 2008).

Lymphocyte proliferation did not significantly differ with seasonal. The lack of seasonal differences was also seen in the 10 month subset of adult horses maintained on forage. Lymphocyte proliferation has minimally been examined in regards to season, thus the lack of seasonal differences is not entirely surprising. However, a study in rhesus monkeys showed that lymphocyte proliferation was elevated in winter compared to summer (Mann et al., 2000). Conversely, a study in humans had found that lymphocyte proliferation was lower in patients with seasonal affective disorder (SAD) than in healthy patients (Song et al., 2015a). This decreased lymphocyte proliferation in SAD patients was corrected by light therapy, suggesting a potential role of photoperiod in lymphocyte proliferation, although the effect of light therapy was only examined in SAD patients (Song et al., 2015a). In the present study, equine lymphocyte proliferation did not appear to be affected by season.

Gene expression of various cytokines including IFN-γ, TNF-α, IL-4, IL-6, and IL-10 mediated by PBMC was also examined to determine any differences seasonally as well as between senior and adult horses. A significant effect of season was seen for IL-6, with July being decreased relative to the other months examined (January, April, October). Despite the deviation of these results compared to the other parameters in which inflammation was
decreased most in winter, this decrease of IL-6 in summer is similar to the results seen by McFarlane et al., 2012 in baboons (McFarlane et al., 2012), as previously mentioned. However, gene expression of TNF-α was significantly increased in July compared to January and April. Overall differences for senior and adult horses were also seen for gene expression of IFN-γ and TNF-α, which are not surprising, given the differences in these cytokine proteins determine by flow cytometry. Additionally, differences in PBMC-mediated gene expression of IFN-γ between senior and adult horses have been found previously, although differences in TNF-α were not significant (Adams et al., 2015).

No differences were seen seasonally or regarding age category for gene expression of any other cytokines. Initially the lack of similarity in results between anti-inflammatory IL-10 and the pro-inflammatory cytokines (IFN-γ, TNF-α, and IL-6) may appear unexpected. However, despite the fact that IL-10 is regulated in part by NF-κB (Cao et al., 2006), which also regulates many of the pro-inflammatory cytokines including IFN-γ (Sica et al., 1997), TNF-α (Collart et al., 1990), and IL-6 (Son et al., 2008), the IL-10 regulation by NF-κB is primarily mediated by dendritic cells and macrophages (Cao et al., 2006; Saraiva and O'Garra, 2010). In the present study, PBMC gene expression was analyzed, where IL-10 is primarily regulated by the MAF/ERK pathway (in T-cells) (Saraiva and O'Garra, 2010). Also, IL-10 gene expression of PBMC has previously been examined with no differences between senior and adult horses being found (Adams et al., 2015).

Stress hormone levels of cortisol and ACTH were also analyzed to determine any seasonal differences which may be potentially influencing factors on immune parameters. Cortisol exhibited seasonal effects, being highest in April and lowest in January and October. This aligns with the results of Cordero et al. (2012) in which cortisol was highest.
in spring for healthy horses (Cordero et al., 2012), although a study the following year demonstrated no seasonal cortisol changes in ponies (Borer-Weir et al., 2013). ACTH was most elevated in October and lowest in January and April, as expected. ACTH is known to exhibit robust seasonal patterns, becoming quite elevated in the fall (McFarlane et al., 2011). Additionally, ACTH was significantly higher in senior horses, which again was expected, particularly since many senior horses develop pituitary pars intermedia dysfunction (PPID), an endocrine disorder causing hypertrichosis and resulting in excess production of ACTH (McFarlane, 2011). Since basal ACTH is used clinically to diagnose PPID, it was included in the potential influencing factors model in order to determine whether circulating levels of ACTH and potentially the presence of PPID are influencing factors on immune function. Among the various immune parameters, ACTH appeared to significantly affect only IFN-γ MFI, thus ACTH does not appear to have a significant role in regulating immune function in general.

Body weight also appeared to have significant effects on each of the following: %IFN-γ, %TNF-α, and lymphocyte proliferation. Specifically, body weight exhibited a negative relationship with %IFN-γ and %TNF-α, which was unexpected, given that changes in body weight and body fat in the old horse have been positively associated with PBMC-mediated inflammation (Adams et al., 2009). In the present study however the negative relationships were only slight (with every kg of increased body weight resulting in a decrease of %IFN-γ and %TNF-α by 0.025 and 0.063, respectively), and body weights were determined using a weight tape, which may not have been the most accurate measure of changes in weight.

The low temperatures in winter and/or short length of daylight also may contribute to the decreased inflammation in winter. Short day length has been shown to decrease
immune response in Siberian hamsters (Bilbo et al., 2002). However, these are not likely to be primary contributing factors, as robust seasonal rhythms in other species have been found for animals housed indoors at room temperature year-round (McFarlane et al., 2012).

Another potential factor that may affect the seasonal changes in inflammation is the variation in forage the horses consumed throughout the year. Horses were maintained on pasture but were fed mixed-grass hay in the winter months. Although forage was not tested throughout the year, it is thought that perhaps the nonstructural carbohydrate (NSC) content of the hay was lower than what the horses received from the grass pasture, yielding lower levels of inflammation in winter, as horses fed higher levels of NSC were found to have increased serum TNF-α (Suagee et al., 2011).

In conclusion, this study demonstrates seasonal patterns in pro-inflammatory cytokines of both the adult horse and the senior horse exhibiting inflamm-aging and immunosenescence. It appears that body weight may play a role, but the seasonal control of the immune system appears quite complex and likely involves various aspects of the immune-neuro-endocrine system (Weil et al. 2015; Haldar & Ahmad 2010) and may also include forage type. The seasonal variations of inflammatory cytokine production, particularly for the already immune-compromised senior horses, may yield understanding of when horses may be more prone to infectious disease. For example, horses may be at greater risk for disease in spring following the decreased inflammation in winter, as has been shown in rhesus monkeys (Mann et al., 2000). These seasonal variations in inflammatory cytokines may also have management implications, such as not vaccinating during winter, as the immune system appears to be suppressed during those months and would likely not elicit as strong of a protective response. However, more research needs to
be performed to determine what these seasonal inflammatory changes may specifically mean for the health of the senior horse.
Table 2.1. Ambient temperatures and length of daylight by representative month from each season.

<table>
<thead>
<tr>
<th>Season</th>
<th>Ambient Temperature (°C)</th>
<th>Length of Daylight (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>January</td>
<td>-10</td>
<td>10h 09m</td>
</tr>
<tr>
<td>April</td>
<td>17.5</td>
<td>13h 29m</td>
</tr>
<tr>
<td>July</td>
<td>25</td>
<td>14h 21m</td>
</tr>
<tr>
<td>October</td>
<td>4.2</td>
<td>10h 51m</td>
</tr>
</tbody>
</table>
Figure 2.1. Lymphocyte production of pro-inflammatory cytokine interferon(IFN)-γ after stimulation with PMA/ionomycin for n=8 senior (≥20 years) and n=8 adult (9-12 years) horses for each season. Both (A) percent of lymphocytes producing IFN-γ (%IFN-γ) and (B) IFN-γ mean fluorescence intensity (MFI) are shown, with significant differences (p<0.05) between seasons being signified by different lettering (a, b). \( P_{\text{age}} < 0.0001 \) denotes the p-value for overall differences between senior and adult horses for IFN-γ production, while \( P_{\text{season}} = 0.336 \) denotes the overall p-value for differences between seasons.
Figure 2.2. Lymphocyte production of pro-inflammatory cytokine tumor necrosis factor (TNF)-α after stimulation with PMA/ionomycin for n=8 senior (≥20 years) and n=8 adult (9-12 years) horses for each season. Both (A) percent of lymphocytes producing TNF-α and (B) TNF-α mean fluorescence intensity (MFI) are shown, with significant differences (p<0.05) between seasons being signified by different lettering (a, b, c). $P_{\text{age}}$ denotes the p-value for overall differences between senior and adult horses for TNF-α production, while $P_{\text{season}}$ denotes the overall p-value for differences between seasons.
Figure 2.3. Lymphocyte proliferation of n=8 senior (≥20 years) and n=8 adult (9-12 years) horses for each season. Peripheral blood mononuclear cells were stimulated with mitogen concanavalin A and incubated 96 hours to determine lymphocyte proliferation index. $P_{age}$ denotes the p-value for overall differences between senior and adult horses in lymphocyte proliferation index, while $P_{season}$ denotes the overall p-value for differences between seasons.
Figure 2.4. Peripheral blood mononuclear cell-mediated gene expression of 8 senior (≥20 years) and 8 adult horses. Real-time polymerase chain reaction was performed to determine relative quantities (RQ) of (A) interferon(IFN)-γ, (B) tumor necrosis factor(TNF)-α, (C) interleukin(IL)-6, (D) IL-4, and (E) IL-10 throughout the year (January, April, July, and October). Page denotes the p-value for overall differences between senior and adult horses in gene expression, while P_month denotes the overall p-value for differences between months. Significant differences (p<0.05) between months are signified by different lettering (a, b).
Figure 2.5. Potential hormone influences on seasonal inflammatory changes of n=8 senior (≥20 years) and n=8 adult (9-12 years) horses. Serum cortisol (A) and plasma adrenocorticotropic hormone (ACTH) (B) were examined throughout the year (January, April, July, and October) to determine whether these factors may influence any seasonal inflammatory changes. Significant differences (p<0.05) between seasons are denoted by different lettering (a, b, c).  $P_{age}$ denotes the p-value for overall differences between senior and adult horses in gene expression, while $P_{month}$ denotes the overall p-value for differences between months.
Figure 2.6. Body weights of n=8 senior (≥20 years) and n=8 adult (9-12 years) horses seasonally. Body weights were examined throughout the year (January, April, July, and October) to determine whether it may influence any seasonal inflammatory changes. Any significant differences (p<0.05) between seasons are denoted by different lettering. $P_{age}$ denotes the p-value for overall differences between senior and adult horses in gene expression, while $P_{month}$ denotes the overall p-value for differences between months.
CHAPTER 3
RELATIONSHIPS OF INFLAMM-AGING WITH NUTRITIONAL STATUS, BODY COMPOSITION, AGE, AND PITUITARY PARS INTERMEDIA DYSFUNCTION IN A SENIOR HORSE POPULATION.

Abstract

Similar to aged humans, senior horses (≥20 years) exhibit chronic low-grade inflammation systemically, known as inflamm-aging. In addition to inflamm-aging, senior horses present with sarcopenia and often the endocrinopathy pituitary pars intermedia dysfunction (PPID). Despite the concurrence of these phenomena, the relationships inflamm-aging may have with measures of nutritional status, body composition, age, and pituitary function in the horse remain unknown. Thus, an exploratory study of a population of n=42 similarly-managed senior horses was conducted. Serum was collected to determine vitamin, mineral, and fatty acid content. Peripheral blood mononuclear cells were also isolated to determine inflammatory cytokine production of interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) following stimulation with a mitogen, as well as to determine gene expression of interleukin(IL)-1β, IL-6, IL-10, IFN-γ, and TNF-α. Serum IL-6 and C-reactive protein were determined by enzyme-linked immunosorbent assay. Whole blood was collected for hematological and biochemical analysis. Body composition was evaluated via ultrasound and muscle scoring for all 42 horses as well as by deuterium oxide dilution for a subset of n=10 horses. Pituitary function was determined by measuring basal adrenocorticotropic hormone concentrations as well as by thyrotropin releasing hormone stimulation testing (to determine PPID status). Results showed various relationships between inflammatory markers and the other variables measured. Most notably,
docosadienoic acid (C22:2n6c), docosapentaenoic acid (C22:5n3c), and folate were positively associated with numerous inflammatory parameters (P≤0.05). Being positive for PPID was negatively associated with vitamin B12 and positively associated with percentage of eosinophils (P≤0.05). No relationships between inflammation and body composition were found, however age was associated with multiple parameters, particularly with numerous inflammatory cytokines and fatty acids. In summary, inflamm-aging exhibited relationships with various other parameters examined, particularly with certain fatty acids. This exploratory study provides insights into physiological changes associated with inflamm-aging in the senior horse.

Introduction

Senior horses (≥20 years), like aged humans, exhibit chronic low-grade inflammation that occurs systemically; this phenomenon is known as inflamm-aging (Franceschi et al., 2000). In humans, the term inflamm-aging was coined (Franceschi et al., 2000) after discovering that despite the generalized immunodepression previously known to occur with aging, peripheral blood mononuclear cells (PBMC) produced elevated levels of inflammatory cytokines in the elderly when compared to their younger counterparts (Fagiolo et al., 1993). Various other systemic increases in markers of inflammation such as interleukin(IL)-1, IL-6, tumor necrosis factor-alpha (TNF-α), C-reactive protein (CRP), and serum amyloid A (SAA) have also been associated with old age in humans (Franceschi et al., 2000; Ostan et al., 2015). These increases in pro-inflammatory cytokines and other inflammatory markers in turn increase systemic inflammation. Inflamm-aging in the horse is likewise characterized by increased pro-inflammatory cytokine production by monocytes and lymphocytes of old horses when compared to young horses. Specifically,
old horses have increased levels of circulating IL-1β, IL-15, IL-18, and TNF-α in whole blood, as well as increased production of IFN-γ and TNF-α by lymphocytes and monocytes after stimulation with a mitogen (Adams et al., 2008; Adams et al., 2009). A vast body of human literature has shown systemic inflammation to be an underlying condition predisposing people to various diseases including Alzheimer’s disease, atherosclerosis, macular degeneration, and degenerative arthritis (Franceschi and Campisi, 2014). Inflamm-aging, therefore, is considered to be a key predictor of morbidity and mortality (Franceschi and Campisi, 2014); however, what exactly inflamm-aging means for the physiology of the horse remains unknown. Furthermore, the causes of inflamm-aging in both humans and horses remain relatively unknown (Franceschi and Campisi, 2014).

In many species, various vitamins, minerals, and fatty acids have been associated with inflammation, whether pro- or anti-inflammatory. Many studies have shown Vitamin D to be anti-inflammatory in humans and rats (Abbas, 2016; Capri et al., 2006; Moore et al., 2005). A recent human study in China showed folic acid, the synthetic form of folate, to be anti-inflammatory as well, even improving cognition of patients with Alzheimer’s disease (Chen et al., 2016). Mineral supplements including zinc (Zhu et al., 2016) and selenium (Brummer et al., 2013) in piglets and horses, respectively, have also exhibited various effects on immunity. In human patients experiencing a systemic inflammatory response, most micronutrients decreased with the exception of iron, which was shown to increase (Thurnham and Northrop-Clewes, 2016). In a human population study, many polyunsaturated fatty acids (PUFAs) were associated with a lower inflammatory profile, in that a negative correlation was found between PUFAs and pro-inflammatory cytokines, while a positive correlation was found between PUFAs and anti-inflammatory cytokines.
Additionally, omega-3 supplementation in the horse has demonstrated potential immunomodulatory effects at a clinical level (Nogradi et al., 2015). Nutrition has implications for healthspan and life span; however, this relationship requires further elucidation (Dato et al., 2016), particularly in the old horse, where much currently remains unknown (Siciliano, 2002). Furthermore, the numerous associations between inflammation and vitamins, minerals, and fatty acids in various species indicate that nutritional intervention has the potential to alter inflammatory profiles (Dasilva et al., 2016; Dato et al., 2016), which may extend to the senior horse.

In addition to inflamm-aging, old horses experience sarcopenia (age-associated muscle wasting), similarly to other species including humans (Lehnhard et al., 2004; Reed et al., 2015; Schaap et al., 2009; Schaap et al., 2006). In longitudinal human studies, increased inflammatory markers, particularly TNF-α, IL-6, and CRP, were associated with decreased muscle mass and strength (Schaap et al., 2009; Schaap et al., 2006). In a study of over 3000 healthy, aged (70-79 yrs) adults, IL-6 and TNF-α were associated with decreased muscle mass and strength (Visser et al., 2002). In aged cattle, lymphocyte-mediated (primarily CD8+) inflammation was also associated with sarcopenia (Costagliola et al., 2016). In the old horse, decreased muscle mitochondrial density and shifted fiber types (toward a higher percentage of myosin heavy chain types I and IIA) have been associated with aging (Li et al., 2016). Furthermore, a recent study suggests that the pro-inflammatory status associated with inflamm-aging in old horses may decrease exercise-induced satellite cell activity, thereby decreasing the normal process of hypertrophy associated with exercise (Reed et al., 2015). This, in turn, may indicate why maintaining physical fitness in senior horses is more challenging (Reed et al., 2015), a key issue in the
equine industry due to the number of equine athletes competing into their senior years (Malinowski et al., 1997; McKeever, 2016). The relationship between inflamm-aging and sarcopenia therefore requires further study.

While there is little evidence that senior horses have different hematological or biochemical reference ranges than adult horses (Silva and Furr, 2013), it was of interest to determine whether inflammation in the older animal was associated with any particular clinical biomarkers. For example bilirubin, a biomarker of liver function, has variable associations with inflammation in other species, exhibiting anti-inflammatory properties (Moreno-Otero et al., 1994) and even protecting against inflamm-aging in some studies (Zelenka et al., 2016), while exhibiting pro-inflammatory effects in other studies (Qaisiya et al., 2016).

Senior horses also frequently exhibit pituitary pars intermedia dysfunction (PPID), commonly known as equine Cushing’s disease. This endocrinopathy is caused by dopaminergic neurodegeneration of the hypothalamic neurons, causing hypertrophy, hyperplasia, and often adenomas of the pituitary pars intermedia (Durham, 2016; Miller et al., 2008b) and frequently results in hypertrichosis, polydipsia, polyuria, hyperhidrosis, laminitis, muscle atrophy, and abnormal fat distribution (McFarlane, 2011). Endocrinologically this frequently results in increased basal levels of circulating adrenocorticotropin hormone (ACTH), while also resulting in a decreased ability of horses to return to resting levels of ACTH after intravenous injection of thyrotropin releasing hormone (TRH) (Beech et al., 2007). ACTH has been associated with increased gene expression of pro-inflammatory IL-6 in septic foals (Gold et al., 2012); however, the relationship of ACTH with inflammation is not well-characterized. PPID has been
associated with total leukocyte-mediated cytokine dysregulation (McFarlane and Holbrook, 2008). Therefore, it was of interest to examine whether various circulating serum and PBMC-mediated markers of inflammation would be associated with PPID. Further, the exact mechanisms that result in this hypothalamic dopaminergic neurodegeneration found in many old horses remain unknown, and specific markers of inflamm-aging being differentially associated with PPID may yield understanding of underlying mechanisms involved in this pathology.

Thus, an exploratory study to examine the potential relationships between inflammatory parameters and various vitamin, mineral, fatty acid, hematology, biochemistry, body composition, and PPID parameters in senior horses was undertaken. It was hypothesized that inflammm-aging would be inversely associated with markers of muscle mass and with levels of known anti-inflammatory nutrients. It was also hypothesized that specific markers of inflamm-aging would be differentially associated in relation to PPID status and that inflamm-aging would not be associated with hematology or blood biochemistry markers.

**Methods and Materials**

2.1. Animals and Study Design

Forty-two senior horses (mean age = 24.4 ± 3.0 yr [SD]; range = 18-29 yr) were used in this study. Horses were of mixed-breeds and sex (33 mares, 11 geldings) with a mean body condition score (BCS; according to the Henneke scale 1-9) (Henneke et al., 1983) of 5.2 ± 0.8 and body weight (BW) of 531.2 ± 86.9 kg. All horses were free of clinical signs of infectious disease and housed at the University of Kentucky, Department of Veterinary Science Maine Chance Farm in Lexington, Kentucky on pasture during the
winter season. Horses received a diet of 50% oats and 50% vitamin and mineral fortified alfalfa pellets (5.5 kg per day, divided equally into meals fed at 0830 and 1400 h) with mixed grass hay provided ad libitum (Table 3.1). All procedures were approved by the University of Kentucky Institute of Animal Care and Use Committee.

Samples and measurements collected for all (n=42) horses included the following: BW, BCS, muscle mass score, rump ultrasound, and blood samples. A subset of n=10 of these horses also had deuterium oxide assessment of percent body fat and fat free mass (FFM) performed. Horses were weighed (using a portable calibrated large-animal scale) and assessed for BCS using the Henneke scale (1-9) (Henneke et al., 1983) by two experienced assessors. Muscle mass was scored by two assessors using the previously established 1-5 scale (Graham-Thiers and Kronfeld, 2005). Blood was collected within 2 hours after horses had eaten their morning meal. Percent body fat and fat free mass were estimated using ultrasound (Kane, 1987; Lehnhard et al., 2004) and deuterium oxide (Dugdale et al., 2011) methods within one week post blood collection. Furthermore, thyrotropin releasing hormone (TRH) stimulation testing was performed on all horses within 3 hours of their morning meal, with adrenocorticotropic hormone (ACTH) being measured prior to and 10 minutes post intravenous TRH injection, to determine pituitary function, as further described below. Twenty-eight of the 42 horses tested positive for pituitary pars intermedia dysfunction (PPID) according to results from TRH stimulation.

2.2. Blood Sampling

All blood was collected from the jugular vein using an aseptic technique. Heparinized blood was collected to isolate peripheral blood mononuclear cells (PBMCs), which were processed fresh after collection. Serum was collected for analysis of vitamins,
fatty acids, and inflammatory proteins (interleukin [IL]-6, tumor necrosis factor-alpha [TNF-α], and C-reactive protein [CRP]), centrifuged (800g x 10 min x 22°C), and frozen at -20°C until analysis. Serum was also collected to analyze trace mineral content (in royal blue top, serum clot activator [silicone coated] tubes with hemogard closure to enable zinc analysis [Fisher Scientific, Waltham, MA]), centrifuged (800g x 10 min x 22°C), and frozen at -20°C until analysis. EDTA plasma was collected for ACTH analysis, centrifuged (800g x 10 min x 22°C), and frozen at -20°C until analysis. Lithium heparinized whole blood was collected and immediately analyzed for complete blood count with differential and blood chemistry panel analysis.

2.3. PBMC Isolation, IFN-γ and TNF-α Intracellular Staining, and Flow Cytometry

Peripheral blood mononuclear cells were isolated from the collected heparinized blood using a Ficoll-Paque Plus™ (Amersham Biosciences, Piscataway, NJ) density gradient, as has been previously described (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). PBMC were counted using a VICELL™ Counter-XR (Beckman Coulter, Miami, FL) and plated at a concentration of 4x10^6 cells/mL in complete media (RPMI 1640 [Gibco, Grand Island, NY] with 2.5% fetal equine serum [FES; BioWest, Nuaillé, France], 55 μM 2-mercaptoethanol [Gibco], and 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/mL streptomycin [HyClone Pen/Strep/Glutamine solution; Thermo Scientific]) (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). PBMC were aliquoted in duplicate into 24-well plates, with brefeldin A (10 μg/mL; Sigma) added to all wells, and phorbol 12-myristate 13-acetate (PMA; 25 ng/mL; Sigma) and ionomycin (1 μM; Sigma) added to one well per sample. PBMC were incubated 4 hours at 37°C, 5% CO₂, then fixed with 2% paraformaldehyde overnight. To determine pro-inflammatory
cytokine production, PBMC were stained intracellularly with IFN-γ FITC mouse anti-bovine antibody (AbD Serotec, Raleigh, NC; 0.1 mg) and with TNF-α anti-equine monoclonal antibody (HL801; kindly provided by Dr. Rob MacKay, University of Florida) and secondary antibody FITC-conjugated goat F(ab’)2 anti-mouse IgG (H + L) (Invitrogen, Carlsbad, CA; 2 mg/mL) (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). Flow cytometry was performed using a FACS Calibur flow cytometer (Becton Dickinson) and Cell Quest® software (Becton Dickinson) (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006).

2.4. RNA Isolation and Reverse Transcription

Following the incubation in 24-well plates, aliquots (500 μL) of PBMC were centrifuged in 1.5 mL microcentrifuge tubes, resuspended in 1 mL of Trizol® solution, and stored at -80°C, following the manufacturer’s protocol. RNA was isolated by phenol-chloroform extraction and stored at -80°C (Breathnach et al., 2006). Using an Epoch microplate spectrophotometer (BioTek, Winooski, VT), RNA was quantified. Reverse transcription was performed on 1 μg RNA in RNase-free water (41.5 μL total) using Master Mix (16 μL avian myeloblastosis virus [AMV] buffer 5X, 16 μL MgCl2, 4 μL dNTP, 1 μL RNasin, 1 μL oligo dT primer, and 0.5 μL AMV reverse transcriptase per sample; Promega, Madison, WI) and a thermocycler (Bio-Rad, Hercules, CA), with samples incubated at 42°C for 15 minutes and 95°C for 5 minutes (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). Samples of cDNA were stored at -20°C until real time-polymerase chain reaction (RT-PCR) analysis.
2.5. Determination of Cytokine Gene Expression

cDNA samples were thawed at room temperature and loaded into the epMotion 5070 (Eppendorf) with 5 equine specific intron-spanning primers and probes including IFN-γ, IL-1β, IL-6, IL-10, and TNF-α, (Applied Biosystems, Foster City, CA) in addition to Beta-glucuronidase (β-GUS), the housekeeping gene (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). RT-PCR was performed using the 7900HT Fast RT-PCR System (Applied Biosystems), which incubated samples at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15s and 60°C for 60s (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). The reaction volume for each sample was 10 µL of master mix, with 5 µL Sensimix II Probe Kit (Bioline), 0.5 µL assay mix for the gene of interest (primer/probe sets; Applied Biosystems), and 4.5 µL cDNA template (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). Relative changes in cytokine gene expression were quantified using the ΔΔCT method (Livak and Schmittgen, 2001). The average ΔCT of all the wells treated with media alone served as the calibrator for each cytokine. Data are reported as natural logs of relative quantity (RQ) values (RQ = 2 -ΔΔCT) (Livak and Schmittgen, 2001).

2.6. Inflammatory protein ELISAs

Using enzyme-linked immunosorbent assay (ELISA) methods previously described in the horse, serum inflammatory protein concentrations of IL-6 (Burton et al., 2009) and CRP (Lavoie-Lamoureux et al., 2012) were quantified in duplicate. Briefly, IL-6 was measured by coating ELISA plates (Immunoplate Maxisorp, Nalge Nunc Int., Rochester, NY) with a polyclonal goat anti-horse IL-6 antibody (AF1886, R&D Systems, Inc., Minneapolis, MN), blocking plates with phosphate buffered saline (PBS, pH 7.2)
supplemented with 0.5% bovine serum albumin, washing with phosphate buffer (2.5 mmol NaH₂PO₄, 7.5 mmol Na₂HPO₄, 145 mmol NaCl, 0.1% (v/v) Tween 20, pH 7.2), and tagging with biotinylated goat anti-horse IL-6 (AF1886, R&D Systems, Inc., Minneapolis, MN) and a streptavidin–horseradish peroxidase solution (Jackson ImmunoResearch Lab., West Grove, PA) (Burton et al., 2009). A recombinant equine IL-6 (1886-EL, R&D Systems, Inc., Minneapolis, MN) in two-fold dilutions was used to create a standard curve, ranging from 500-4 ng/mL (Burton et al., 2009). Reactions were pigmented and stopped with TMB substrate solution (Thermo Scientific, Rockford, IL) & TMB stop solution (KPL, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), respectively and analyzed in duplicate colorimetrically at 450 nm of absorbance using an ELISA reader (Bio-Rad Laboratories, Inc., Philadelphia, PA). The serum sample dilution was 1:50 or 1:100, with dilution factor taken into account when calculating protein concentrations. The mean intra-assay coefficient of variation was 6.4%.

Serum CRP was quantified using a commercially available equine-specific CRP ELISA kit (Kamiya Biomedical, Seattle, WA), according to manufacturer instructions (Lavoie-Lamoureux et al., 2012), except all serum samples were diluted 1:300 instead of 1:100. The standard curve ranged from 200-6.25 ng/mL. ELISA reader (Bio-Rad) analysis was performed at an absorbance of 450 nm. The mean intra-assay coefficient of variation was 2.5%, while the mean inter-assay coefficient of variation was 0.8%.

2.7. Circulating vitamins and minerals

Serum vitamins and minerals were analyzed at Michigan State University Diagnostic Center for Population and Animal Health (MSU DCPAH), except Vitamin B12 and folate, which were analyzed at Cornell University Animal Health Diagnostic Center.
(AHDC) Endocrinology Laboratory using American Association of Veterinary Laboratory Diagnosticians (AAVLD) validated assays. Serum 25-hydroxyvitamin D was analyzed via radioimmunoassay (RIA) using a commercially available 125 iodine-RIA kit (Dia Sorin, Stillwater, MN). Vitamins A (retinol), E (α-tocopherol), and β-carotene were measured via ultra high pressure liquid chromatography with a C18 column and photodiode array detection (Waters, Milford, MA), following liquid-liquid extraction. Fatty acids, including saturated and unsaturated, were measured via gas chromatography with a SP2556 column and flame ionization detection (PerkinElmer Inc., Waltham, MA), following methyl esterification preparation. Trace minerals including total zinc, selenium, cobalt, copper, iron, manganese, and molybdenum were measured in serum via inductively coupled plasma mass spectrometry (Agilent Technologies, Santa Clara, CA), following direct dilution preparation. Serum vitamin B12 and folate were analyzed via chemiluminescence immunoassay using an Immulite® 2000 (Siemens, Berlin, Germany).

2.8. Muscle measures

Estimated percent body fat, fat weight, and fat free mass (FFM) were determined using ultrasound measurements of rump fat thickness at approximately 11 cm caudal from the tail head and 10 cm off the midline. This measurement of rump fat was then used to calculate the estimated percent body fat according to the equation: % body fat = 5.4*(ultrasound rump fat thickness in cm) + 2.47 (Kane, 1987; Lehnhard et al., 2004). Estimated fat weight and FFM were calculated in turn using the equations: fat weight = body weight*(% body fat); FFM = body weight – fat weight (Lehnhard et al., 2004). Muscle mass scores were also determined by two assessors using the previously published
scale of 1-5 (where 1=lowest; 5=highest), and scores were averaged prior to statistical analysis (Graham-Thiers and Kronfeld, 2005).

Furthermore, percent body fat and fat free mass were determined in ten horses using the deuterium oxide (D₂O) dilution method previously validated in ponies (Dugdale et al., 2011). (The horses assessed were n=5 with high inflammation and n=5 with low inflammation, as determined by %IFN-γ.) Briefly, feed and water were removed from horse pens, and a dose of 0.12 g/kg BW D₂O (Cambridge Isotope Laboratories, Tewksbury, MA) was administered through a temporary catheter in the left jugular vein. (All horses analyzed had a BCS of 4-6, therefore, 0.12*BW could be used for all horses.) Blood samples were collected by venipuncture of the right jugular vein immediately before and 4 hours after D₂O infusion. D₂O was administered into the catheter (16Gx5 ½”; Hospira, Inc.; Lake Forest, IL) with a 0.22 mm filter (Millex®GP Filter Unit, EMD Millipore, Darmstadt, Germany) on the syringe tip to ensure sterility of the procedure, followed by immediate administration of sterile saline (100 mL). Syringes were weighed to determine the exact weight of D₂O administered to each animal. Blood was immediately spun at 2000xg for 10 minutes at 4°C and placed on ice. Plasma was aliquoted with limited air exposure into air-tight, o-ring screw cap vials (Fisher Scientific, Waltham, MA) and immediately placed in freezer. Samples were stored at -80°C until analysis.

Metabolic Solutions (Nashua, NH) performed D₂O content analysis in triplicate using cavity ring-down spectroscopy (CRDS) with a liquid water isotope analyzer automated injection system, version 2 upgrade (Los Gatos Research, Mountain View, CA) as has previously been published (Thorsen et al., 2011). To remove plasma proteins: zinc sulfate monohydrate (5 mg) was added to plasma (25-50 µL), samples were vortexed, and
samples were spun at 6000g for 10 minutes at room temperature (22°C) to precipitate proteins. The supernatant was injected six times, with the last three being averaged to determine values. A standard curve was generated using known values of D₂O. Values were determined as deltas relative to the V-SMOW (Vienna Standard Mean Ocean Water) standard in parts per thousand (ppt). Intra-run variation was <2 delta ppt/mL and inter-run variation is <3.5 delta ppt/mL. D₂O analysis was performed on samples pre and post D₂O administration as well as on D₂O infusate. The calculation of total body water (TBW) adapted from Dugdale et al. (2011) was conducted as follows (Dugdale et al., 2011):

\[
TBW (kg) = \frac{W \times A \times a \times [(\text{delta of dose} - \text{delta of dilution tap water})/(\text{delta of post} - \text{delta of pre})]}{1000/1.04}
\]

Here, \( W = \) water needed to dilute the D₂O infusate dose to enable CRDS measurement (58.9225 mg), \( A = \) amount of D₂O (g) administered to the horse, \( a = \) amount of D₂O infusate dose diluted and measured via CRDS (0.0276 mg), delta of D₂O infusate dose relative to VSMOW (2595.79 ppt/mL), delta of dilution tap water relative to VSMOW (-81.38 ppt/mL), 1000 = necessary to convert g to kg, and 1.04 = factor of overestimation of D₂O method due to deuterium binding to protein and non-exchangeable areas.

Using the adjustment factor of 0.723 adapted by Pace and Rathbun (Pace and Rathbun, 1945), equations were also used to determine percent body fat and fat free mass:

\[
\% \text{ Body fat} = 100 - \left( \frac{\text{TBW}}{\text{BW}} / 0.732 \right)
\]

\[
\text{Fat Free Mass (FFM)} = \frac{\text{TBW}}{0.732}
\]
2.9. Pituitary Function

ACTH levels were determined by Cornell AHDC Endocrinology Laboratory via chemiluminescence immunoassay using an Immulite® 1000 (Siemens, Berlin, Germany), as previously published in the horse (Place et al., 2010).

As a dynamic measure of pituitary function, TRH stimulation was also performed. TRH (Sigma-Aldrich, St. Louis, MO) was stored at -20°C until dissolved in saline. Using sterile techniques in a biochemical hood, TRH was dissolved in 0.9% saline (1 mg/mL), aliquoted into sterile microcentrifuge tubes, and immediately frozen at -80°C (Beech et al., 2007; McFarlane et al., 2006). The morning of TRH testing, the aliquots were thawed at room temperature, and using sterile techniques in a chemical hood 1 mL of TRH solution was drawn into each of the syringes, which were immediately placed on ice. Blood was collected aseptically from the jugular vein in EDTA-containing tubes prior to and 10 minutes post intravenous TRH administration and placed on ice (Diez de Castro et al., 2014). The blood was then centrifuged at 800g for 10 minutes at room temperature and placed back on ice. Plasma was aliquoted and stored at -20°C until shipment on dry ice to Cornell AHDC for ACTH analysis. Horses with ACTH values 10 minutes post TRH injection of >110 pg/mL were considered PPID, as recommended by the Equine Endocrinology Group (Restifo et al., 2016).

2.10. Hematological and Biochemical Analyses

Blood was analyzed by Rood and Riddle Equine Hospital Laboratory, Lexington, Kentucky to determine complete blood count (CBC) with differential and blood chemistry panel. A Beckman Coulter ACT/DIFF hematology machine and Beckman Coulter AU480 serum chemistry analyzer were used to determine CBC and blood chemistry, respectively.
Specifically measured were: hemoglobin (Hgb), packed cell volume (PCV), red blood cells (RBC), white blood cells (WBC), total protein, mature neutrophils (segs), immature neutrophils (bands), lymphocytes (lymph), monocytes (mono), eosinophils (eos), sodium, potassium, chloride, albumin, serum glutamic oxaloacetic transaminase/aspartate aminotransferase (SGOT/AST), alkaline phosphatase, total bilirubin, direct bilirubin, creatine kinase (CK), creatinine, glucose, gamma-glutamyl transferase (GGT), blood urea nitrogen (BUN), phosphorus, calcium, sorbitol dehydrogenase (SDH), and lactate dehydrogenase (LDH).

2.11. Statistical Analysis

Prior to data analysis, WINPEPI DESCRIBE (version 3.07) computer program for epidemiologists (Abramson, 2011) was used to determine outliers. Non-normally distributed data were natural log-transformed to achieve a normal distribution. Outliers at each end of the distribution, defined as values further than five times the median absolute deviation (MAD) from the median were then excluded prior to analysis to achieve normal distribution.

Data were analyzed with SPSS version 24 (IBM Corp, Armonk, NY). Bivariate Pearson correlations were performed among the various parameter values. Partial correlations among the various parameters were also analyzed with age as a covariate. (Breed and sex were not included as covariates, given the population of horses examined were predominantly female [79%] and over half of the horses were Thoroughbreds, with quite a variety of other breeds composing the rest of the population.) The few variables that were not normally distributed following log-transformation (IL-6 ELISA, C22:2n6c, and C22:6n3c) were analyzed using non-parametric
tests: bivariate Spearman correlations and partial Spearman correlations with age as a covariate. For comparisons of deuterium oxide method with ultrasound and muscle scoring to determine body composition, data were analyzed via partial Pearson correlations and with both age and body weight as covariates for n=10 horses that underwent D2O analysis. For all analyses, data with P≤0.05 was considered significant. Results tables depict significant relationships between variables.

Results

3.1. Relationships of Inflammation to Serum Levels of Vitamins, Minerals, and Fatty Acids

Lymphocyte production of IFN-γ and TNF-α were correlated with various serum vitamins, minerals, and fatty acids. Specifically, the percent of lymphocytes producing IFN-γ (% IFN-γ) exhibited a positive correlation with serum folate and selenium and with fatty acids C20:2n6c, C20:4n6c, C22:2n6c, C22:5n3c, and C24:1n9c (P≤0.05), while % IFN-γ exhibited a negative correlation with serum C16, C18:1n9c, and iron (P≤0.05); however, when the model was adjusted for age, only relationships with C22:2n6c and C22:5n3c remained significant (P≤0.05) (Table 3.2). The IFN-γ MFI was positively correlated with folate, selenium, and fatty acids C20:2n6c, C20:4n6c, C22:5n3c, and C24:1n9c (P≤0.05) and was negatively correlated with fatty acid C16 (P≤0.05) (Table 3.2). After adjusting for age, only zinc, C22:2n6c, and C22:5n3c were significant (P≤0.05) (Table 3.2). The percent of lymphocytes producing TNF-α (% TNF-α) exhibited a positive correlation with serum folate, selenium, C20:2n6c, C22:2n6c, C22:5n3c, and C24:1n9c (P≤0.05), and % TNF-α exhibited a negative correlation with C16 and C18:1n9c (P≤0.05). After adjusting for age, only folate, C20:2n6c, C22:2n6c, and C22:5n3c exhibited a significant relationship with % TNF-α (P≤0.05) (Table 3.2). Tumor necrosis factor-α MFI
was positively correlated with serum folate and fatty acid C22:2n6c and negatively correlated with C16 (P≤0.05); after adjusting for age, the relationship of TNF-α MFI with folate and C22:2n6c remained significant (P≤0.05) (Table 3.2).

Peripheral blood mononuclear cell gene expression of various inflammatory cytokines was also correlated with various serum vitamins, minerals, and fatty acids. Gene expression of IFN-γ was positively correlated with serum folate, C20:4n6c, and C22:5n3c (P≤0.05) and was negatively correlated with C16 and C18:1n9c (P≤0.05); after the model was adjusted for age folate, C22:2n6c, and C22:5n3c were significantly associated with IFN-γ RQ (P≤0.05) (Table 3.2). Gene expression of IL-10 (IL-10 RQ) was positively correlated (P≤0.05) with serum folate, selenium, C20:2n6c, C20:4n6c, C22:5n3c, and C24:1n9c and negatively correlated (P≤0.05) with iron, C16, and C18:1n9c (Table 3.2). Folate and C22:5n3c were still positively associated (P≤0.05) with IL-10 RQ after adjusting for age (Table 3.2). Interleukin-1β RQ was negatively correlated (P≤0.05) with zinc (Table 3.2). After adjusting the model for age, zinc remained negatively associated with IL-1β RQ, and fatty acid C16 became positively associated with IL-1β RQ (P≤0.05) (Table 3.2). Interleukin-6 RQ exhibited a positive correlation with fatty acids C18, C18:3n3c, and C22:5n3c and showed a negative correlation with C16. Upon including age in the model, C16:1n7c, C18, C18:3n3c, and C22:5n3c were positively correlated with IL-6, while C18:1n9c exhibited a negative correlation with IL-6 (P≤0.05) (Table 3.2). Tumor necrosis factor-α RQ was positively correlated (P≤0.05) with folate, selenium, C20:2n6c, C22:2n6c, C22:4n6c, and C22:5n3c; upon including age in the model, all of these correlations remained significant except with selenium and C22:4n6c (P>0.05).
Serum inflammatory proteins also correlated with some vitamins, minerals, and fatty acids. Specifically, Serum CRP exhibited a directly proportional relationship (P≤0.05) with copper and an inverse relationship (P≤0.05) with vitamin B12 and selenium; after adjusting for age, copper and vitamin B12 retained significant relationships with CRP (P≤0.05). IL-6 exhibited a directly proportional relationship (P≤0.05) with folate, C16:1n7c, C18:3n6c, C20:1n9c, and C22:3n3c, with all relationships remaining significant (P≤0.05) after adjusting for age, as well as C22:2n6c and C20:3n3c become positively and negatively correlated (P≤0.05) with serum IL-6, respectively.

In summary, C22:2n6c, C22:5n3c, and folate particularly stood out as being positively associated with markers of inflammation after taking age into account as a covariate. Docosadienoic acid (C22:2n6c) exhibited a positive correlation with % IFN-γ, IFN-γ MFI, % TNF-α, TNF-α MFI, IFN-γ RQ, TNF-α RQ, and serum IL-6. Docosapentaenoic acid (DPA; C22:5n3c) was positively correlated with % IFN-γ, IFN-γ MFI, % TNF-α, IFN-γ RQ, IL-10 RQ, IL-6 RQ, and TNF-α RQ. Folate was also positively correlated with % TNF-α, TNF-α MFI, IFN-γ RQ, IL-10 RQ, TNF-α RQ, and serum IL-6. Some other fatty acids were correlated (P≤0.05) with two inflammatory parameters, including: C20:2n6c with % TNF-α and TNF-α RQ (positive correlation) and C16:1n7c with IL-6 RQ and serum IL-6 (positive).

3.2. Relationships of Inflammation to Hematological (Complete Blood Count [CBC]) and Biochemical Parameters

Lymphocyte production of IFN-γ and TNF-α were correlated with various measures of overall health, as determined by CBC and blood chemistry panel measures. Specifically, % IFN-γ was positively correlated (P≤0.05) with direct bilirubin, hemoglobin,
total bilirubin, and WBC counts (Table 3.3). Upon adjusting the model for age, all parameters remained positively associated with % IFN-γ (P≤0.05) (Table 3.3). Interferon-γ MFI exhibited a negative correlation (P≤0.05) with eosinophils; this relationship was retained when adjusted for age, and mature neutrophils also became negatively correlated (P≤0.05) with IFN-γ MFI upon age-adjustment (Table 3.3). Interferon-γ MFI exhibited a positive correlation (P≤0.05) with calcium, lymphocytes, direct bilirubin, and total bilirubin, which was also retained when adjusted for age for all parameters except calcium (Table 3.3). Percent TNF-α was positively correlated with direct bilirubin, total bilirubin, and WBC, while % TNF-α was negatively correlated with eosinophils (P≤0.05) (Table 3.3). After adjusting the model for age, the relationships of % TNF-α with WBC and total bilirubin as well as between % TNF-α and eosinophils remained significant (P≤0.05) (Table 3.3). Tumor necrosis factor-α MFI was negatively correlated (P≤0.05) with sodium, SDH, and SGOT/AST and was positively correlated (P≤0.05) with direct and total bilirubin. Only the relationships of TNF-α MFI with SGOT/AST and total bilirubin remained after adjusting for age (P≤0.05) (Table 3.3).

Peripheral blood mononuclear cell gene expression of inflammatory cytokines was also correlated with various measures examined by CBC and chemistry panel. Interferon-γ RQ was positively correlated (P≤0.05) with WBC, direct bilirubin, and total bilirubin, and was negatively correlated with eosinophils. All relationships except total bilirubin were retained following age adjustment, and the positive correlation between IFN-γ RQ and lymphocytes became significant (P≤0.05) (Table 3.3). Interleukin-10 RQ was positively associated (P≤0.05) with WBC, direct bilirubin, and total bilirubin and was negatively associated with SDH. Following age adjustment, the relationship of IL-10 RQ with
eosinophils became significant in addition to the relationship between IL-10 and WBC remaining significant (P≤0.05). Interleukin-1β RQ was positively associated with mature neutrophils and negatively associated with CK, hemoglobin, and lymphocytes (P≤0.05); following age adjustment, all relationships remained significant, as well as the negative correlation between IL-1β RQ and creatinine becoming significant (P≤0.05). Interleukin-6 RQ was positively correlated (P≤0.05) with WBC and lymphocytes and negatively correlated (P≤0.05) with neutrophils and SDH, with all relationships except WBC (P≤0.05) being retained following age adjustment. Tumor necrosis factor-α RQ was positively associated with direct bilirubin and negatively correlated with LDH, SDH, and SGOT/AST (P≤0.05); only the relationship with SGOT/AST remained significant following age-adjustment (P≤0.05).

Additionally, serum inflammatory proteins exhibited relationships with various aspects of immune health examined via CBC and chemistry panel. Serum CRP was positively correlated with SDH and negatively correlated with BUN and creatinine. After model adjustment for age, relationships with BUN and creatinine remained significant, with the negative correlation between CRP and SGOT/AST also becoming significant (P≤0.05) (Table 3.3). Serum IL-6 was negatively correlated (P≤0.05) with alkaline phosphatase, CK, LDH, and SGOT/AST, and was positively correlated with phosphorus, neutrophils, and total protein (P≤0.05). All relationships were retained (P≤0.05) following model adjustment for age, as well as the negative correlation between serum IL-6 and RBC becoming significant (P≤0.05) (Table 3.3).

In summary, various CBC and chemistry measures were significantly correlated with inflammation following age-adjustment. Specifically, inflammation was positively
correlated with total bilirubin (% IFN-γ, IFN-γ MFI, % TNF-α, TNF-α MFI), WBC (% IFN-γ, % TNF-α, IFN-γ RQ, IL-10), and direct bilirubin (% IFN-γ, IFN-γ MFI, IFN-γ RQ) (P≤0.05). Inflammation was negatively correlated with eosinophils (IFN-γ MFI, % TNF-α, IFN-γ RQ, IL-10), SGOT/AST (TNF-α MFI, CRP, TNF-α RQ, serum IL-6), and CK (IL-1β & serum IL-6) (P≤0.05). Lymphocytes, neutrophils, and hemoglobin all exhibited both negative and positive relationships with inflammatory markers, specifically: lymphocytes (IFN-γ MFI, IFN-γ RQ, IL-6 RQ – positive correlation; IL-1β – negative correlation), neutrophils (IL-1β RQ, serum IL-6 – positive; IFN-γ MFI, IL-6 RQ – negative), and hemoglobin (% IFN-γ -positive; IL-1β RQ-negative).

3.3. Relationships of Inflammation to Body Composition Measures

Relationships between inflammation and body composition were also determined. Serum IL-6 exhibited a directly proportional relationship with body weight both prior to (R=0.321; p=0.038) and following age-adjustment (R=0.551; P≤0.001). Serum IL-6 also appeared to be positively correlated with fat free mass as estimated by ultrasound prior to (R=0.322; p=0.037) and following age-adjustment of the model (R=0.534; P≤0.001); however, upon taking body weight into the model as a covariate in addition to age, the association between IL-6 and FFM disappeared (R=0.011; p=0.947). No other measures of body composition (including average muscle score, BCS, body weight, % body fat via ultrasound, FFM via ultrasound, and for the subset of n=10 horses % body fat via D2O and FFM via D2O) exhibited relationships (P>0.05) with the inflammatory measures examined.
3.4. Relationships of PPID to Inflammation, Vitamins, Minerals, Fatty Acids, Hematological, Biochemical, and Body Composition Measures

The presence or absence of the endocrinopathy PPID was associated with various vitamin, mineral, fatty acid, CBC, chemistry panel, and body composition parameters examined. Pituitary pars intermedia dysfunction status was positively associated (P≤0.05) with basal ACTH and folate and was negatively associated with vitamin B12 (Table 3.4). Adjusting the model for age yielded directly proportional relationships (P≤0.05) between PPID status and basal ACTH, serum CRP, and eosinophils (Table 3.4). After adjusting for age, PPID status was also negatively correlated (P≤0.05) with RBC, creatinine, vitamin B12, and fatty acid C20:4n6c (Table 3.4).

Basal ACTH, a known indicator of pituitary function, also exhibited some relationships with the various parameters examined. Basal ACTH was negatively correlated (P≤0.05) with vitamin B12. When age adjustment was taken into account, basal ACTH exhibited a significant positive correlation with fatty acid C16, while exhibiting a significant negative correlation with direct bilirubin (P≤0.05) (Table 3.4).

3.5. Relationships of Age to Inflammation, Vitamins, Minerals, Fatty Acids, Complete Blood Count, Chemistry Panel, Body Composition, and PPID Measures

Even within this population of senior horses, age was associated with various vitamin, mineral, fatty acid, hematological, biochemical, body composition, and PPID parameters. Among the inflammatory parameters, age was positively correlated with the percent of lymphocytes producing IFN-γ and TNF-α, as well as with gene expression of IFN-γ, IL-10, and TNF-α (P≤0.05) (Table 3.5). Vitamin E, selenium, and fatty acids C18:2n6c, C20:4n6c, and C24:1n9c also exhibited positive correlations with age, while
fatty acids C16, C16:1n7c, C18:1n7c, C18:1n9c, and C20:1n9c exhibited negative correlations with age (P≤0.05) (Table 3.5). Among CBC and chemistry panel parameters, only SDH was inversely proportional to age (P≤0.05) (Table 3.5). Various measures of body composition exhibited negative correlations with age including BCS, body weight, FFM as estimated by ultrasound, and muscle score (P≤0.05) (Table 3.5). And within this group of old horses, both PPID status and basal ACTH were directly proportional to age (P≤0.05) (Table 3.5).

3.6. Comparison of D2O Methods with Ultrasound and Muscle Scoring in Determining Body Composition

When examining D2O methods vs. ultrasound for the subset of 10 horses with age and body weight as covariates, results generally showed poor correlation (P>0.05) (Table 3.6). However, FFM measurements via D2O and muscle score were strongly correlated (R=0.895; p=0.001) (Table 3.6).

Discussion

The occurrence of inflamm-aging in senior horses has been well-established (Adams et al., 2008; Adams et al., 2009). However, what remains to be determined are the implications of inflamm-aging on the overall health of the horse and how markers of inflamm-aging may be related to other parameters associated with nutritional status, health, and wellbeing. It was expected that inflamm-aging would be inversely associated with muscle measures and known anti-inflammatory nutrients, while specific markers of inflammation would be differentially associated with PPID status due to cytokine dysregulation. Thus, an old horse population was examined to determine whether various
vitamin, mineral, fatty acid, hematological, biochemical, body composition, and PPID parameters showed any significant associations with certain inflammatory measures.

In this study docosadienoic acid (C22:2n6c), docosapentaenoic acid (DPA; C22:5n3c), and folate were all positively associated with numerous inflammatory parameters. Docosadienoic acid has exhibited antioxidant activity in previous *in vitro* research, exhibiting some of the highest cyclooxygenase enzyme inhibition among the numerous fatty acids examined (Henry et al., 2002). The results showing that C22:2n6c then is positively associated with inflammation is somewhat surprising, given that oxidative stress and inflammation frequently occur together; however, little research on C22:2n6c has been conducted thus far. Docosapentaenoic acid was also positively associated with various inflammatory markers despite being an omega-3 fatty acid, which are generally considered to be anti-inflammatory. In a study examining metabolic syndrome in obese adolescents, changes in DPA specifically were positively associated with changes in anti-inflammatory adiponectin and were negatively associated with pro-inflammatory leptin and leptin/adiponectin ratio, indicating that DPA tends to be associated with an anti-inflammatory status (Masquio et al., 2016). A recent human study of ulcerative colitis (a type of inflammatory bowel disease), showed an interesting caveat in that serum DPA concentrations were higher in patients with pro-inflammatory ulcerative colitis compared to healthy controls, although the opposite relationship was found at the tissue level (Wiese et al., 2016). Similarly to these studies involving other inflammatory conditions, inflamm-aging appears to be associated with altered fatty acid metabolism. Additionally, in this study, markers of inflammation were positively associated with folate concentrations, although some previous studies have suggested folate to have anti-
inflammatory properties (Chen et al., 2016; Cianciulli et al., 2016), whereas other studies have found no association (Cao et al., 2016). One possible explanation for these perhaps unexpected associations between markers of inflammation and (anti-inflammatory) folate and fatty acid concentrations is that these anti-inflammatory compounds have been released from tissues into the bloodstream in an effort to moderate the elevated systemic inflammation inherent with inflamm-aging, i.e. a compensatory effect.

Some hematological and biochemical parameters were also associated with markers of inflammation in this study. Specifically direct bilirubin, total bilirubin, and WBC were positively associated with various inflammatory parameters, while SGOT/AST, eosinophils, and CK were negatively associated with inflammatory parameters. The positive correlations between bilirubin and inflammation are somewhat expected, as some studies have shown bilirubin to be associated with neuro-inflammation (Liu et al., 2016a; Qaisiya et al., 2016). However in a rodent model examining inflamm-aging specifically, mild hyperbilirubinemia was suggested to be protective against inflamm-aging (Zelenka et al., 2016), and another study found a negative association between serum bilirubin and the number of pro-inflammatory CD8+ T cells (Moreno-Otero et al., 1994). SGOT/AST has also previously been associated with inflammation (Tiwari et al., 2016), with experimentally-induced reductions in inflammation likewise being associated with decreases in SGOT/AST (Seif El-Din et al., 2016), which is in contrast to the negative correlation between SGOT/AST and inflammation found in this study. The relationship of inflammation to liver disease as a whole is complex in that increasing age and inflamm-aging in humans have been associated with an increased prevalence of liver disease; however, in the very elderly (>70 years), the incidence of liver disease is very low.
The complexity of the relationship between liver disease and inflammation may to some degree explain these seemingly contradictory results for bilirubin and SGOT/AST in relation to inflammation. Furthermore, SGOT/AST is not a specific biomarker for liver damage, as it can also reflect muscle (and other soft tissue) damage, which may further explain the inconclusiveness of these results. Creatine kinase (CK), a common biomarker of muscle damage, exhibited an inverse relationship with inflammation as well. This was somewhat surprising, considering myositis and myopathies are generally associated with inflammation. However, studies have also shown decreased levels of CK in association with several inflammatory rheumatic diseases as well as with inflammatory bowel disease tissue (Kitzenberg et al., 2016; Lee et al., 2000; Sanmarti et al., 1996), supporting the results of the current study. Furthermore, CK and SGOT/AST values were greater than the reference range for over half of the horses sampled, suggesting that old horses may exhibit elevated levels of the muscle damage biomarkers, potentially due to sarcopenia. White blood cell counts being positively correlated with inflammation is well-documented. Eosinophils (percentage of WBC count) being negatively correlated with inflammation is not surprising given that elevated levels of eosinophils, which are associated with parasitic infections, elicit a Th2 immune response that could be decreasing the inflammatory response associated with aging. However, the horses had been dewormed within a month prior to the study.

Body composition parameters showed few correlations with the systemic inflammatory markers examined. This was somewhat unexpected, given the association of both inflamm-aging and sarcopenia with aging. And no relationships between inflammation and muscle measurements remained after adjusting for age, and in the case
of FFM estimated by ultrasound, adjusting for body weight as well. This indicates that some other aspect of the aging process may be contributing to sarcopenia aside from systemic inflamm-aging; however, if investigated in a population of both old and young horses, a relationship between inflamm-aging and sarcopenia may become apparent.

Muscle composition as determined by D$_2$O analysis (for n=10 horses) did not exhibit strong correlations when compared to ultrasound methods after modeling with body weight and age as covariates. This may give further reasons for the general lack of relationships between inflammation and body composition parameters discussed previously for the n=42 horses. However, the strong positive correlation between D$_2$O analysis and muscle scoring even after taking body weight and age into account suggests muscle scoring as a valuable method of muscle assessment.

PPID measures, including basal ACTH and PPID status as defined by TRH testing, were also associated with some of the various parameters analyzed in this study. Notably, vitamin B12 exhibited a negative correlation with PPID status regardless of including age as a covariate and also exhibited a negative correlation with basal ACTH. Vitamin B12 is key to proper functioning of the brain and nervous system, with case studies showing improvements in nervous system function after treatment with B12 for those deficient in this vitamin (Kumar, 2004; Shyambabu et al., 2008). A recent human study has shown vitamin B12 to be negatively associated with inflammation (Al-Daghri et al., 2016), similarly to the current study in which vitamin B12 was negatively correlated with CRP. Studies have also found that B12 deficiencies may be associated with Parkinson’s disease (Orozco-Barrios et al., 2009) and with increased risk for white matter hyperintensities (de van der Schueren et al., 2016), a predictor of Alzheimer’s disease (Provenzano et al., 2013).
Human patients with active Cushing’s disease have exhibited decreased levels of vitamin B12, while recovered patients did not, suggesting a role of vitamin B12 in disease state (Faggiano et al., 2005). In the present study, vitamin B12 levels for all horses were within the normal range except for two horses that exceeded normal range; thus the horses did not appear to be deficient in this nutrient. However, the results of the present study showing a negative relationship of PPID (or equine Cushing’s disease) with vitamin B12 support those previously published in the human with Cushing’s disease (Faggiano et al., 2005). Eosinophils were also positively correlated with PPID status, which is not surprising due to the fact that PPID horses exhibit higher levels of IL-8 (McFarlane and Holbrook, 2008), a chemoattractant of eosinophils (Erger and Casale, 1995). Furthermore, PPID horses tend to have higher fecal egg counts than do similarly aged healthy horses, which may also account for the higher percentage of eosinophils in PPID horses (McFarlane et al., 2010).

Age itself was examined in relation to the various parameters to determine where it might have the strongest associations. The modeling for other parameters incorporated age as a covariate due to: 1) the number of relationships it appeared to impact and 2) the fact that inflammation was the primary focus of this study within a group of senior horses, and it was not desired to have the potentially confounding variable of age as a component of inflamm-aging. It is known that the body undergoes various changes when entering senior years, which may have effects on numerous variables. However, to better understand the process of aging in the senior horse, relationships between age and the various parameters were determined. When examining the various inflammatory measures, many parameters were positively correlated with age as expected, based on previous studies regarding inflamm-aging in the horse and other species (Adams et al., 2008; Franceschi et
al., 2007). Numerous fatty acids being negatively correlated with age was also unsurprising, as these results are similar to those found in humans (Ferrucci et al., 2006). The positive correlation between age and both vitamin E and selenium may suggest either a protective effect in which horses with higher levels of vitamin E and selenium tend to live longer, or an inability of senescent cells to uptake these nutrients, thereby leaving them sequestered in circulation. A recent mouse model publication found that selenium deficiency was associated with longevity, despite having decreased healthspan (delayed wound healing as well as earlier onset of age-associated decreased glucose tolerance, decreased insulin sensitivity, and osteoporosis, etc.) (Wu et al., 2016). This demonstrates that the relationship between age and selenium may be rather complex. Numerous body composition parameters including muscle measures were negatively correlated with age as expected, since horses experience sarcopenia. PPID measures (including basal ACTH and PPID status, as defined by TRH testing) were also associated with increased age, which is expected, as PPID is generally an endocrinopathy of the senior horse (McFarlane, 2011), and presumably would become more common with increasing age.

This exploratory study demonstrates the various complex relationships between inflammatory parameters and various vitamin, mineral, fatty acid, hematological, biochemical, body composition, and PPID parameters in senior horses. Further study of the various relationships determined, particularly the relationship of inflammatory markers with fatty acids and folate as well as the relationship of PPID status with vitamin B12 and eosinophils, will aid understanding of the process of aging in the horse, with the goal of promoting longevity and healthspan.
Acknowledgments

The authors would like to thank Dr. David Wagner of Metabolic Solutions, Nashua, NH for his assistance with D₂O analysis, including his invaluable and ready correspondence to ensure the accuracy of the D₂O section in the Methods & Materials. The authors would also like to thank Michigan State University Diagnostic Center for Population and Animal Health, particularly Justin Zyskowski and Cheryl Engfehr, for assistance in understanding methods used in determining vitamin, mineral, and fatty acid analysis. Furthermore, the authors would like to thank Rood & Riddle Equine Hospital laboratory for assistance with CBC and chemistry panel methods.
### Table 3.1. Nutrient composition (DM basis) of feed and hay

<table>
<thead>
<tr>
<th>Component</th>
<th>Oats</th>
<th>Alfalfa Pellets</th>
<th>Grass Hay</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM, %</td>
<td>92</td>
<td>90.8</td>
<td>92.6</td>
</tr>
<tr>
<td>DE, Mcal/kg</td>
<td>3.34</td>
<td>3.01</td>
<td>1.94</td>
</tr>
<tr>
<td>CP, %</td>
<td>12.6</td>
<td>13.6</td>
<td>14.7</td>
</tr>
<tr>
<td>ADF, %</td>
<td>11.1</td>
<td>21.6</td>
<td>43.8</td>
</tr>
<tr>
<td>NDF, %</td>
<td>24.7</td>
<td>37.3</td>
<td>66.1</td>
</tr>
<tr>
<td>Ca, %</td>
<td>0.13</td>
<td>0.82</td>
<td>0.54</td>
</tr>
<tr>
<td>P, %</td>
<td>0.4</td>
<td>0.59</td>
<td>0.42</td>
</tr>
<tr>
<td>Mg, %</td>
<td>0.14</td>
<td>0.33</td>
<td>0.29</td>
</tr>
<tr>
<td>K, %</td>
<td>0.53</td>
<td>1.13</td>
<td>1.46</td>
</tr>
<tr>
<td>Na, %</td>
<td>0.028</td>
<td>0.206</td>
<td>0.078</td>
</tr>
<tr>
<td>Fe, ppm</td>
<td>91</td>
<td>313</td>
<td>265</td>
</tr>
<tr>
<td>Zn, ppm</td>
<td>35</td>
<td>128</td>
<td>36</td>
</tr>
<tr>
<td>Cu, ppm</td>
<td>9</td>
<td>56</td>
<td>11</td>
</tr>
<tr>
<td>Mn, ppm</td>
<td>49</td>
<td>128</td>
<td>115</td>
</tr>
</tbody>
</table>

1Analyzed by Equi-Analytical Laboratories (Ithaca, NY).
Table 3.2. Comparisons of various inflammatory measures to serum levels of vitamins, minerals, and fatty acids

<table>
<thead>
<tr>
<th>Inflammatory Parameter</th>
<th>Comparison Parameter</th>
<th>Pearson correlations</th>
<th>Age-adjusted correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Correlation Coefficient</td>
<td>p-value</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-0.343</td>
<td>0.028</td>
</tr>
<tr>
<td>% IFN-γ</td>
<td>C16:0</td>
<td>0.327</td>
<td>0.048</td>
</tr>
<tr>
<td></td>
<td>C20:2n6c</td>
<td>0.333</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>C20:4n6c</td>
<td>0.380</td>
<td>0.013</td>
</tr>
<tr>
<td></td>
<td>C22:2n6c</td>
<td>0.335</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>C22:5n3c</td>
<td>0.405</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>C24:1n9c</td>
<td>0.364</td>
<td>0.019</td>
</tr>
<tr>
<td></td>
<td>Folate</td>
<td>0.309</td>
<td>0.047</td>
</tr>
<tr>
<td></td>
<td>Iron</td>
<td>-0.351</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>Selenium</td>
<td>0.386</td>
<td>0.013</td>
</tr>
<tr>
<td>IFN-γ MFI</td>
<td>C16:0</td>
<td>0.361</td>
<td>0.020</td>
</tr>
<tr>
<td></td>
<td>C20:2n6c</td>
<td>0.320</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td>C20:4n6c</td>
<td>0.345</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>C22:2n6c</td>
<td>0.286</td>
<td>0.066</td>
</tr>
<tr>
<td></td>
<td>C22:5n3c</td>
<td>0.405</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>C24:1n9c</td>
<td>0.338</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>Folate</td>
<td>0.347</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td>Selenium</td>
<td>0.362</td>
<td>0.020</td>
</tr>
<tr>
<td></td>
<td>Zinc</td>
<td>0.244</td>
<td>0.135</td>
</tr>
<tr>
<td>% TNF-α</td>
<td>C16:0</td>
<td>-0.432</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>C18:1n9c</td>
<td>-0.390</td>
<td>0.017</td>
</tr>
<tr>
<td></td>
<td>C20:2n6c</td>
<td>0.368</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td>C22:2n6c</td>
<td>0.362</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>C22:5n3c</td>
<td>0.458</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>C24:1n9c</td>
<td>0.342</td>
<td>0.028</td>
</tr>
<tr>
<td></td>
<td>Folate</td>
<td>0.398</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>Selenium</td>
<td>0.339</td>
<td>0.030</td>
</tr>
<tr>
<td>TNF-α MFI</td>
<td>C16:0</td>
<td>-0.330</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>C22:2n6c</td>
<td>0.366</td>
<td>0.019</td>
</tr>
<tr>
<td></td>
<td>Folate</td>
<td>0.410</td>
<td>0.008</td>
</tr>
<tr>
<td>Ln(IFN-γ RQ)</td>
<td>C16:0</td>
<td>-0.377</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>C18:1n9c</td>
<td>-0.381</td>
<td>0.020</td>
</tr>
<tr>
<td></td>
<td>C20:4n6c</td>
<td>0.313</td>
<td>0.044</td>
</tr>
<tr>
<td></td>
<td>C22:2n6c</td>
<td>0.257</td>
<td>0.101</td>
</tr>
<tr>
<td></td>
<td>C22:5n3c</td>
<td>0.455</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>Folate</td>
<td>0.404</td>
<td>0.008</td>
</tr>
</tbody>
</table>
Table 3.2. (cont.) Comparisons of various inflammatory measures to serum levels of vitamins, minerals, and fatty acids\(^{1,2}\)

<table>
<thead>
<tr>
<th>Inflammatory Parameter</th>
<th>Comparison Parameter</th>
<th>Pearson correlations</th>
<th>Age-adjusted correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Correlation Coefficient</td>
<td>p-value</td>
</tr>
<tr>
<td>Ln(IL-10 RQ)</td>
<td>C16:0</td>
<td>-0.312</td>
<td>0.047(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>C18:1n9c</td>
<td>-0.386</td>
<td>0.018(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>C20:2n6c</td>
<td>0.339</td>
<td>0.028(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>C20:4n6c</td>
<td>0.322</td>
<td>0.038(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>C22:5n3c</td>
<td>0.417</td>
<td>0.007(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>C24:1n9c</td>
<td>0.320</td>
<td>0.041(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>Folate</td>
<td>0.460</td>
<td>0.002(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>Iron</td>
<td>-0.305</td>
<td>0.050(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>Selenium</td>
<td>0.365</td>
<td>0.019(^\dagger)</td>
</tr>
<tr>
<td>Ln(IL-6 RQ)</td>
<td>C16:0</td>
<td>0.258</td>
<td>0.118</td>
</tr>
<tr>
<td></td>
<td>Zinc</td>
<td>-0.354</td>
<td>0.031(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>C16:0</td>
<td>-0.311</td>
<td>0.048(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>C16:1n7c</td>
<td>0.241</td>
<td>0.150</td>
</tr>
<tr>
<td></td>
<td>C18:0</td>
<td>0.364</td>
<td>0.018(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>C18:1n9c</td>
<td>-0.484</td>
<td>0.002(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>C18:3n3c</td>
<td>0.300</td>
<td>0.060</td>
</tr>
<tr>
<td></td>
<td>C22:5n3c</td>
<td>0.455</td>
<td>0.003(^\dagger)</td>
</tr>
<tr>
<td>Ln(TNF-α RQ)</td>
<td>C20:2n6c</td>
<td>0.407</td>
<td>0.008(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>C22:2n6c</td>
<td>0.468</td>
<td>0.002(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>C22:4n6c</td>
<td>0.335</td>
<td>0.032(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>C22:5n3c</td>
<td>0.370</td>
<td>0.017(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>Folate</td>
<td>0.401</td>
<td>0.008(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>Selenium</td>
<td>0.356</td>
<td>0.023(^\dagger)</td>
</tr>
<tr>
<td>Ln(CRP ELISA)</td>
<td>Copper</td>
<td>0.417</td>
<td>0.006(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>Selenium</td>
<td>-0.338</td>
<td>0.031(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>Vitamin B12</td>
<td>-0.321</td>
<td>0.038(^\dagger)</td>
</tr>
<tr>
<td>Ln(IL-6 ELISA)</td>
<td>C16:1n7c</td>
<td>0.342</td>
<td>0.038(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>C18:3n6c</td>
<td>0.339</td>
<td>0.028(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>C20:1n9c</td>
<td>0.361</td>
<td>0.020(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>C20:3n3c</td>
<td>-0.222</td>
<td>0.164</td>
</tr>
<tr>
<td></td>
<td>C22:2n6c</td>
<td>0.289</td>
<td>0.063</td>
</tr>
<tr>
<td></td>
<td>C22:3n3c</td>
<td>0.329</td>
<td>0.036(^\dagger)</td>
</tr>
<tr>
<td></td>
<td>Folate</td>
<td>0.351</td>
<td>0.023(^\dagger)</td>
</tr>
</tbody>
</table>

1A cross (\(^\dagger\)) is used to denoted P≤0.05 for Pearson correlations. An asterisk (*) is used to denote P≤0.05 for age-adjusted models.

2Spearman correlations and age-adjusted non-parametric analysis were performed for C22:2n6c and IL-6 ELISA due to non-normal distribution.

3IFN-γ = interferon-γ; % gated = percent of lymphocytes producing the cytokine; MFI = mean fluorescence intensity; TNF-α = tumor necrosis factor-α; IL = interleukin; CRP = C-reactive protein; ELISA = enzyme-linked immunosorbent assay

78
Table 3.3. Comparisons of various inflammatory measures to complete blood count and chemistry panel measures\textsuperscript{1-3}

<table>
<thead>
<tr>
<th>Inflammatory Parameter</th>
<th>Comparison Parameter</th>
<th>Pearson correlations</th>
<th>Age-adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Correlation Coefficient</td>
<td>p-value</td>
</tr>
<tr>
<td>% IFN-γ</td>
<td>D. Bili</td>
<td>0.413</td>
<td>0.006(^†)</td>
</tr>
<tr>
<td></td>
<td>Hgb</td>
<td>0.350</td>
<td>0.025(^†)</td>
</tr>
<tr>
<td></td>
<td>T. Bili</td>
<td>0.416</td>
<td>0.007(^†)</td>
</tr>
<tr>
<td></td>
<td>WBC</td>
<td>0.470</td>
<td>0.002(^†)</td>
</tr>
<tr>
<td>IFN-γ MFI</td>
<td>Calcium</td>
<td>0.312</td>
<td>0.050(^†)</td>
</tr>
<tr>
<td></td>
<td>D. Bili</td>
<td>0.378</td>
<td>0.014(^†)</td>
</tr>
<tr>
<td></td>
<td>Eosinophils</td>
<td>-0.419</td>
<td>0.006(^†)</td>
</tr>
<tr>
<td></td>
<td>Lymphocytes</td>
<td>0.377</td>
<td>0.015(^†)</td>
</tr>
<tr>
<td></td>
<td>Seg</td>
<td>-0.293</td>
<td>0.063</td>
</tr>
<tr>
<td></td>
<td>T. Bili</td>
<td>0.408</td>
<td>0.008(^†)</td>
</tr>
<tr>
<td>% TNF-α</td>
<td>D. Bili</td>
<td>0.372</td>
<td>0.015(^†)</td>
</tr>
<tr>
<td></td>
<td>Eosinophils</td>
<td>-0.329</td>
<td>0.033(^†)</td>
</tr>
<tr>
<td></td>
<td>T. Bili</td>
<td>0.424</td>
<td>0.006(^†)</td>
</tr>
<tr>
<td></td>
<td>WBC</td>
<td>0.404</td>
<td>0.008(^†)</td>
</tr>
<tr>
<td>TNF-α MFI</td>
<td>D. Bili</td>
<td>0.358</td>
<td>0.022(^†)</td>
</tr>
<tr>
<td></td>
<td>Sodium</td>
<td>-0.319</td>
<td>0.042(^†)</td>
</tr>
<tr>
<td></td>
<td>SDH</td>
<td>-0.374</td>
<td>0.025(^†)</td>
</tr>
<tr>
<td></td>
<td>SGOT/AST</td>
<td>-0.345</td>
<td>0.027(^†)</td>
</tr>
<tr>
<td></td>
<td>T. Bili</td>
<td>0.423</td>
<td>0.007(^†)</td>
</tr>
<tr>
<td>Ln(IFN-γ RQ)</td>
<td>D. Bili</td>
<td>0.404</td>
<td>0.008(^†)</td>
</tr>
<tr>
<td></td>
<td>Eosinophils</td>
<td>-0.322</td>
<td>0.038(^†)</td>
</tr>
<tr>
<td></td>
<td>Lymphocytes</td>
<td>0.288</td>
<td>0.068</td>
</tr>
<tr>
<td></td>
<td>T. Bili</td>
<td>0.365</td>
<td>0.019(^†)</td>
</tr>
<tr>
<td></td>
<td>WBC</td>
<td>0.452</td>
<td>0.003(^†)</td>
</tr>
<tr>
<td>Ln(IL-10 RQ)</td>
<td>D. Bili</td>
<td>0.368</td>
<td>0.017(^†)</td>
</tr>
<tr>
<td></td>
<td>Eosinophils</td>
<td>-0.299</td>
<td>0.054</td>
</tr>
<tr>
<td></td>
<td>SDH</td>
<td>-0.325</td>
<td>0.050(^†)</td>
</tr>
<tr>
<td></td>
<td>T. Bili</td>
<td>0.330</td>
<td>0.035(^†)</td>
</tr>
<tr>
<td></td>
<td>WBC</td>
<td>0.389</td>
<td>0.011(^†)</td>
</tr>
<tr>
<td>Ln(IL-1β RQ)</td>
<td>CK</td>
<td>-0.362</td>
<td>0.028(^†)</td>
</tr>
<tr>
<td></td>
<td>Creatinine</td>
<td>-0.289</td>
<td>0.074</td>
</tr>
<tr>
<td></td>
<td>Hgb</td>
<td>-0.397</td>
<td>0.014(^†)</td>
</tr>
<tr>
<td></td>
<td>Lymphocytes</td>
<td>-0.448</td>
<td>0.004(^†)</td>
</tr>
<tr>
<td></td>
<td>Seg</td>
<td>0.431</td>
<td>0.006(^†)</td>
</tr>
<tr>
<td>Ln(IL-6 RQ)</td>
<td>Lymphocytes</td>
<td>0.384</td>
<td>0.013(^†)</td>
</tr>
<tr>
<td></td>
<td>SDH</td>
<td>-0.401</td>
<td>0.014(^†)</td>
</tr>
<tr>
<td></td>
<td>Seg</td>
<td>-0.350</td>
<td>0.025(^†)</td>
</tr>
<tr>
<td></td>
<td>WBC</td>
<td>0.321</td>
<td>0.038(^†)</td>
</tr>
</tbody>
</table>
Table 3.3. (cont.) Comparisons of various inflammatory measures to serum levels of vitamins, minerals, and fatty acids\(^1,2\)

<table>
<thead>
<tr>
<th>Inflammatory Parameter</th>
<th>Comparison Parameter</th>
<th>Pearson correlations</th>
<th>Age-adjusted correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Correlation Coefficient</td>
<td>p-value</td>
</tr>
<tr>
<td>Ln(TNF-α RQ)</td>
<td>D. Bili</td>
<td>0.317</td>
<td>0.041†</td>
</tr>
<tr>
<td></td>
<td>LDH</td>
<td>-0.363</td>
<td>0.018†</td>
</tr>
<tr>
<td></td>
<td>SDH</td>
<td>-0.387</td>
<td>0.018†</td>
</tr>
<tr>
<td></td>
<td>SGOT/AST</td>
<td>-0.468</td>
<td>0.002†</td>
</tr>
<tr>
<td>Ln(CRP ELISA)</td>
<td>BUN</td>
<td>-0.447</td>
<td>0.003†</td>
</tr>
<tr>
<td></td>
<td>Creatinine</td>
<td>-0.505</td>
<td>0.001†</td>
</tr>
<tr>
<td></td>
<td>SDH</td>
<td>0.331</td>
<td>0.045†</td>
</tr>
<tr>
<td></td>
<td>SGOT/AST</td>
<td>-0.285</td>
<td>0.067</td>
</tr>
<tr>
<td>Ln(IL-6 ELISA)</td>
<td>Alk. Phosphatase</td>
<td>-0.433</td>
<td>0.005†</td>
</tr>
<tr>
<td></td>
<td>CK</td>
<td>-0.417</td>
<td>0.008†</td>
</tr>
<tr>
<td></td>
<td>LDH</td>
<td>-0.426</td>
<td>0.005†</td>
</tr>
<tr>
<td></td>
<td>Phosphorus</td>
<td>0.311</td>
<td>0.045†</td>
</tr>
<tr>
<td></td>
<td>RBC</td>
<td>-0.296</td>
<td>0.057</td>
</tr>
<tr>
<td></td>
<td>Seg</td>
<td>0.378</td>
<td>0.015†</td>
</tr>
<tr>
<td></td>
<td>SGOT/AST</td>
<td>-0.514</td>
<td>0.001†</td>
</tr>
<tr>
<td></td>
<td>Total Protein</td>
<td>0.401</td>
<td>0.008†</td>
</tr>
</tbody>
</table>

\(^1\)Complete blood count analysis by Rood and Riddle Equine Hospital (Lexington, KY).

\(^2\) A cross (†) is used to denote P≤0.05 for Pearson correlations. An asterisk (*) is used to denote P≤0.05 for age-adjusted models.

\(^3\) Spearman correlations and age-adjusted non-parametric analysis were performed for IL-6 ELISA due to non-normal distribution.

\(^4\)IFN-γ = interferon-γ; % = percent of lymphocytes producing the cytokine; MFI = mean fluorescence intensity; TNF-α = tumor necrosis factor-α; IL = interleukin; CRP = C-reactive protein; ELISA = enzyme-linked immunosorbent assay; D. Bili = direct bilirubin; Hgb = hemoglobin; T. Bili = total bilirubin; WBC = white blood cells; Seg = mature neutrophils; SDH = sorbitol dehydrogenase; CK = creatine kinase; LDH = lactate dehydrogenase; SGOT/AST = serum glutamic oxaloacetic transaminase/aspartate aminotransferase; BUN = blood urea nitrogen; Alk. Phosphatase = alkaline phosphatase; RBC = red blood cells.
Table 3.4. Comparisons of measures of PPID to various inflammatory, vitamin, mineral, fatty acid, complete blood count, chemistry panel, and body composition measures1-3

<table>
<thead>
<tr>
<th>PPID Parameter</th>
<th>Comparison Parameter</th>
<th>Pearson correlations</th>
<th>Age-adjusted Correlation</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Correlation Coefficient</td>
<td>p-value</td>
<td>Correlation Coefficient</td>
</tr>
<tr>
<td>PPID Status</td>
<td>RBC</td>
<td>-0.205</td>
<td>0.193</td>
<td>-0.311</td>
</tr>
<tr>
<td></td>
<td>Eosinophils</td>
<td>0.269</td>
<td>0.084</td>
<td>0.311</td>
</tr>
<tr>
<td></td>
<td>Creatinine</td>
<td>-0.228</td>
<td>0.146</td>
<td>-0.383</td>
</tr>
<tr>
<td></td>
<td>ln(CRP ELISA)</td>
<td>0.185</td>
<td>0.240</td>
<td>0.368</td>
</tr>
<tr>
<td></td>
<td>Vitamin B12</td>
<td>-0.547</td>
<td>&lt;0.001†</td>
<td>-0.556</td>
</tr>
<tr>
<td></td>
<td>Folate</td>
<td>0.307</td>
<td>0.048‡</td>
<td>0.201</td>
</tr>
<tr>
<td></td>
<td>C20:4n6c</td>
<td>-0.078</td>
<td>0.621</td>
<td>-0.308</td>
</tr>
<tr>
<td></td>
<td>ln(Basal ACTH)</td>
<td>0.595</td>
<td>&lt;0.001†</td>
<td>0.482</td>
</tr>
<tr>
<td>Ln(Basal ACTH)</td>
<td>C16:0</td>
<td>0.113</td>
<td>0.486</td>
<td>0.527</td>
</tr>
<tr>
<td></td>
<td>D. Bili</td>
<td>-0.165</td>
<td>0.304</td>
<td>-0.331</td>
</tr>
<tr>
<td></td>
<td>Vitamin B12</td>
<td>-0.333</td>
<td>0.033‡</td>
<td>-0.300</td>
</tr>
</tbody>
</table>

1Complete blood count analysis by Rood and Riddle Equine Hospital (Lexington, KY).
2Pituitary pars intermedia dysfunction (PPID) status is determined by thyrotropin releasing hormone stimulation, with adrenocorticotropic hormone (ACTH) ≥110 pg/mL being considered PPID and ACTH<110 pg/mL considered non-PPID.
3A cross (†) is used to denote P≤0.05 for Pearson correlations. An asterisk (*) is used to denote P≤0.05 for age-adjusted models.
4PPID = Pituitary par intermedia dysfunction; ACTH = adrenocorticotropic hormone; RBC = red blood cells; CRP = C-reactive protein; ELISA = enzyme-linked immunosorbent assay; D. Bili = direct bilirubin
Table 3.5. Comparisons of various inflammatory, vitamin, mineral, fatty acid, complete blood count, chemistry panel, body composition, and PPID measures to age

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pearson correlations with Age</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>% IFN-γ</td>
<td>0.382</td>
<td>0.013*</td>
</tr>
<tr>
<td>% TNF-α</td>
<td>0.436</td>
<td>0.004*</td>
</tr>
<tr>
<td>Ln(IFN-γ RQ)</td>
<td>0.423</td>
<td>0.005*</td>
</tr>
<tr>
<td>Ln(IL-10 RQ)</td>
<td>0.434</td>
<td>0.004*</td>
</tr>
<tr>
<td>Ln(TNF-α RQ)</td>
<td>0.330</td>
<td>0.033*</td>
</tr>
<tr>
<td>C16:0</td>
<td>-0.529</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>C16:1n7c</td>
<td>-0.332</td>
<td>0.044*</td>
</tr>
<tr>
<td>C18:1n7c</td>
<td>-0.410</td>
<td>0.008*</td>
</tr>
<tr>
<td>C18:1n9c</td>
<td>-0.501</td>
<td>0.002*</td>
</tr>
<tr>
<td>C18:2n6c</td>
<td>0.551</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>C20:1n9c</td>
<td>-0.372</td>
<td>0.017*</td>
</tr>
<tr>
<td>C20:4n6c</td>
<td>0.377</td>
<td>0.014*</td>
</tr>
<tr>
<td>C24:1n9c</td>
<td>0.454</td>
<td>0.003*</td>
</tr>
<tr>
<td>Selenium</td>
<td>0.438</td>
<td>0.004*</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0.491</td>
<td>0.001*</td>
</tr>
<tr>
<td>SDH</td>
<td>-0.410</td>
<td>0.012*</td>
</tr>
<tr>
<td>BCS</td>
<td>-0.454</td>
<td>0.003*</td>
</tr>
<tr>
<td>Body weight</td>
<td>-0.607</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>FFM (ultrasound)</td>
<td>-0.602</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Muscle Score</td>
<td>-0.309</td>
<td>0.047*</td>
</tr>
<tr>
<td>PPID Status</td>
<td>0.462</td>
<td>0.002*</td>
</tr>
<tr>
<td>Ln(Basal ACTH)</td>
<td>0.454</td>
<td>0.003*</td>
</tr>
</tbody>
</table>

1Complete blood count analysis by Rood and Riddle Equine Hospital (Lexington, KY).
2All parameters with P≤0.05 are shown in this table.
3IFN-γ = interferon-gamma; % = percent of lymphocytes producing the cytokine; TNF-α = tumor necrosis factor-α; IL = interleukin; SDH = sorbitol dehydrogenase; BCS = body condition score; FFM = fat free mass; PPID = pituitary pars intermedia dysfunction; ACTH = adrenocorticotropic hormone
Table 3.6. Comparison of deuterium oxide methods of body composition determination with body composition determinations via ultrasound and muscle scoring\textsuperscript{1,2}

<table>
<thead>
<tr>
<th>Body Composition Parameter</th>
<th>Comparison Parameter</th>
<th>Pearson correlations adjusted for Age &amp; Body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Muscle Score}</td>
<td>% Body Fat (ultrasound)</td>
<td>0.035, 0.929</td>
</tr>
<tr>
<td></td>
<td>% Body Fat (D\textsubscript{2}O)</td>
<td>-0.529, 0.143</td>
</tr>
<tr>
<td></td>
<td>FFM (ultrasound)</td>
<td>-0.031, 0.937</td>
</tr>
<tr>
<td></td>
<td>FFM (D\textsubscript{2}O)</td>
<td>0.895, 0.001*</td>
</tr>
<tr>
<td>% Body Fat (ultrasound)</td>
<td>% Body Fat (D\textsubscript{2}O)</td>
<td>-0.618, 0.076§</td>
</tr>
<tr>
<td></td>
<td>FFM (ultrasound)</td>
<td>-0.651, 0.057§</td>
</tr>
<tr>
<td></td>
<td>FFM (D\textsubscript{2}O)</td>
<td>0.375, 0.321</td>
</tr>
<tr>
<td>FFM (D\textsubscript{2}O)</td>
<td>% Body Fat (ultrasound)</td>
<td>0.375, 0.321</td>
</tr>
<tr>
<td></td>
<td>% Body Fat (D\textsubscript{2}O)</td>
<td>-0.683, 0.043*</td>
</tr>
<tr>
<td></td>
<td>FFM (ultrasound)</td>
<td>-0.124, 0.751</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Correlations with age and body weight as covariates are displayed; correlation p-values denoted with (*) indicate $P \leq 0.05$, while (§) indicate $P \leq 0.10$.
\textsuperscript{2}BCS = body condition score; D\textsubscript{2}O = deuterium oxide; FFM = fat free mass
CHAPTER 4

PERIPHERAL BLOOD MONONUCLEAR CELL-MEDIATED INFLAMM-AGING AND IMMUNOSENESCENCE OF AGED HORSES WITH VS. WITHOUT PITUITARY PARS INTERMEDIA DYSFUNCTION (PPID).

Abstract

Pituitary pars intermedia dysfunction (PPID) is an endocrine disorder commonly affecting senior equids (≥20 years). This endocrine disease results in various clinical signs, including hypertrichosis and immunosuppression. The senior horse population is also affected by immune changes associated with advanced age, including immunosenescence (decreased immune response) and inflamm-aging (low-grade, systemic inflammation). These age-associated changes occur both systemically and specifically mediated by peripheral blood mononuclear cells (PBMC). To determine whether the presence of PPID affects these already immune-compromised senior horses, PBMC-mediated cytokine expression and production as well as lymphocyte proliferation were compared in age-matched PPID and non-PPID horses. It was hypothesized that PPID affects cell-mediated immunity differentially from the process of aging alone.

Heparanized blood was collected from n=6 PPID (mean age ± SEM = 24.7±0.9 years) and n=6 non-PPID horses (23.3±1.0 years) of mixed breeds and sex. PPID Status was determined using thyrotropin releasing hormone (TRH) stimulation testing, with adrenocorticotropin hormone (ACTH) levels <110 pg/mL at 10 minutes post TRH injection (i.v.) being considered non-PPID horses. Ficoll gradient centrifugation was used to isolate PBMC from heparinized blood. Intracellular staining for pro-inflammatory
cytokines and flow cytometry were then performed on cells stimulated with Phorbol 12-myristate 13-acetate (PMA; 4 hr, 5% CO₂, 37°C) and phytohemagglutinin (PHA; 72 hr, 5% CO₂, 37°C). Reverse transcription and real time-polymerase chain reaction (RT-PCR) were also performed on PMA-stimulated PBMC to determine gene expression of interleukin(IL)-2, IL-4, IL-6, interferon(IFN)-γ, and tumor necrosis factor(TNF)-α. Lymphocyte proliferation was also analyzed using flow cytometry for PBMC stained with carboxyfluorescein succinimidyl ester (CFSE) and stimulated with concanavalin A (96 hr, 5% CO₂, 37°C). SigmaPlot version 12.3 was used to perform t-tests between PPID and non-PPID horses for the various immune measures, with a two-way ANOVA with repeated measures used to perform lymphocyte proliferation analysis. Data with p<0.05 were considered significant.

No significant differences between PPID and non-PPID horses were found for any of the various immune measures including intracellular staining, gene expression, and lymphocyte proliferation (p>0.05). Therefore, the immunosuppression and increased susceptibility to infections associated with PPID do not appear to affect lymphocyte-mediated inflamm-aging or immunosenescence in the senior horse. Further research is warranted to elucidate the mechanisms underlying PPID in order to better manage and even prevent PPID in the horse.

**Introduction**

Pituitary pars intermedia dysfunction (PPID) is an endocrine disease prevalent among equids, in which 15-30% of the senior horse population is affected (≥20 years) (McFarlane, 2011). This endocrine disease is caused by dopaminergic neurodegeneration of the
pituitary pars intermedia, leading to elevated levels of circulating adrenocorticotropin hormone (ACTH), α-melanocyte stimulating hormone (α-MSH), and other peptides cleaved from the prohormone pro-opiomelanocortin (POMC) (McFarlane, 2011). The elevated levels of these hormones and peptides are then thought to contribute to the clinical signs of PPID, although the exact mechanisms remain largely unclear (Hart et al., 2016). These clinical signs of PPID include: hypertrichosis, polydipsia, polyuria, hyperhidrosis, muscle atrophy, laminitis, abnormal fat distribution, immunosuppression and opportunistic infections, behavioral abnormalities, reproductive infertility, and neurologic impairment (McFarlane, 2011).

Senior horses are also known to exhibit age-related immune changes including: inflamm-aging (increased low-grade inflammation systemically) and immunosenescence (decreased immune response) (Adams et al., 2008). Many of these age-related immune changes occur not only in circulation but also are specifically mediated by peripheral blood mononuclear cells (PBMC) (Adams et al., 2015; Adams et al., 2008). Given that senior horses are already immune-compromised, the concurrent presentation of the PPID in these senior horses may further decrease their ability to respond effectively to pathogens, leading to the immunosuppression and susceptibility to opportunistic infections characteristic of the PPID horse.

Furthermore, given the high prevalence of this endocrine disease within the senior horse population, understanding how the disease affects immunity of the aged horse already experiencing inflamm-aging and immunosenescence, may improve management of these immune-compromised horses. According to McFarlane et al. (2015), PPID horses have exhibited decreased neutrophil function (McFarlane et al., 2015). However, effects of
PPID on PBMC function have not yet been determined. Thus, whether cell-mediated immunity differs between age-matched PPID and non-PPID horses was examined, particularly in relation to PBMC cytokine gene expression, lymphocyte inflammatory cytokine production, and lymphocyte proliferation. It was hypothesized that PPID affects cell-mediated immunity differentially from the process of aging alone.

**Methods and Materials**

2.1. *Animals*

Six aged, PPID horses (mean age ± SEM = 24.7±0.9 years) and six aged, non-PPID horses (23.3±1.0 years) of mixed breeds and sex were used in this study. PPID status was determined by thyrotropin releasing hormone (TRH) stimulation testing, examining ACTH concentration in EDTA plasma 10 minutes post intravenous injection, with horses with ACTH levels above 110 pg/mL being considered PPID, as recommended by the Equine Endocrinology Group (Restifo et al., 2016). The ACTH analysis was performed by Cornell Animal Health Diagnostic Center, Endocrinology Laboratory using chemiluminescence immunoassay (Immulite® 1000; Siemens, Berlin, Germany)(Place et al., 2010). All PPID horses had ACTH responses to TRH stimulation of at least 500 pg/mL during non-fall testing as well as clinical signs of PPID, an indication these horses had more advanced stages of PPID. Mean ACTH levels ± SEM were 875.3±127.2 and 61.3±6.9 pg/mL for PPID and non-PPID horses, respectively. None of the horses in this study had received treatment for PPID during or within a year prior to sampling. Horses were maintained on pasture and mixed grass hay at the University of Kentucky, Department of Veterinary Science Maine Chance Farm. To maintain body weight, all non-PPID and three PPID horses regularly received a mixture of 50% oats, 50% alfalfa pellets, while the other three
PPID horses received only hay. Differences in diet were taken into consideration with statistical analysis below. All procedures were approved by the University of Kentucky Institute of Animal Care and Usage Committee.

2.2. Sample Collection & Culture Preparation.

All horses were screened using TRH testing in May in order to determine PPID status using the established ACTH reference range for non-fall months (Restifo et al., 2016). Four months following endocrine testing, peripheral blood samples were collected from all horses to evaluate immune measures. Heparinized blood was collected aseptically by jugular venipuncture to isolate peripheral blood mononuclear cells using a Ficoll-Paque Plus™ (Amersham Biosciences, Piscataway, NJ) density gradient. A VICELL™ Counter-XR (Beckman Coulter, Miami, FL) was used to enable plating of cells at a concentration of 4x10^6 and stimulating with phorbol 12-myristate 13-acetate (PMA; 25 ng/mL; Sigma) and ionomycin (1 μM; Sigma) for 4 hours, 37^\circ C, 5% CO₂, prior to intracellular staining and RNA isolation (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006; Siard et al., 2016).

Cells used for proliferation analysis were fluorescently tagged with carboxyfluorescein succinimidyl ester (CFSE), plated at a concentration of 2x10^6/mL media, and stimulated with concanavalin A (2.5, 5, and 10 μL) for 96 hours, 37^\circ C, 5%CO₂, as previously described (Adams et al., 2008). The only exception was the use of fetal equine serum (Biowest, Nuaillé, France) instead of fetal bovine serum (FBS) to quench the CFSE reaction and for subsequent washes.

PBMC were also frozen in liquid nitrogen in media containing FBS and dimethyl sulfoxide (DMSO) for later analysis, at which point they were thawed, washed, stimulated
with phytohemagglutinin (PHA; 50 µg/mL), and incubated for 72 hours, 37°C, 5%CO₂ (the last 4 hr of which, Brefeldin A was added) prior to intracellular staining. The concentration of PHA used for stimulation was based on titrations performed prior to sample analysis.

2.3. IFN-γ and TNF-α Intracellular Staining

PMA-stimulated PBMC were fixed with 2% paraformaldehyde (Sigma), and incubated at 4°C overnight prior to intracellular staining. Cells were then treated with saponin buffer [PBS supplemented with 1% fetal bovine serum, 0.1% saponin (Sigma), and 0.1% sodium azide (Sigma)] and stained for IFN-γ and TNF-α using IFN-γ FITC mouse anti-bovine antibody (AbD Serotec, Raleigh, NC; 0.1 mg; 1:100 dilution) or TNF-α anti-equine monoclonal antibody (HL801; kindly provided by Dr. Rob MacKay, University of Florida; 1:10 dilution) and secondary antibody FITC-conjugated goat F(ab’)₂ anti-mouse IgG (H + L) (Invitrogen; 2 mg/mL; 1:1000 dilution) respectively, as previously described (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006; Siard et al., 2016). PHA-stimulated PBMC were likewise intracellularly stained according to the same protocol for IFN-γ.

2.4. Flow Cytometry

For PMA-stimulated, intracellularly-stained PBMC, a Becton Dickenson FASCalibur Flow cytometer and Cell Quest® software were used to determine percent of lymphocytes producing each cytokine and to determine mean fluorescence intensity (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006; Siard et al., 2016).

For PHA-stimulated, intracellularly-stained PBMC, a flow cytometer (Becton Dickenson) was also used to determine percent of lymphocytes producing IFN-γ and to determine IFN-γ MFI.
For lymphocyte proliferation, a flow cytometer (Becton Dickenson) and ModFit LT™ 3.0 were used to determine lymphocyte proliferation index, based on generational divisions (Adams et al., 2008).

2.5. RNA Isolation, Reverse Transcription, and Real-Time Polymerase Chain Reaction

Following the 4 hour stimulation with PMA/ionomycin, PBMC were resuspended in Trizol® solution and stored at -80°C until RNA isolation. RNA isolation was performed using a phenol-chloroform extraction (Breathnach et al., 2006). An Epoch microplate spectrophotometer (BioTek, Winooski, VT) was used to determine amounts of RNA in each sample, prior to RNA reverse transcription. To perform reverse transcription, a thermocycler (Applied Biosystems, Foster City, CA) and master mix reagents (16 μL avian myeloblastosis virus [AMV] buffer 5X, 16 μL MgCl₂, 4 μL dNTP, 1 μL RNasin, 1 μL oligo dT primer, and 0.5 μL AMV reverse transcriptase per sample; Promega, Madison, WI) were used, incubating samples at 42°C for 15 minutes and 95°C for 5 minutes (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). Following reverse transcription, samples were stored at -20°C. Real-time polymerase chain reaction was then performed, implementing use of an epMotion 5070 (Eppendorf) to automatically aliquot cDNA samples and 5 equine specific intron-spanning primers and probes, in addition to using the 7900HT Fast RT-PCR System (Applied Biosystems), which incubated samples for 10 min at 95°C, followed by 40 cycles of 95°C for 15s and 60°C for 1 min (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). The primers and probes analyzed included: IFN-γ, IL-2, IL-4, IL-6, and TNF-α, in addition to Beta-glucuronidase (β-GUS), the housekeeping gene (Applied Biosystems) (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). Following linear regression, the ΔΔCT method was used to
determine relative quantities for each cytokine \( RQ = 2^{-\Delta\Delta CT} \) (Livak and Schmittgen, 2001). The calibrator for each cytokine was the average \( \Delta CT \) of all the unstimulated samples (one per horse).

2.5. Statistical Analysis.

Statistical analysis was performed using SigmaPlot version 12.3 (Systat Inc., Richmond, CA). T-tests comparing PPID and non-PPID horses were performed to determine any differences in inflammation due to PPID status. (PHA-stimulated inflammatory data only included \( n=5 \) PPID horses due to one horse having insufficient cells to stimulate with PHA as well as perform the other assays.) Proliferation data was analyzed using a two-way ANOVA with repeated measures to determine any differences between PPID and non-PPID horses at the three different concentrations of concanavalin A mitogen used to stimulate the PBMC. Bar graphs (mean +/- standard deviation) were used when comparing PPID and Non-PPID horses for the various parameters. T-tests were also used to evaluate differences between the \( n=3 \) PPID horses receiving feed and \( n=3 \) PPID horses not receiving feed. (As there were no differences between fed and non-fed horses in various parameters, all data for PPID horses were grouped together for analysis when making comparisons with Non-PPID horses.) All data was normally distributed according to the Shapiro-Wilk normality test, with some data requiring log-transformation to achieve normality. Data with \( p<0.05 \) were considered statistically significant, while \( p<0.10 \) were considered trends.
Results

3.1. Inflammatory Cytokine Intracellular Staining

Various comparisons of immune measures mediated by PBMC were compared in PPID and age-matched non-PPID horses. After examining flow cytometry data for PPID and non-PPID horses, no significant differences were seen for either PMA or PHA-stimulated lymphocytes in regard to the percent of lymphocytes producing inflammatory IFN-γ (Fig. 4.1A&C) and mean fluorescence intensity (Fig. 4.1B&D) (p>0.05). Likewise, flow cytometry data for PPID and non-PPID horses did not significantly differ for percentage of lymphocytes producing pro-inflammatory TNF-α or for TNF-α MFI (Fig. 4.2A&B) (p>0.05).

3.2. Lymphocyte Proliferation

Lymphocyte proliferation also did not show significant differences between PPID and non-PPID horses (p=0.282); however, a difference in proliferation in relation to mitogen concentration was present (p<0.001), as expected, with the 2.5 µL concentration of Con A yielding significantly lower lymphocyte proliferation than the other two concentrations (Fig. 4.3).

3.3. Real-Time Polymerase Chain Reaction Gene Expression

PCR results also did not display differences between PPID and non-PPID horses (p>0.05); however, due to the variation associated with PCR data, it is possible that in a larger data set differences may occur (Fig. 4.4A-E).

3.4. Feeding Status Analysis of PPID Horses

Within the PPID horse group, n=3 horses received feed (50% alfalfa pellets, 50% oats) regularly while n=3 horses were fed hay alone, in order to maintain body weight. T-
tests were run between fed horses and those that did not receive feed, and again, no significant differences were found. This may be in part due to the sample size; however, in order to sample a group of healthy horses with naturally occurring signs of PPID and TRH stimulation testing results to support advanced PPID status, sample size was limited. And, in order not to change their diets for the purposes of this study, which may elicit changes in immune parameters, horses were sampled while continuing maintenance diets.

**Discussion**

The PPID horse is known to experience immunosuppression and increased susceptibility to opportunistic infections as one of the clinical manifestations of this endocrinopathy. Despite the reported increases in bacterial infections, such as dermatophilosis, pneumonia, and abscesses found in PPID horses (McFarlane, 2011), few studies have been performed to determine the specific mediators for these deficiencies in immunity. Furthermore, senior horses (the demographic in which PPID primarily occurs) exhibit inflamm-aging and immunosenescence, which are specifically mediated by PBMC as well as occurring systemically (Adams et al., 2008). Thus, PBMC were examined as a potential source of immunosuppression in the PPID, aged horse.

In this study, no differences were observed in any of the immune parameters analyzed in which PBMC were specifically examined regarding: intracellular staining, cytokine gene expression, and proliferation. Thus, the cell-mediated responses of adaptive immunity do not seem to be affected by PPID status of the horse. Although PPID has been associated with immunosuppression, it appears this compromise of immunity is not mediated by PBMC. Some work has shown total leukocyte cytokine dysregulation in PPID horses (McFarlane and Holbrook, 2008) as well as evidence that neutrophils contribute to
the effects of PPID on immune function of the aged horse (McFarlane et al., 2015). Specifically, neutrophils from aged, PPID horses have exhibited decreased oxidative burst activity and decreased adhesion, which may contribute to immunosuppression (McFarlane et al., 2015).

Of note, PMA-stimulated neutrophils from PPID and control horses did not significantly differ in the McFarlane et al, 2015 study, although neutrophils that were unstimulated or stimulated submaximally exhibited significantly decreased adhesion in PPID horses compared to non-PPID horses (McFarlane et al., 2015). In the current study, using an optimal dose of PHA to stimulate PBMC also does not identify significant differences between PPID and non-PPID horses in flow cytometry measures of either percentage of lymphocytes producing IFN-γ or IFN-γ MFI. Additionally, the proliferation assay was performed using concanavalin A at various concentrations, and likewise no significant differences were found between PPID and non-PPID horses regarding lymphocyte proliferation. Therefore, the lack of significant differences between PPID and non-PPID horses in relation to lymphocyte function is likely not a matter of the type of mitogen.

Some of the peptides produced by cleavage of POMC have established immunomodulatory effects, which may help compose a neuro-endocrine-immune axis (Bohm and Luger, 2004; Brazzini et al., 2003). Alpha-MSH particularly has long been established as a potent anti-inflammatory, anti-oxidant agent (Bhardwaj et al., 1996; Catania et al., 1999; Luger and Brzoska, 2007; Oktar et al., 2004). Mechanistically, α-MSH downregulates nuclear factor (NF)-κβ activity stimulated by lipopolysaccharide (LPS) (Luger et al., 2000). Furthermore, this hormone has specifically exhibited anti-
inflammatory effects on PBMC in humans through increasing IL-10 gene expression and protein production; however monocytes, not T lymphocytes, were responsible for the anti-inflammatory response elicited by the addition of α-MSH (Bhardwaj et al., 1996). Since only the PCR data in the current study included monocytes as well as lymphocytes, this may in part explain the lack of differences between PPID and non-PPID horses. In regards to PCR data, as mentioned previously, no significant differences were found for PPID vs. non-PPID; however, due to the variability of PCR data, some differences may become significant if the sample size were increased. Studies examining monocytes in the PPID horse specifically have not yet been performed to determine whether the increased levels of α-MSH in the PPID horse may yield monocyte-mediated anti-inflammatory effects similar to studies examining human monocytes (Bhardwaj et al., 1996; Catania et al., 1999; Yang et al., 2015). In addition to direct anti-inflammatory effects of α-MSH alone, McFarlane et al. (2015) found that in horses the ratio of insulin to α-MSH was positively correlated with oxidative burst activity (McFarlane et al., 2015), indicating that a relationship of relative hormone amounts may influence immunity of the PPID horse.

ACTH, the other key hormone cleaved from POMC, has been positively associated with IL-6 expression in septic foals (Gold et al., 2012); however the relationship between ACTH and inflammation has not been clearly established. Additionally, much of the circulating ACTH is thought to be biologically inactive due to the fact that PPID horses do not appear to exhibit elevated cortisol levels despite their increased levels of ACTH (McFarlane et al., 2015).

Still, more research is needed regarding the pathogenesis of PPID and its effects on the immune system, particularly how this endocrinopathy is associated with
immunosuppression and susceptibility to opportunistic infections in the aged horse. Furthermore, it is not yet understood what causes the dopaminergic neurodegeneration of the pituitary pars intermedia. A variety of factors including oxidative stress, infectious disease, nutritional deficiencies, or even inflammation may play a role in the development of this endocrine dysfunction, but clearly more research is warranted.
Figure 4.1. Lymphocyte production of IFN-γ *in vitro* for PPID vs. Non-PPID horses. Percentage of PMA-stimulated lymphocytes (A) producing IFN-γ and (B) mean fluorescence intensity of IFN-γ for n=6 PPID and n=6 age-matched non-PPID horses. Percentage of PHA-stimulated lymphocytes (C) producing IFN-γ and (D) mean fluorescence intensity of IFN-γ for n=5 PPID and n=6 age-matched non-PPID horse.
Figure 4.2. Lymphocyte production of TNF-α \textit{in vitro} for PPID vs. Non-PPID horses. Percentage of lymphocytes (A) producing TNF-α and (B) mean fluorescence intensity of TNF-α for \(n=6\) PPID and \(n=6\) age-matched non-PPID horses.
Figure 4.3. Lymphocyte proliferation index of PPID vs. Non-PPID horses. Peripheral blood mononuclear cells (PBMC) from n=6 PPID and n=6 age-matched Non-PPID horses were stained with CFSE and stimulated with various concentrations (2.5, 5, 10 µl/mL) of concanavalin A mitogen for 96 hours to determine proliferation. A two-way ANOVA with repeated measures was performed. $P_{PPID} = 0.103$ and $P_{trt} < 0.001$. PPPID refers to the p-value for the difference between PPID and non-PPID horses; $P_{trt}$ refers to the p-value for the differences between concanavalin A mitogen concentrations for stimulation of PBMC.
Figure 4.4. PBMC-mediated cytokine gene expression of PPID vs. Non-PPID horses. RT-PCR was performed on PMA/ionomycin-stimulated PBMC from n=6 PPID and n=6 Non-PPID horses to determine gene expression of (A) IFN-γ, (B) IL-2, (C) IL-4, (D) IL-6, and (E) TNF-α (logged).
CHAPTER 5
EFFECTS OF POLYPHENOLS INCLUDING CURCUMINOIDS, RESVERATROL, QUERCETIN, PTEROSTILBENE, AND HYDROXYPTEROSTILBENE ON LYMPHOCYTE PRO-INFLAMMATORY CYTOKINE PRODUCTION OF SENIOR HORSES IN VITRO

Veterinary Immunology and Immunopathology, 2016: 173, 50-59

Abstract

Senior horses (aged ≥20 years) exhibit increased chronic, low-grade inflammation systemically, termed inflamm-aging. Inflammation is associated with many afflictions common to the horse, including laminitis and osteoarthritis, which are commonly treated with the non-steroidal anti-inflammatory drugs (NSAIDs) flunixin meglumine and phenylbutazone. Although these NSAIDs are effective in treating acute inflammatory problems, long-term treatment with NSAIDs can result in negative side effects. Thus, bioactive polyphenols including curcuminoids, resveratrol, quercetin, pterostilbene, and hydroxypterostilbene were investigated to determine their effectiveness as anti-inflammatory agents in vitro. Heparinized blood was collected via jugular venipuncture from senior horses (n=6; mean age=26 ± 2 years), and peripheral blood mononuclear cells (PBMC) were isolated using a Ficoll density gradient. PBMC were then incubated 22 hours at 37°C, 5% CO₂ with multiple concentrations (320, 160, 80, 40, 20, 10 µM) of all five polyphenols (curcuminoids, resveratrol, quercetin, pterostilbene, and hydroxypterostilbene), dissolved in DMSO to achieve the aforementioned concentrations. PBMC were stimulated the last 4 hours of the incubation period with phorbol 12-myristate 13-acetate (PMA)/ionomycin and Brefeldin A (BFA). A Vicell-XR counter evaluated cell
viability following incubation. PBMC were stained intracellularly for interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) and analyzed via flow cytometry. Data was analyzed by one-way analysis of variance (ANOVA). Viability of PBMC incubated with various compound concentrations were compared with PBMC incubated with DMSO alone (positive control) to determine at what concentration each compound caused cytotoxicity. The highest concentration at which cell viability did not significantly differ from the positive control was: 20 µM for curcuminoids, 40 µM for hydroxypterostilbene, 80 µM for pterostilbene, and 160 µM for quercetin and resveratrol. Flunixin meglumine and phenylbutazone were then evaluated within this range of optimal concentrations for the polyphenol compounds (160, 80, 40, 20 µM) to compare the polyphenols to NSAIDs at equivalent concentrations. The highest concentration at which viability did not significantly differ from the positive control was: 40 µM for flunixin meglumine and 160 µM for phenylbutazone. All five polyphenols and flunixin meglumine significantly decreased lymphocyte production of IFN-γ, while only hydroxypterostilbene, pterostilbene, quercetin, and resveratrol significantly reduced lymphocyte production of TNF-α compared to the positive control (p<0.05). Polyphenols performed similarly to or more effectively than common NSAIDs in reducing lymphocyte production of inflammatory cytokines of the senior horse in vitro. This study therefore supports the further investigation of polyphenols to determine whether they may be effective anti-inflammatory treatments for chronic inflammation in the horse.

**Introduction**

The senior horse population is growing globally, with horses 20 years or older comprising an estimated 7.6% of the population in the United States (USDA., 2006) and
horses ≥15 years old comprising 29% of the population in the United Kingdom (Ireland et al., 2011a). In addition to increasing lifespan, healthspan must be taken into account. With increased age, an increase in inflammation at both the gene expression [interleukin (IL)-1β, IL-15, IL-18 and TNF-α] and protein levels [tumor necrosis factor-alpha (TNF-α)] has been characterized in the horse (Adams et al., 2008; Katepalli et al., 2008). This increased inflammation with aging indicates that horses, like many other species, exhibit inflamm-aging or low-grade, chronic inflammation that occurs systemically with aging (Franceschi et al., 2007). Although clearly established clinical conditions of the horse associated with inflamm-aging have not yet been discovered, human studies have shown that inflammation is characterized as a risk factor for developing various diseases and is associated with increased morbidity and mortality (Freund et al., 2010). Inflamm-aging in the horse therefore likely has detrimental effects.

In addition to systemic inflamm-aging, many horses develop various chronic inflammatory conditions over the course of their lives, ranging from laminitis to osteoarthritis. These conditions are frequently treated with common non-steroidal anti-inflammatory drugs (NSAIDs), such as flunixin meglumine and phenylbutazone. NSAIDs are quite useful for treating acute inflammatory conditions; however, long-term use can result in various harmful side effects, such as stomach ulcers, gastric oxidative stress, hypoalbuminemia, neutropenia, liver damage, and kidney damage (Bessone, 2010; Ingrasciotta et al., 2015; Martinez Aranzales et al., 2015; McConnico et al., 2008; Monreal et al., 2004; Reed et al., 2006). Thus, various anti-inflammatory polyphenols including curcuminoids, resveratrol, quercetin, pterostilbene, and hydroxypterostilbene were
investigated to determine their effects on inflammation of the senior horse in vitro as an alternative to the use of NSAIDs in treating chronic inflammation.

Polyphenol compounds have been shown to exhibit anti-inflammatory properties in various capacities both in vitro and in vivo, including decreasing peripheral blood mononuclear cell (PBMC) inflammatory gene expression, as reviewed by Afman et al. (Afman et al., 2014). Furthermore, the polyphenolic compound curcumin has decreased PBMC production of pro-inflammatory IL-8 (Literat et al., 2001). Curcumin has traditionally been used in Asia since ancient times to treat various maladies including many inflammatory conditions (Jeenger et al., 2015); moreover, this polyphenol has recently gained popularity in research and the health market, for its various anti-inflammatory, antioxidant, anti-tumorigenic properties (Franck et al., 2008; Schaaf et al., 2009; Venkatesan and Chandrakasan, 1995). Curcumin is derived from the root of Curcuma longa, and comprises 77% of the curcuminoids found in turmeric, making curcumin the primary curcuminoid (Kumar et al., 2010). Resveratrol is a phytoalexin found most notably in red wine, which has shown anti-inflammatory activities both dependent and independently of NF-κB activation (a key transcription factor in pro-inflammatory cytokine expression) (Birrell et al., 2005; Kumar and Sharma, 2010; Samsami-Kor et al., 2015). Moreover, resveratrol supplementation has decreased NF-kB activity in PBMC as well as decreasing pro-inflammatory high sensitivity C-reactive protein (hs-CRP) and TNF-α in plasma (Samsami-Kor et al., 2015). Resveratrol has been found specifically to act as an antioxidant in equine neutrophils in vitro, as well (Kohnen et al., 2007). Quercetin is an antioxidant flavonoid found in various plants including teas and onions and has been shown to decrease inflammation in high-fat diet mice (Das et al., 2013) and high-fructose
diet rats (Vazquez Prieto et al., 2015). Pterostilbene, an analog of resveratrol and the primary antioxidant component of blueberries, has demonstrated anti-inflammatory properties, decreasing inflammation produced by adipocytes as well as decreasing inflammation produced as a result of myocardial ischemia and reperfusion (Hsu et al., 2013; Lv et al., 2015; Wang et al., 2015). Hydroxypterostilbene, an analog of pterostilbene found in the plant Sphaerophysa salsula, has recently been shown to decrease the pro-inflammatory enzyme COX-2 and to downregulate multiple pro-inflammatory pathways including PI3K/Akt and MAPK signaling (Cheng et al., 2014). These various polyphenol compounds possessing anti-inflammatory properties are of interest as alternative anti-inflammatory therapies to the traditional long-term use of NSAIDs in treating chronic inflammatory conditions.

Thus, we hypothesize that polyphenols including curcuminoids, resveratrol, quercetin, pterostilbene, and hydroxypterostilbene will significantly reduce inflammation of the senior horse in vitro, similarly to common NSAIDs phenylbutazone and flunixin meglumine. PBMC will particularly be examined for the following reasons: PBMC have been shown to contribute to chronic, low-grade inflammation (Adams et al., 2008); are easily accessible (Literat et al., 2001); and previous research has demonstrated the effects of polyphenols on PBMC (Afman et al., 2014; Literat et al., 2001; Samsami-Kor et al., 2015). This study is the first step in determining the ability of polyphenols to decrease inflammation in the horse in vivo.

Thus far, clearly established clinical conditions of the horse associated with inflamm-aging have not been discovered. Based on human studies, however, it is anticipated that decreasing inflamm-aging may be beneficial to healthspan and lifespan.
Furthermore, the horse exhibiting inflamm-aging was used as a model to determine whether polyphenols could decrease chronic inflammation of the horse. NSAIDs were used as a comparison to polyphenols in determining how various anti-inflammatory treatments (including both nutraceuticals and pharmaceuticals) affected circulating inflammatory cytokine production by PBMCs in the horse. The use of polyphenols could then potentially have further implications for the management of chronic inflammatory conditions, such as osteoarthritis and recurrent laminitis in the horse.

**Methods and Materials**

2.1. Animals

Six senior horses (mean age = 26 ± 2 years) were used in this study, exhibiting no clinical abnormalities (eg nasal discharge, cough) upon physical assessment. When examining basal adrenocorticotropin hormone (ACTH), three of the horses were pituitary pars intermedia dysfunction (PPID) negative [with plasma levels within normal range (9-35 pg/mL) as measured by Cornell Animal Health Diagnostic Center, Endocrinology Laboratory], and three were PPID positive (plasma levels >35 pg/mL) (Place et al., 2010); however, no significant differences in inflammatory parameters were found between PPID and non-PPID horses.

2.2. Sample Collection, PBMC Isolation, and Culture Preparation

Heparinized blood was collected via jugular venipuncture from n=6 senior horses, and PBMCs were isolated using a Ficoll-Paque Plus™ (Amersham Biosciences, Piscataway, NJ) density gradient and purified with sterile Phosphate Buffered Saline (PBS) (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). A VICELL™ Counter-XR (Beckman Coulter, Miami, FL) counted cells and determined cell viability using
Trypan blue staining prior to plating. PBMCs were plated at a concentration of $4 \times 10^6$ cells/mL with cRPMI media [RPMI 1640 (Gibco, Grand Island, NY) supplemented with 2.5% fetal equine serum (FES; Sigma-Aldrich, St. Louis, MO), 55 μM 2-mercaptoethanol (Gibco), and 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/mL streptomycin (HyClone Pen/Strep/Glutamine solution; Thermo Scientific)] (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006).

2.3. Compound Preparation

The day prior to peripheral blood collection, stock solutions (0.16 M) of polyphenolic compounds [curcuminoids (Natsol Laboratories Pvt. Ltd., Hyderabad, India), resveratrol (Biological Prospects® LLC, Lexington, KY), pterostilbene (Biological Prospects® LLC), 3-hydroxypterostilbene (Natsol Laboratories), and quercetin (Natsol Laboratories)] and NSAIDs [flunixin meglumine (Phoenix Pharmaceuticals, Inc., St. Joseph, MO; 50 mg/mL injectable solution) and phenylbutazone paste (Phoenix Pharmaceutical, Inc.; 0.33 g/mL)] were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich). The stock solution for each of the polyphenols and NSAIDS were diluted with DMSO to make a total of 6 working solutions (such that an addition of 2 μL/mL media would yield final concentrations of 320, 160, 80, 40, 20, and 10 μM) for each of the polyphenolic compounds and NSAIDs (Qureshi et al., 2012). The working solutions were then frozen at -20°C overnight until use the following day.

The compound concentrations were determined based on previous work (Quereschi et al 2013) in which resveratrol, pterostilbene, and quercetin at concentrations ranging 320-10 μM were shown to significantly inhibit macrophage inflammatory cytokine production. In vivo these optimal concentrations ranging from 20-160 μM would equate to 76.7-614.0
µM concentration in plasma, (given that the 20-160 µM was for 4x10⁶ PBMC/mL, old horses tend to have ~2.5x10⁷ PBMC per 15 mL blood, and previous work showing that horses have 6.33 L plasma/100 kg BW and 10.31 L blood total/100 kg BW (Marcilese et al., 1964)).

2.4. Cell Viability after Incubation with Compounds

After plating the PBMCs with c-RPMI, DMSO (2 µL/mL media) was added to two wells per horse to act as controls, while the polyphenol and NSAID stock solutions (2 µL/mL media) were each added to a well to achieve final concentrations of 320, 160, 80, 40, 20, and 10 µM for each compound. PBMCs were incubated with the compounds 22 hours at 37°C, 5% CO₂. At 18 hours, Brefeldin A (10 µg/mL; Sigma) was added to all wells, and all wells except one of the DMSO controls per horse were stimulated with phorbol 12-myristate 13-acetate (PMA; 25 ng/mL; Sigma) and ionomycin (1 µM; Sigma) (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). Cell counts and viability for each well were then determined using the Vicell Counter-XR to determine whether cells treated with the various compounds differed from those treated with DMSO and PMA (positive control). The highest compound concentration at which viability did not differ from the positive control was determined to be the optimal concentration.

2.5. IFN-γ and TNF-α Intracellular Staining and Flow Cytometry

Following incubation, aliquots (200 µL each) of cells from each well were placed into duplicate 96-well V-bottom plates. Both plates were centrifuged, resuspended in 2% paraformaldehyde (Sigma), and stored at 4°C overnight. Cells were then washed in saponin buffer [PBS supplemented with 1% fetal bovine serum (FBS), 0.1% saponin, and 0.1% sodium azide (Sigma)] and IFN-γ FITC mouse anti-bovine antibody (AbD Serotec,
Raleigh, NC; 0.1 mg) was added at a 1:100 dilution in saponin buffer to one plate, while TNF-α anti-equine monoclonal antibody (HL801; kindly provided by Dr. Rob MacKay, University of Florida) at a 1:10 dilution in saponin buffer was added to the other plate (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). Both plates were incubated 30 minutes on ice and washed in saponin buffer. The cells in the IFN-γ–stained plate were then resuspended in FACS Flow (Becton Dickinson, San Jose, CA), while those in the TNF-α plate were stained with secondary antibody FITC-conjugated goat F(ab’)2 anti-mouse IgG (H + L) (Invitrogen; 2 mg/mL) at a 1:1000 dilution in saponin buffer and incubated an additional 30 minutes on ice (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006). Following incubation, the plate was washed in saponin buffer and resuspended in FACS Flow. Both plates were transferred to 5-mL round-bottom tubes for flow cytometric analysis. Using a FACS Calibur flow cytometer (Becton Dickinson) and Cell Quest® software (Becton Dickinson), samples were analyzed, and lymphocyte populations were gated on cell size and granularity to determine percent IFN-γ- and percent TNF-α-positive lymphocytes as well as mean fluorescence intensities (MFI) of lymphocytes (Adams et al., 2008; Adams et al., 2009; Breathnach et al., 2006).

2.6. **Statistical Analysis.**

SigmaPlot version 10.0 and SigmaStat version 3.5 (Systat Inc., Richmond, CA) were used for data analysis. Data not normally distributed was log-transformed first in an effort to achieve a normal distribution; however, if normality still could not be achieved, data was analyzed on ranks. Normally distributed data was shown using bar graphs (mean +/- standard deviation), while data not achieving normal distribution was shown with box
plots (median, 25th, and 75th percentile). For all analyses performed, data with p<0.05 was considered statistically significant.

One-way analysis of variance (ANOVA) tests were performed to determine cell viability using the Holm-Sidak method for normally distributed data, while Kruskal-Wallis one-way ANOVA on ranks using the Tukey test was implemented for data not normally distributed. Two-way ANOVA tests using the Holm-Sidak method was employed to analyze inflammatory cytokine data at the various concentrations for each compound.

For comparisons of the optimal concentrations of each polyphenol and NSAID to each other, one-way ANOVAs with Holm-Sidak method were used to measure the viability and the various inflammatory cytokine parameters for normally distributed data, while Kruskal-Wallis one-way ANOVAs on ranks using Dunn’s method were implemented for data not normally distributed.

Results

3.1. Cell viability for polyphenols at varying concentrations

Polyphenolic compounds curcuminoids, hydroxysterolbene, pterostilbene, quercetin, and resveratrol exhibit anti-inflammatory properties but can also be cytotoxic at high concentrations, thus Vicell counting was performed to determine cell viability after the incubation with compounds and stimulation with PMA and ionomycin. Results show that with increased compound concentrations, cell viability decreased for all compounds, as expected (Fig. 5.1). Each of the concentrations of compound for all six horses were compared to the positive control DMSO PMA in an effort to determine the highest non-cytotoxic concentration for each compound, designated the optimal concentration. Results show that the highest non-cytotoxic concentrations for each of the compounds were the
following: (A) curcuminoids 20 µM, (B) hydroxypterostilbene 40 µM, (C) pterostilbene 80 µM, (D) quercetin 160 µM, and (E) resveratrol 160 µM (Fig. 5.1).

3.2. Intracellular staining and flow cytometry for polyphenols at varying concentrations

The effect of polyphenols (at concentrations ranging from 320 to 10 µM in two-fold dilutions) on percentages of lymphocytes producing IFN-γ and TNF-α when stimulated with PMA and ionomycin were examined. No significant interactions were observed between % IFN-γ and % TNF-α for any of the polyphenols except curcuminoids, thus the asterisks in Figure 5.2B-E are grouped over the bars for % IFN-γ and % TNF-α for each concentration, as there was an overall comparison of inflammatory cytokines for each of the compound concentrations to the positive control (DMSO/PMA). Results indicate that a concentration-dependent effect was present, with higher concentrations of compounds yielding decreased pro-inflammatory cytokine production, (some of which is due to the decreased cell viability with increasing concentration exhibited in Figure 5.1). Significant differences (p<0.05) relative to the positive control for inflammatory cytokines IFN-γ and TNF-α were found for (A) curcuminoids at all concentrations but 10 µM; (B) hydroxypterostilbene at all concentrations but 10 µM; (C) pterostilbene at 320, 160, 80 µM; (D) quercetin at 160 µM; and (E) resveratrol at 320, 160 µM (Fig. 5.2). Data for curcuminoids (Fig. 5.2A) and hydroxypterostilbene (Fig. 5.2B) were log-transformed to achieve equal variance and normal distribution, respectively; however, for comparison to the other compounds, these data are represented graphically without log-transformation.

3.3. Cell viability and intracellular staining for NSAIDs

Since NSAIDs are often used to treat inflammation, the polyphenol compounds were also compared with the NSAIDs flunixin meglumine and phenylbutazone commonly
used in the horse. In the same manner as previously described, cell viabilities were determined and intracellular staining and flow cytometry for IFN-γ and TNF-α production by lymphocytes was quantified. Parameters for NSAIDs were only measured at concentrations ranging from 20 to 160 µM in two-fold dilutions, since the optimal concentrations for the various polyphenols had fallen within this range. Results are shown for n=5 horses, as one horse was excluded as an outlier. Results for flunixin meglumine showed a significant difference (p<0.05) in cell viability at 80 and 160 µM compared to the positive control (Fig. 5.3A), while phenylbutazone did not exhibit a difference in cell viability at any of the concentrations tested (Fig. 5.3C). For lymphocyte production of IFN-γ and TNF-α, a significant difference (p<0.05) was exhibited for flunixin meglumine at 40, 80, and 160 µM compared to the positive control (Fig. 5.3B), while phenylbutazone did not have any significant differences for the concentrations tested (Fig. 5.3D). Again, no significant interactions were observed between % IFN-γ and % TNF-α for either of the NSAIDs, thus the asterisks in Figure 5.3B are grouped over the bars for % IFN-γ and % TNF-α for each concentration, as there was an overall comparison of inflammatory cytokines for each of the compound concentrations to the positive control (DMSO/PMA).

3.4. Intracellular staining for optimal concentrations

Optimal concentrations, as determined by the highest concentrations of each compound at which cell viability did not significantly differ (p>0.05) from the positive control, were compared among the various compounds to highlight the effects of the compounds relative to each other. Optimal concentrations for each of the compounds and NSAIDs were: phenylbutazone – 160 µM, flunixin meglumine – 40 µM, curcuminoids – 20 µM, hydroxypterostilbene – 40 µM, pterostilbene – 80 µM, quercetin – 160 µM, and
resveratrol – 160 µM. All compounds except phenylbutazone significantly reduced (p<0.05) IFN-γ compared to the positive control, both by decreasing the percent of lymphocytes producing IFN-γ (Fig. 5.4A) and the amount of IFN-γ produced on average per cell [mean fluorescence intensity (MFI)] (Fig. 5.4B). The percent of lymphocytes producing TNF-α was reduced (p<0.05) for hydroxypterostilbene (40 µM), pterostilbene (80 µM), quercetin (160 µM), and resveratrol (160 µM) (Fig. 5.4C). TNF-α MFI was significantly decreased (p<0.05) for all compounds including both NSAIDs when compared to the positive control (Fig. 5.4D).

For comparisons among the compounds for percent of lymphocytes producing IFN-γ (%IFN-γ), DMSO/PMA (the positive control) and phenylbutazone exhibited similar levels (p>0.05) (Fig. 5.4A). Phenylbutazone and flunixin meglumine did not differ statistically (p>0.05), although flunixin did decrease % IFN-γ significantly compared to the positive control (p<0.05) (Fig. 5.4A). Flunixin meglumine, curcuminoids, and quercetin performed similarly in reduction of % IFN-γ (Fig. 5.4A). Curcuminoids also performed statistically similarly to hydroxypterostilbene, pterostilbene, and resveratrol (p>0.05), which exhibited the greatest reductions in % IFN-γ (Fig. 5.4A).

Among the compounds for IFN-γ MFI, the positive control DMSO/PMA and phenylbutazone produced similar amounts of IFN-γ on average per cell (p>0.05) (Fig. 5.4B). Phenylbutazone also performed statistically similarly to flunixin meglumine, curcuminoids, hydroxypterostilbene, and quercetin (p>0.05) (Fig. 5.4B). The latter four compounds additionally exhibited similar reductions in IFN-γ MFI to pterostilbene and resveratrol, decreasing IFN-γ MFI most significantly (Fig. 5.4B).
For comparisons among the compounds for percent of lymphocytes producing TNF-α (% TNF-α), the positive control (DMSO/PMA), phenylbutazone, flunixin meglumine, and curcuminoids performed statistically similarly (p>0.05) (Fig. 5.4C). However, phenylbutazone, flunixin meglumine, curcuminoids, hydroxypterostilbene, pterostilbene, quercetin, and resveratrol also did not differ significantly (p>0.05) from each other for % TNF-α (Fig. 5.4C).

Among the compounds for TNF-α MFI, all polyphenols and NSAIDs decreased TNF-α MFI compared to DMSO/PMA (p<0.05) (Fig 5.4D). Phenylbutazone, flunixin meglumine, and quercetin similarly decreased TNF-α MFI, while flunixin meglumine also exhibited similar levels of TNF-α MFI to curcuminoids and hydroxypterostilbene (Fig. 5.4D). Curcuminoids, hydroxypterostilbene, pterostilbene, and resveratrol produced similar amounts of TNF-α on average per cell, decreasing TNF-α MFI most significantly compared to the positive control (p<0.05) (Fig. 5.4D).

3.5. Viability of optimal concentrations

Optimal concentrations were determined as the highest concentration within each compound titration at which the percent viability of PBMC did not differ (p>0.05) from the control, DMSO/PMA. However, since optimal concentrations were then selectively analyzed in comparison to the positive control for IFN-γ and TNF-α, PBMC viability for the chosen optimal concentrations was also evaluated to determine whether they still did not significantly differ from the positive control when collectively analyzed. Hydroxypterostilbene (40 µM) and pterostilbene (80 µM) significantly differed (p<0.05) in viability when compared to the positive control (Fig. 5.5). No other differences in viability between treatments were found.
Discussion

Polyphenols and other nutraceuticals have recently gained popularity in many species including horses for garnering health benefits without many of the side effects associated with pharmaceuticals (Belcaro et al., 2010a; Horohov, 2012; Paller et al., 2015; Reed et al., 2006). Polyphenols including curcuminoids, hydroxypterostilbene, pterostilbene, quercetin, and resveratrol have demonstrated anti-inflammatory activity in previous studies. Although various in vitro and in vivo studies have been performed in other species demonstrating the anti-inflammatory effects of polyphenols (Luna et al., 2007; Qureshi et al., 2012; Vazquez Prieto et al., 2015; Venkatesan and Chandrakasan, 1995), little research has been performed in the horse despite the common occurrence of inflammatory-associated conditions (Goodrich and Nixon, 2006). Furthermore, senior horses, like elderly humans, exhibit inflam-aging, and thus provide an excellent model of elevated inflammation in which to test the efficacy of anti-inflammatory compounds.

In the present study, the effects of polyphenols on inflammatory cytokine production in vitro have been examined. Results indicate that lymphocyte production of pro-inflammatory cytokines decreased when treated with polyphenols compared to the positive control of cells stimulated with PMA/ionomycin and treated with DMSO. All optimal concentrations of compounds (highest concentration at which viability was not diminished within each compound titration curve when compared to the positive control) decreased lymphocyte production of inflammatory cytokines (p<0.05). This demonstrates that curcuminoids, hydroxypterostilbene, pterostilbene, quercetin, and resveratrol can significantly reduce inflammation in vitro without becoming toxic to PBMCs.
Other studies have found similar results, with resveratrol, pterostilbene, and quercetin decreasing TNF-α, IL-1β, IL-6, and iNOS expression as well as TNF-α secretion upon stimulation of macrophage cell line RAW264.7 with LPS (Qureshi et al., 2012). The same study found that resveratrol, pterostilbene, and quercetin decreased NF-κB activation of the human kidney line HEK293 cells, as well (Qureshi et al., 2012). This study exhibited similar findings to our results in that resveratrol and pterostilbene decreased inflammation similarly to or better than quercetin (Qureshi et al., 2012). Notably, in the present study, pterostilbene (80 µM) at half the concentration of resveratrol and quercetin (160 µM) still decreased inflammation significantly. Curcumin and resveratrol have been shown to decrease inflammation through modulating the NF-κB pathway at various stages (Buhrmann et al., 2011; Duarte et al., 2010; Samsami-Kor et al., 2015). Additionally, curcumin has exhibited more potent anti-inflammatory activity than resveratrol in equine neutrophils (Derochette et al., 2013), which supports our results in which curcuminoids (20 µM) reduced inflammation similarly to resveratrol (160 µM). Although little research has been performed thus far on hydroxypterostilbene, it has been found to decrease production of pro-inflammatory COX-2, as well as downregulating upstream PI3K/Akt and MAPK, more effectively than its counterpart pterostilbene (Cheng et al., 2014). This aligned with our results, showing potent anti-inflammatory action of hydroxypterostilbene (40 µM), even at half the concentration of pterostilbene (80 µM). The variation observed in optimal compound concentrations suggests that compounds exhibiting anti-inflammatory effects at lower concentrations have greater bioactivity _in vitro_, such as curcuminoids and hydroxypterostilbene, while those exhibiting anti-inflammatory effects at higher
concentrations like quercetin and resveratrol may be less bioactive in reducing inflammation.

Furthermore, these polyphenols reduced inflammation at least as effectively as common NSAIDs flunixin meglumine and phenylbutazone. In fact, phenylbutazone did not significantly reduce lymphocyte production of IFN-\(\gamma\) or TNF-\(\alpha\) at any of the concentrations tested, whereas all polyphenols did. Phenylbutazone did, however, reduce TNF-\(\alpha\) MFI when compared to the positive control. Phenylbutazone may not have performed as well as expected due to the fact that phenylbutazone paste was suspended in DMSO before addition to PBMCs (since paste is the formulation most commonly used in the horse). Using an intravenous formulation of phenylbutazone, however, may have yielded significant decreases in inflammation on more parameters, since the effects of PBMC incubation with paste may exhibit masked effects when compared to a more pure form.

Additionally, the mechanisms of action for these polyphenols suppress inflammation by inhibiting the gene expression of inflammatory cytokines and other proteins, while NSAIDs primarily work to treat the symptoms of inflammation, inhibiting the pro-inflammatory COX enzyme (Beretta et al., 2005). Polyphenols particularly modulate inflammation via NF-\(\kappa\)B and MAPK signaling (Buhrmann et al., 2011; Busch et al., 2012; Comalada et al., 2005; Kumar and Sharma, 2010; Vazquez Prieto et al., 2015). Curcumin, quercetin, and resveratrol have also been shown to activate Sirtuin 1 (SIRT1), which is involved in deacetylation of histones and transcription factors. (Busch et al., 2012; Chung et al., 2010). One study compared phenylbutazone with curcumin and resveratrol, finding that both of the polyphenols decreased NF-\(\kappa\)B activation at far smaller
concentrations than phenylbutazone, thereby suggesting the greater potency of polyphenols to modulate the source of inflammation when compared to phenylbutazone (Takada et al., 2004). Another study compared flunixin meglumine and phenylbutazone, determining that while both NSAIDs inhibited COX-1, flunixin meglumine also decreased NF-kB activation to some degree, indicating that flunixin meglumine may function to decrease inflammation by a COX-independent pathway as well (Bryant et al., 2003). This may also explain why our study found flunixin meglumine to be the more effective NSAID. Nevertheless negative side effects, particularly gastrointestinal problems, have been associated with long-term NSAID use (Luna et al., 2007; Reed et al., 2006; Yoon and Baek, 2005). Thus, polyphenol nutraceuticals may provide a more proactive approach to treating inflammation, without the various side effects, though more research is needed in this area, particularly in vivo.

Although these results suggest significant anti-inflammatory activity of the polyphenols examined in vitro, bioavailability and bioactivity of these compounds in the horse in vivo must also be taken into account. Little research has been performed thus far in this area, as the use of these naturally-occurring polyphenols as anti-inflammatory treatments in the horse is novel. From research in other species, oral bioavailability of the polyphenol compounds are as follows: curcuminoids – 0.47% (Gutierres et al., 2015), pterostilbene – 35-80% (Azzolini et al., 2014; Kapetanovic et al., 2011), quercetin – 17% (Ader et al., 2000), resveratrol – 20% (Kapetanovic et al., 2011), and hydroxypterostilbene – yet to be determined. For NSAIDs phenylbutazone and flunixin meglumine, the common routes of administration (and the formulations used in this study) were oral paste and i.v. injectable, respectively; the bioavailability of oral phenylbutazone in the horse is
approximately 70% (Tobin et al., 1986), while flunixin meglumine was assumed to be 100%, as it is administered intravenously. Upon taking the bioavailability of these compounds into account, the extrapolated amount of the compounds needing to be administered orally based on our results would be the following: curcuminoids – 269.36, pterostilbene – 4.40-10.07, quercetin – 48.89, resveratrol – 31.37, flunixin meglumine (i.v.) – 3.38, and phenylbutazone – 12.11 mg/kg body weight. These extrapolated concentrations, while shown to be anti-inflammatory in vitro, need to be examined in vivo to determine their anti-inflammatory effects in the horse.

Furthermore, some of these amounts may appear formidable, however it is necessary to note that studies have been conducted in vivo with much smaller amounts yielding biologically beneficial effects. Curcumin administered orally (90 mg/kg) to diabetic rats has improved glucose tolerance and insulin sensitivity (Gutierres et al., 2015), and curcumin given at a low dose of 8 mg/kg in pigs has been shown to decrease transport stress in various biological markers (Wei et al., 2010). Pterostilbene (40 mg/kg) administered orally has yielded powerful antioxidant results in diabetic rats (Amarnath Satheesh and Pari, 2006), and our results suggest that the dose could potentially be lowered and still exhibit beneficial effects. Quercetin (20 mg/kg) given to rats has been shown to decrease adipose inflammation (Vazquez Prieto et al., 2015). Resveratrol administered at a dose of 20 mg/kg to diabetic rats decreased blood lipid and sugar levels (Balata et al., 2016). Although hydroxypterostilbene has been researched little thus far, a study in mice found that hydroxypterostilbene (10 mg/kg, i.p.) exhibited anti-tumor properties including decreased levels of COX-2 (Cheng et al., 2014). Flunixin meglumine has exhibited analgesic properties at a dose of only 1.1 mg/kg i.v. (Foreman and Ruemmler, 2011).
Phenylbutazone at the clinical dose of 4.4 mg/kg has been shown to decrease lameness in the horse when administered i.v. (Foreman and Ruemmler, 2011) as well as decreasing clinical signs of osteoarthritis when administered orally (Doucet et al., 2008). Given the body of literature in other species regarding the biological effects of polyphenols in vivo, it is anticipated that doses lower than those anticipated by the results of this in vitro study may be effective biologically, and perhaps reduce chronic inflammation. Some of these effects may be mediated by polyphenol metabolites not taken into account when studies have examined bioavailability of these compounds previously. Furthermore, many of these polyphenols are known to have poor bioavailability, thus research is currently underway to formulate more bioavailable versions or targeting systems of many of these polyphenolic compounds (Balata et al., 2016; Belcaro et al., 2010a; Nahar et al., 2015; Swaminathan et al., 2016).

In vivo research in the horse examining the anti-inflammatory effects of these polyphenols is clearly warranted. Future research should focus on determining the pharmacokinetics of these compounds in the horse. This will make possible the establishment of a recommended dose, which can then be examined for anti-inflammatory and antioxidant effects in vivo. Safety of the polyphenols should also be monitored in the horse when establishing dose, although long-term tolerability has already been reportedly high in other species for some polyphenols, such as curcumin and resveratrol (Belcaro et al., 2010a; Tome-Carneiro et al., 2012).

In conclusion, our research suggests the polyphenol compounds curcuminoids, resveratrol, quercetin, pterostilbene, and hydroxypterostilbene show potential as anti-inflammatory therapies for chronic inflammation in the horse. More research is warranted
in vivo in the horse to determine the extent to which these compounds may be effective as alternatives to the use of NSAIDs when treating long-term inflammatory conditions.

Acknowledgments

The authors would like to thank Dr. Patrick Lawless of Equithrive® for providing the polyphenol compounds. The authors would also like to thank Mr. Lynn Ennis and the farm staff at the University of Kentucky Maine Chance Farm for their assistance and care of the horses. The authors also recognize the William Robert Mills Endowment, Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, for funding this project.
Figure 5.1. Effect of compound concentrations (ranging from 10 – 320 μM in two-fold dilutions; denoted as C-10—C-320) on % viability of PBMC of n=6 senior horses following incubation with DMSO or compounds and PMA/ionomycin. Compounds include A) Curcuminoids, B) Hydroxypterostilbene, C) Pterostilbene, D) Quercetin, and E) Resveratrol. Bar graphs show mean +/- SD, while box plots show the median, 25th, and 75th percentile. Bars denoted with an asterisk (*) signify a significant difference (p<0.05) in that compound concentration relative to DMSO/PMA, the positive control.
Figure 5.2. Effect of compound concentration on lymphocyte production of pro-inflammatory cytokines interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) using an in vitro model with cells from n=6 senior horses. Compounds include A) Curcuminoids, B) Hydroxypterostilbene, C) Pterostilbene, D) Quercetin, and E) Resveratrol. Compound concentrations ranged from 10 – 320 μM in two-fold dilutions (denoted C-10—C-320), with DMSO/PMA serving as a positive control for inflammation. Bars denoted with an asterisk (*) signify a significant difference (p<0.05) in that compound concentration relative to DMSO/PMA, the positive control. Bars denoted with an asterisk  (** ) indicate no interaction between the cytokines, but an overall significant difference (p<0.05) of inflammatory cytokine production when comparing the compound concentrations to the positive control.
Figure 5.3. Effect of compound concentration on cell viability and lymphocyte production of IFN-\(\gamma\) and TNF-\(\alpha\) for the common NSAIDs flunixin meglumine (FM) and phenylbutazone (Bute). Concentrations ranged from 20 – 160 \(\mu\)M in two-fold dilutions and were compared with DMSO/PMA as a positive control. Bar graphs show mean +/- SD, while box plots show the median, 25\textsuperscript{th} and 75\textsuperscript{th} percentile. Bars denoted with an asterisk (*) signify a significant difference (p<0.05) in that concentration relative to DMSO/PMA, the positive control. Bars denoted with an asterisk ( * ) indicate no interaction between the cytokines, but an overall significant difference (p<0.05) of inflammatory cytokine production when comparing the FM to the positive control.
Figure 5.4. Comparison of the positive control of DMSO/PMA to optimal concentrations for each compound in examining inflammation. Optimal concentrations were determined as the highest concentration of each compound where viability was not significantly different from the positive control. Comparisons of the percentage of lymphocytes producing IFN-γ (A) and TNF-α (C) as well as the mean fluorescence intensity (MFI) for IFN-γ (B) and TNF-α (D) were compared for the optimal concentrations of each compound to the positive control. Bar graphs show mean +/- SD, while box plots show the median, 25th, and 75th percentile. Bars denoted with different letters significantly differ from each other (p<0.05), while those with the same letters do not differ statistically. Compounds are abbreviated [Phenylbutazone (Bute), Flunixin meglumine (FM), Curcuminoids (Cur), Hydroxypterostilbene (Hyd), Pterostilbene (Pter), Quercetin (Quer), and Resveratrol (Res)] followed by the concentration (μM).
Figure 5.5. Effect of optimal compound concentrations on % viability of PBMC following incubation with PMA/ionomycin. Bars denoted with different letters are significantly different from each other (p<0.05), while those with the same letters do not statistically differ. Compounds are abbreviated [Phenylbutazone (Bute), Flunixin meglumine (FM), Curcuminoids (Cur), Hydroxypterostilbene (Hyd), Pterostilbene (Pter), Quercetin (Quer), and Resveratrol (Res)] followed by the concentration (µM).
CHAPTER 6
EFFECTIVENESS OF CURCUMIN IN MODULATING CHRONIC INFLAMMATION OF THE SENIOR HORSE

Abstract

Senior horses (≥20 years) exhibit inflamm-aging, or systemic, chronic, low-grade inflammation associated with aging, similarly to senior humans. In humans, inflamm-aging is associated with morbidity and mortality, with many diseases having underlying inflammatory components. In addition to inflamm-aging, many horses exhibit chronic inflammation, but treating these conditions with non-steroidal anti-inflammatory drugs (NSAIDs) long-term can have negative side effects. As a potential alternative to NSAIDs, an in vivo study was conducted to examine the effects of dietary polyphenolic compound curcumin, found in the root of Curcuma longa, on inflammation in horse, particularly the senior horse experiencing inflamm-aging. Senior horses (n=30) were supplemented with either curcumin (5.6 mg/kg BW of Longvida® containing 20% optimized curcumin) or placebo (4.48 mg/kg BW of background components of Longvida® without curcumin) for 90 days. The effects of curcumin were examined to determine peripheral blood mononuclear cell (PBMC)-mediated cytokine production & gene expression using flow cytometry & RT-PCR, respectively. Prostaglandin E2 (PGE2) production was determined using ELISA to examine the effects of curcumin on the inflammatory cyclooxygenase-2 pathway. Endocrine function was examined as well, specifically basal insulin, basal adrenocorticotropic hormone (ACTH), and ACTH in response to thyrotropin releasing hormone stimulation (a test to determine whether horses exhibit pituitary pars intermedia dysfunction, a common endocrinopathy in older horses). Horses were clinically examined
pre and post study to examine body temperature, heart rate, respiratory rate, and overall health status. Horse body weight and body condition score was also monitored monthly throughout the study. Complete blood count & blood chemistry panels were run pre and post study to monitor health status, as well. Blood was also collected post study to determine curcumin and curcumin metabolite concentrations. No significant differences (p>0.05) were seen between treatment groups over the course of the study for any of the parameters measured. Curcumin and metabolites were only detected in quantifiable amounts for 2/15 horses supplemented with curcumin. In this study, horses supplemented with curcumin did not appear to experience anti-inflammatory effects in relation to the placebo group. These negative results may be due to any number of factors including low dosage and/or low bioavailability of curcumin. Further research is needed to determine if a higher dosage of curcumin can demonstrate bioactive effects in the horse, particularly in regards to decreasing chronic inflammation.

Introduction

Senior horses comprise a significant percentage of the equine population, with estimates ranging from 11.4 to 15% in the United States (≥20 years old) (Malinowski et al., 1997; USDA, 2016) and estimates as large as 29% in the United Kingdom (≥15 years) (Ireland et al., 2011a). However, like humans, senior horses tend to exhibit both inflamm-aging (chronic, low-grade systemic inflammation systemically with aging) and immunosenescence (deterioration of the immune system with aging, particularly adaptive immunity), leading to a generally immune-compromised state (Adams et al., 2008; Franceschi et al., 2000; Franceschi et al., 2007). Inflamm-aging has been characterized in both gene expression and proteins in whole blood as well as specifically being mediated
by lymphocytes and monocytes (Adams et al., 2008; Adams et al., 2009). Thus far, clearly
established clinical conditions of the horse associated with inflamm-aging have not been
discovered. Based on human studies, however, inflamm-aging is associated with morbidity
and mortality (Adriaensen et al., 2015; Franceschi and Campisi, 2014).

Common treatments for localized and chronic inflammation of the horse include
non-steroidal anti-inflammatory drugs (NSAIDs), such as phenylbutazone and flunixin
meglumine. These NSAIDs are a non-specific cyclooxygenase (COX) inhibitors, meaning
that they inhibit both COX-1 and COX-2; this has specifically been shown in horses
(Brideau et al., 2001; Duz et al., 2015). Inhibition of COX-1 is frequently associated with
secondary problems, most notably gastric ulcers (Carvajal et al., 2004; Monreal et al.,
2004), since COX-1 protects the glandular lining of the stomach. Phenylbutazone
specifically has created oxidative stress in the stomach mucosa of horses (Martinez
Aranzales et al., 2014). Other adverse effects of NSAIDs may include liver and kidney
damage, hypoalbuminemia, and neutropenia (Bessone, 2010; Ingrasciotta et al., 2015;
McConnico et al., 2008; Reed et al., 2006). NSAIDs targeting COX-2 have been developed;
however, even these NSAIDs are not intended for ongoing use. For example, firocoxib
(Equioxx®, Merial), one of the most commonly used COX-specific NSAIDs in horses, has
a maximum of 14 days of intended use and must be prescribed by a veterinarian, according
to the manufacturer.

Despite the benefits of using NSAIDs for treating acute inflammatory conditions,
long-term use of NSAIDs in treating chronic conditions can have detrimental effects. Thus,
the potential of anti-inflammatory phytonutrient curcumin to mitigate chronic
inflammation of the senior horse was examined.
Curcumin has been used in traditional Asian medicine for treating various inflammatory diseases (Jeenger et al., 2014; Jurenka, 2009); however, recently curcumin has gained a scientific reputation as a potent anti-inflammatory, antioxidant, and anti-tumorigenic compound (Franck et al., 2008; Schaaf et al., 2009; Venkatesan and Chandrakasan, 1995). This polyphenol comprises 0.3% to 5.4% of raw turmeric, the cooking spice which is derived from the rhizomes of *Curcuma longa* (Jeenger et al., 2015; Khan and Abourashed, Hoboken, New Jersey). In food-grade curcumin, the compound curcumin itself constitutes 77%, while other curcuminoids demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC) only make up 17% and 6%, respectively (Kumar et al., 2010).

The number of mechanisms by which curcumin is thought to act is vast (Di Pierro et al., 2013). However, many anti-inflammatory activities of curcumin are thought to be orchestrated through down-regulating the nuclear factor–kappa B (NF-κB) and COX pathways. For the NF-κB pathway: Curcumin has been found to decrease IκB kinase (IKK) activity, thereby decreasing IκBα phosphorylation, which in turn decreases NF-κB activation, thus decreasing its gene products, such as IκBα, interleukin(IL)-6, IL-12, tumor necrosis factor-alpha (TNF-α), and COX-2 (Bharti et al., 2003; Nanji et al., 2003). The COX pathway may actually be a specific cascade of the NF-κB pathway, as it has been suggested that NF-κB upregulates COX-2 expression (Kim et al., 2006). COX-2 catalyzes the conversion of arachidonic acid into pro-inflammatory prostaglandin E2 (PGE2) (Chizzolini and Brembilla, 2009; Park and Conteas, 2010). COX-1 is constitutively expressed, while COX-2 is inducible. Curcumin has been shown to selectively inhibit COX-2, without interfering with COX-1 (Goel et al., 2001).
The effectiveness of curcumin as an anti-inflammatory agent has been extensively exemplified in vitro. Since curcumin is a lipophilic molecule, it can easily penetrate the phospholipid bilayer of cells and may even be capable of mediating some of its actions through influencing lipid rafts of cell membranes (Derochette et al., 2013; Lin et al., 2014). Some studies have shown that curcumin decreases inflammation and oxidative stress when taken orally (Venkatesan and Chandrakasan, 1995). However, curcumin is rapidly metabolized into curcumin sulfate or curcumin glucuronide after oral administration (Kumar et al., 2010). Thus, it is often unable to reach immune cells while still in its bioactive state.

Since this issue of bioavailability is a known hurdle, various studies have been conducted in an endeavor to generate more bioactive analogs of curcumin. Stabilizing curcumin by solid lipid curcumin particle (SLCP)™ technology has met with success in the formulation of Longvida® optimized curcumin, which employs a lipophilic matrix to deliver curcumin into the bloodstream and target tissues (Gota et al., 2010). Longvida® has been shown to improve mood and working memory (Cox et al., 2015) and to decrease pathophysiology associated with Alzheimer’s disease (decreasing amyloid plaques, insoluble β-amyloid peptide, and soluble Tau dimers)(Begum et al., 2008; Ma et al., 2013), while decreasing inflammation [interleukin(IL)-1β, inducible nitric oxide synthase (iNOS), and upstream c-Jun N-terminal kinase (JNK)](Begum et al., 2008). In vitro studies have also shown that Longvida® decreases inflammation by decreasing nitric oxide, prostaglandin E2, and IL-6 via downregulating NF-κB activity(Nahar et al., 2014). Recently, oral supplementation with Longvida® has also demonstrated anti-inflammatory effects, decreasing the degree of systemic inflammation (IL-8, TNF-α) associated with
exercise-induced muscle damage (McFarlin et al., 2016). This body of evidence taken together asserts that solid lipid particle curcumin has great potential as an anti-inflammatory treatment.

In relation to the potential of curcumin use in the horse specifically, a few studies have been performed examining curcumin as an anti-inflammatory and antioxidant. Most studies have primarily focused on its in vitro effects, which have yielded favorable results, suggesting the use of curcumin for its anti-inflammatory and anti-oxidant properties (Derochette et al., 2013; Franck et al., 2006; Franck et al., 2008). Our lab has found curcumin to have potent anti-inflammatory effects in vitro specifically in the senior horse (Siard et al., 2016). One small scale study in vivo has been performed in the horse with some beneficial effects, showing decreased IL-1β and IL-1RN expression and apparent, though not statistically significant, reductions in COX-2, TNF-α, and IL-6 expression in osteoarthritic mares (Farinacci et al., 2009a). However, numerous limitations were present, including a short study duration of only fifteen days, small sample size (n=7), and no placebo group to serve as a control (Farinacci et al., 2009a). Moreover, no studies have been conducted in the senior horse.

Thus, we performed the first large-scale study to determine the effectiveness of curcumin in the senior horse in vivo, comparing its anti-inflammatory action to a control placebo group. It was hypothesized that treatment with curcumin would decrease inflammation at both the gene expression and protein levels in senior horses. Specifically, this study was performed to determine the effects of curcumin supplementation on PBMC-mediated inflammatory cytokine expression and production, COX-1 and COX-2 gene expression, and PGE2 production in senior horses. It is anticipated that decreasing
inflamm-aging may be beneficial to healthspan and lifespan. Furthermore, the horse exhibiting inflam-aging was used as a model to determine whether polyphenols could decrease chronic inflammation.

**Methods and Materials**

2.1. *Animals and Supplementation.*

Thirty senior horses (mean age = 26.2±3.2 years) of mixed breeds and both sexes were fed a mixture of 50% alfalfa pellets and 50% oats twice per day (at 0830 and 1400 hours) at maintenance. The horses were housed on pasture (with mixed grass hay supplemented during early spring) at the University of Kentucky, Department of Veterinary Science, Woodford Farm. The study was conducted from late March to late June 2016.

Treatment groups were determined by blocking horses based on body condition score (BCS), age, inflammatory status as determined by percent of lymphocytes producing IFN-γ (see Sections 2.3 & 2.4), and pituitary function using a thyrotropin releasing hormone (TRH) test (see 2.7). BCS was determined using the Henneke scale of 1-9 (Henneke et al., 1983).

The horses daily received 5.6 mg/kg BW of Longvida® supplement (containing 20% of optimized curcumin; Verdure Sciences, Noblesville, IN) (n=15) (Farinacci et al., 2009a) or 4.48 mg/kg BW of background placebo (n=15) for 90 days. This dose of Longvida® curcumin was chosen by extrapolating from human Longvida® studies with biological effects (Cox et al., 2015; McFarlin et al., 2016). Longvida® and placebo were provided by Verdure Sciences. Longvida® and placebo supplements were suspended in
soybean oil (approximately 25 mL) and administered orally by syringe once per day with the morning meal.

A veterinarian who was blinded in regard to treatment groups examined the horses prior to and post study to determine heart rate, respiratory rate, rectal temperature, and overall health status. All procedures were in accordance with the University of Kentucky’s Institute of Animal Care and Usage Committee.

2.2. Sample Collection.

Heparinized blood was collected from horses for PBMC isolation prior to receiving any treatments (Day 0) and on Days 15, 30, 60, and 90 after receiving treatment. Serum also collected pre and post study prior to feeding and treatment to determine basal insulin concentrations. TRH stimulation testing was performed pre and post study, with EDTA plasma collected pre and 10 minutes post TRH injection to determine ACTH concentrations. Blood was also collected into citrate tubes post study to determine curcumin and curcumin metabolite concentrations. All blood sampling (except serum for basal insulin, as noted) occurred approximately 1.5 hours after being fed (similar to a previous study in the horse (Farinacci et al., 2009a)). Horse body weights and BCS (by three assessors) were also recorded pre study and monthly thereafter for the duration of the study.

2.3. PBMC Isolation & Culture.

Peripheral blood mononuclear cells (PBMC) were isolated and purified from heparinized blood using a Ficoll-Paque Plus™ (Amersham Biosciences, Piscataway, NJ) density gradient and Phosphate Buffered Saline (PBS), respectively (Adams et al., 2008;
Breathnach et al., 2006; Siard et al., 2016). PBMC were counted using a VICELL™ Counter-XR (Beckman Coulter, Miami, FL) in order to plate cells at a concentration of $4 \times 10^6$ cells/mL media [RPMI 1640 (Gibco, Grand Island, NY) supplemented with 2.5% fetal equine serum (FES; Sigma-Aldrich, St. Louis, MO), 100 μM 2-mercaptoethanol (Gibco), and 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/mL streptomycin (HyClone Pen/Strep/Glutamine solution; Thermo Scientific)] (Adams et al., 2008; Breathnach et al., 2006; Siard et al., 2016). PBMC were plated in duplicate, with all wells receiving Brefeldin A (10 μg/mL; Sigma), while only one well per sample was stimulated with phorbol 12-myristate 13-acetate (PMA; 25 ng/mL; Sigma) and ionomycin (1 μM; Sigma) (Adams et al., 2008; Breathnach et al., 2006; Siard et al., 2016). The cells were then incubated at 37°C, 5% CO₂ for 4 hours prior to intracellular staining and RNA isolation (Adams et al., 2008; Breathnach et al., 2006; Siard et al., 2016).

2.4. Intracellular Staining and Flow Cytometry.

Following incubation, PBMC were transferred into duplicate 96-well V-bottom plates, fixed with 2% paraformaldehyde (Sigma), and stored overnight at 4°C. Cells were then perforated and washed with saponin buffer [PBS supplemented with 1% fetal bovine serum (FBS), 0.1% saponin, and 0.1% sodium azide (Sigma)]. One plate was intracellularly stained with IFN-γ FITC mouse anti-bovine antibody (AbD Serotec, Raleigh, NC; 0.1 mg). The second plate was stained with TNF-α anti-equine monoclonal antibody (HL801; kindly provided by Dr. Rob MacKay, University of Florida) and secondary antibody FITC-conjugated goat F(ab’)₂ anti-mouse IgG (H + L) (Invitrogen; 2 mg/mL). Following intracellular staining, cells from both plates were resuspended in FACS Flow and analyzed using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) and Cell Quest®.
software (Becton Dickinson). Data were gated on lymphocyte populations to determine lymphocyte mediated production of IFN-γ and TNF-α.

2.5. RNA Isolation, Reverse Transcription, and Real-Time Polymerase Chain Reaction.

Following the 4 hour incubation, PBMC were resuspended in Trizol® solution and stored at -80°C until RNA was isolated using phenol-chloroform extraction. Following quantification with an Epoch microplate spectrophotometer (BioTek, Winooski, VT), RNA (1 μg) was reverse transcribed in a thermocycler (Bio-Rad, Hercules, CA) after the addition of master mix (16 μL avian myeloblastosis virus (AMV) buffer 5X, 16 μL MgCl₂, 4 μL dNTP, 1 μL RNasin, 1 μL Oligo dT primer, and 0.5 μL AMV Reverse Transcriptase per sample; Promega, Madison, WI) to the samples. The thermocycler incubated samples at 42°C for 15 minutes and 95°C for 5 minutes. The cDNA samples then underwent RT-PCR after the epMotion 5070 (Eppendorf) loaded plates with the samples and 11 equine specific intron-spanning primers and probes (Applied Biosystems), specifically IFN-γ, TNF-α, IL-1β, IL-6, IL-12, IL-4, IL-10, Tbet, GATA3, COX-1, and COX-2 in addition to Beta-glucuronidase (BGUS), the housekeeping gene. During RT-PCR, samples were incubated at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15s and 60°C for 60s using the 7900HT Fast RT-PCR System (Applied Biosystems, Foster City, CA). The ΔΔCT method (Livak and Schmittgen 2001) will be used to determine relative changes in cytokine gene expression, with data being reported as relative quantity (RQ) values according to the equation $RQ = 2^{-\Delta\Delta CT}$. The average ΔCT of all media alone samples on Day 0 will serve as the calibrator for each cytokine.
2.6. PGE2 ELISA.

Serum was collected on Days 0, 30, and 90 to determine prostaglandin E2 content. Serum was assayed using a Parameter PGE2 competitive ELISA (R&D Systems, Minneapolis, MN), according to manufacturer protocol (Shah et al., 2010). This PGE2 ELISA has previously been used to analyze equine samples due to the consistency of arachidonic acid derivative PGE2 among species (de Grauw et al., 2006; de Grauw et al., 2009; Lucia et al., 2013).

Briefly, calibrator diluent was added to non-specific binding wells (200 µL) and zero standard wells (150 µL). Standards, controls, and samples (ranging from undiluted serum to 1:18, with most samples being diluted 1:3) were added to wells in duplicate (150 µL), followed by primary antibody solution (50 µL; in all wells but non-specific binding). Plates were shaken for 1 hour (500 ± 50 rpm; 0.12” orbit), followed by the addition of PGE2 conjugate (50 µL) and an additional 2 hour incubation on a shaker. Plates were then washed four times, and substrate solution (200 µL) was added, with plates being incubated at room temperature in the dark for 30 minutes. Stop solution (100 µL) was then added, and absorbance of each sample was measured at 450 nm with a 540 nm correction using a colorimetric ELISA microplate reader (Bio-Rad Laboratories, Inc., Philadelphia, PA). The mean intra-assay coefficient of variation was 7.2%.

2.7. Endocrine Function.

The horses were sampled to determine pituitary function, particularly examining the presence of pituitary pars intermedia dysfunction (PPID), an endocrinopathy caused by hyperplasia of the pituitary pars intermedia. Thyrotropin releasing hormone (TRH) stimulation was performed to determine pituitary function by exogenous injection of TRH
(1 mg/mL saline per horse; i.v.; Sigma-Aldrich) and analyzing EDTA plasma ACTH concentrations prior to and at 10 minutes post injection with TRH. EDTA plasma was shipped to the Cornell Animal Health Diagnostic Center (AHDC) for ACTH analysis using chemiluminescence immunoassay (Immulite® 1000; Siemens, Berlin, Germany)(Place et al., 2010). TRH stimulation was performed prior to and post study, approximately 1.5 hours post feeding and supplementation with curcumin or placebo.

The horses were also sampled prior to feeding to determine serum basal insulin pre and post study, with serum samples being analyzed by Cornell AHDC using a commercially available radioimmunoassay for human insulin (RIA) (EMD Millipore Corp, Billerica, MA) that has previously been validated for use in equine samples.

2.8. Plasma Amyloid-β ELISA.

EDTA plasma was collected on Days 0 and 90 and frozen at -80°C until analyzed for amyloid-β (Aβ) peptide concentrations by Dr. Michael Paul Murphy, Sanders-Brown Center on Aging, University of Kentucky. A two-site sandwich ELISA was used to measure Aβx-42 in plasma samples, as previously described (Beckett et al., 2010). (Aβ is well-conserved among higher mammals (Johnstone et al., 1991), thus a human ELISA was used.) Briefly, an Immulon 4HBX plate (Dynex-Thermo Fisher) coated with 0.5 µg/well of 2.1.3 monoclonal (end specific for Aβ42) capture antibody (Das et al., 2001; Kukar et al., 2005), was incubated at 4° C overnight prior to blocking with Synblock (AbD Serotec, Raleigh, NC), according to manufacturer protocol. Biotinylated 4G8 (against Aβ17-24; BioLegend, San Diego, CA) antibody was used for antigen detection, followed by incubation with 0.1 µg / ml of neutravidin-HRP (Pierce Biotechnologies; Rockford, IL).
TMB reagent (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and 6% o-phosphoric acid solution were used to develop and stop the reaction, sequentially. A multi-well plate reader (Bio Tek, Winooski, VT) read the plates at 450 nm. The Aβ peptide standard curve samples and plasma samples were run at least in duplicate.

2.9. CBC with Differential & Chemistry Panel.

Lithium heparinized whole blood was collected at Day 0 and Day 90 and sent to Rood and Riddle Equine Hospital Laboratory to analyze complete blood count with differential and blood chemistry panel pre and post study to examine overall health of the horses. This specifically measured hemoglobin (Hgb), packed cell volume (PCV), red blood cells (RBC), white blood cells (WBC), total protein, mature neutrophils (seg), immature neutrophils (bands), lymphocytes (lymph), monocytes (mono), eosinophils (eos), sodium, potassium, chloride, albumin, serum glutamic oxaloacetic transaminase/aspartate aminotransferase (SGOT/AST), alkaline phosphatase, total bilirubin (T. Bili), direct bilirubin (D. bili), creatine kinase (CK), creatinine, glucose, gamma-glutamyl transferase (GGT), blood urea nitrogen (BUN), phosphorus, calcium, sorbitol dehydrogenase (SDH), and lactate dehydrogenase (LDH).

2.10. Curcumin and Curcumin Metabolite Measurement.

Plasma, buffy coat, and red blood cells were collected post study approximately 1.5 hours post treatment and feeding using tubes containing acid citrate dextrose additives (Solution A - 22.0 g/L trisodium citrate, 8.0 g/L citric acid, 24.5 g/L dextrose; BD Vacutainer®, Franklin Lakes, NJ) and immediately placed on ice. Within 1 hour of collection, blood was centrifuged at 1500g for 15 minutes at 4°C. Aliquots of plasma, buffy
coat, and red blood cell samples were then stored at -80°C until shipped to Dr. Navindra Seeram at the University of Rhode Island for analysis via ultra fast liquid chromatography tandem mass spectrometry (UFLC-MS/MS). UFLC-MS/MS was used to determine content of curcumin, demethoxycurcumin (DMC), bisdemethoxycurcumin (BDMC), curcumin sulfate, curcumin glucuronide, and ferulic acid for n=3 placebo and all n=15 curcumin-receiving horses.

Briefly, UFLC-MS/MS was performed with a modified protocol from analyzing polyphenol quantities in maple-derived foods (Liu et al., 2016b) to determine curcumin and curcumin metabolite concentrations in buffy coat and red blood cells. Prominence UFLC (SHIMADZU, Marlborough, MA) with a Waters XBridge® BEH C18 column (100 mm × 2.1 mm i.d., 2.5 μm. Milford, MA, USA) were used to perform chromatography, while mass spectrometry was performed using a QTRAP 4500 system (Applied Biosystems, Framingham, MA) with electrospray ionization. Samples were analyzed in Multiple Reaction Monitoring mode using Analyst 1.6.3 software and MultiQuant 3.0.1 software. The calibration curve ranged from 1-1000 ng/mL for curcumin and ferulic acid and from 0.1-1000 ng/mL for DMC, BDMC, curcumin sulfate, and curcumin glucuronide and were analyzed using linear regression following x² transformation. Urolithin B was used as the internal standard. Acetonitrile was used to denature proteins. Curcumin compounds were extracted from 200 µL of sample (buffy coat or RBC). The mobile phase consisted of 43% acetonitrile with 0.1% formic acid. The flow rate was 0.2 mL/min with a total run time of 9 min and an injection volume of 10 µL. The assay validation was performed in a forthcoming publication in regards to specificity, accuracy and precision.
(intra-day and inter-day), recovery and matrix effect, and stability using n=6 horses and all three blood fractions (plasma, buffy coat, and red blood cells).

2.11. Statistical Analysis.

To determine sample size for treatment groups, SigmaPlot version 10.0 and SigmaStat version 3.5 were employed using mean difference and standard deviation from a data set in a previous study in which a group receiving supplement was compared to a group receiving placebo. The power level was set at 0.8 and alpha was 0.05.

In this study SigmaPlot version 12.0 was used to perform two-way analysis of variance (ANOVA) tests with repeated measures to compare treatment groups over time for Aβ, ACTH, insulin, PGE2, flow cytometry, and normally-distributed CBC/Chem data.

SAS 9.4 software was used to analyze body weight, PCR gene expression, and some CBC/Chem parameters (Bands, Monocytes, Potassium, SGOT/AST, CK, Phosphorous, SDH, and GGT), as these data were not normally distributed when analyzed in SigmaPlot. However, SAS is more robust and met skewness and kurtosis requirements for each of the variables, (although some required log-transformation). Data was analyzed using a mixed procedure examining time point, treatment group, and time point x treatment group as fixed effects, with time point as the repeated variable. PGE2 analysis only included data from n=12 curcumin and n=11 placebo-receiving horses, as some were excluded due to high coefficients of variation.

For all analyses, data with p<0.05 were considered statistically significant and p<0.10 were considered trends.
Results

3.1. Inflammatory cytokine and PGE2 production.

Inflammatory cytokine production by lymphocytes was determined using flow cytometry. Specifically, percent of lymphocytes producing IFN-γ (Fig. 6.1A) and TNF-α (Fig. 6.1C) as well as mean fluorescence intensity of lymphocytes for each of each of these cytokines (Fig. 6.1B&D) was examined. Similar patterns in results for these cytokine measures were observed. Particularly, no differences between curcumin and placebo-treated horses were found (p>0.05). Significant differences over time were observed (p<0.001) with an overall increase in inflammatory cytokine production over the course of the study. However, no significant interactions between treatment group and time were found (p>0.05).

Curcumin and placebo-treated groups were also compared in relation to serum concentrations of pro-inflammatory prostaglandin E2 (Fig. 6.2) using a commercial ELISA kit (R&D Systems), and no differences were seen between treatment groups (p=0.509) or over time (p=0.673), as well as no interaction of treatment group with time (p=0.877).

3.2. Real-Time Polymerase Chain Reaction Gene Expression.

Gene expression mediated by PBMC was determined by RT-PCR (Table 6.1). Many variables showed significant differences over time, including: Log(COX-1), GATA3, Log(IFN-γ), IL-1β, IL-4, Log(IL-6), Log(IL-10), Log(IL-12), Log(Tbet), and Log(TNF-α) (p<0.05) (Table 6.1). Overall, these variables tended to decrease at Day 30 and increase again thereafter. Log(COX-2) and Log(IL-6) also showed differences between treatment groups (p<0.05) (Fig. 6.3A&B); however, there were no treatment over time
interactions (p>0.05) (Table 6.1). Additionally, the differences in treatment groups for Log(COX-2) and Log(IL-6) appeared to largely be present at the beginning of the study, prior to supplementation (Fig. 6.3A&B).

3.3. Endocrine Parameters.

Endocrine measures were also examined pre and post study to determine any differences in regards to supplementation with curcumin. Plasma ACTH was analyzed both basally and 10 minutes post TRH injection (T-10). No differences between curcumin and placebo treatment groups were found (p>0.05) (Fig. 6.4). Basal ACTH exhibited a trend over time (p=0.066) (Fig. 6.4A), and T-10 ACTH exhibited significant differences over time (p=0.020), with both these measures of ACTH increasing during the study (Fig. 6.4B); however, no interactions between treatment groups over time were observed (p>0.05).

No significant differences in treatment groups were seen for serum insulin concentrations (p>0.05) (Fig. 6.5). Over time, insulin concentrations significantly increased (p<0.001), but no interactions between treatment group and time were observed (p>0.05) (Fig. 6.5).

3.4. Plasma Aβ concentrations.

Plasma Aβ was also examined as it has been associated with Alzheimer’s disease in humans, and some similarities have been suggested between PPID of the horse and Alzheimer’s disease in humans, as both are neurodegenerative diseases occurring primarily in the elderly. Additionally, Longvida® curcumin has helped decrease some pathophysiology associated with Alzheimer’s disease in a mouse model (Ma et al., 2013). No differences in plasma Aβ concentrations were seen in treatment groups overall (p>0.05)
Significant differences were observed over time (p<0.001), with Aβ concentrations increasing after 90 days of treatment with curcumin or placebo (Fig. 6.6). A trend was observed for differences between treatment groups over time (p=0.094) (Fig. 6.6). Plasma Aβ concentrations at baseline of placebo-receiving horses were marginally less than those for curcumin-treatment horses, while post-study Aβ values were slightly greater for placebo-treated horses than for curcumin-treated horses, suggesting that curcumin may decrease the extent to which Aβ values would otherwise increase. However, this interaction was only a trend with no significant differences between treatment group either pre or post study and no significant overall treatment effect (p>0.05).

3.5. Complete Blood Count and Blood Chemistry Analysis.

For CBC and blood chemistry panel parameters, no differences were found in regard to treatment group alone (p>0.05). Many variables among CBC and blood chemistry significantly differed with time (p<0.05) including: PCV, RBC, WBC, Log(GGT), phosphorus, total protein, calcium, sodium, albumin, alkaline phosphatase, direct bilirubin, total bilirubin, glucose, BUN, and LDH. Among these, albumin was the only variable exhibiting a significant interaction with treatment group over time (p<0.05), with placebo-receiving horses having increased albumin levels over time, while curcumin-receiving horses did not (Fig. 6.7A). Direct bilirubin also exhibited a trend (p=0.066) for interactions between treatment groups and time point (Fig. 6.7B). The placebo group appeared to have elevated direct bilirubin prior to the study, but these concentrations dropped during the study, while the curcumin-supplemented horses remained at similar levels (Fig. 6.7B).
3.6. Body Weight, BCS, TPR, and Overall Health Parameters.

No significant changes were seen in body weight either over time or in regard to treatment group (p>0.05) (Fig. 6.8A). Average body condition scores differed only in regard to time (p<0.05) and not treatment group (p>0.05), with BCS increasing over time (Fig. 6.8B). No adverse side effects were observed for horses supplemented with curcumin in comparison to the placebo group regarding body temperature, respiratory rate, heart rate, nasal discharge, or overall health (data not shown).

3.7. Curcumin & Curcumin Metabolite Measurement.

Buffy coat and red blood cell (RBC) fractions of the blood were each analyzed to determine curcumin, DMC, BDMC, curcumin sulfate, curcumin glucuronide, and ferulic acid for n=3 placebo and all n=15 curcumin-receiving horses. The placebo horses were analyzed post study to ensure that they were negative for curcumin and metabolites, and as expected, the horses were negative for all 6 compounds in both blood fractions. Additionally, the curcumin-receiving horses were all analyzed post study, and only 2 horses had quantifiable levels of curcumin, DMC, and BDMC (detected in the RBC fraction from one horse, and both RBC and buffy coat from the other horse).

Discussion

Curcumin has exhibited anti-inflammatory effects in a multitude of studies; however, very few studies have been performed in the horse examining curcumin as an anti-inflammatory. Previously our lab has characterized the in vitro anti-inflammatory effects of curcumin on PBMC from senior horses (≥20 years) experiencing chronic, systemic inflammation known as inflamm-aging (Siard et al., 2016). However, only two
small-scale studies (without control groups) have previously explored the anti-inflammatory effects of dietary curcumin on the horse in vivo (Farinacci et al., 2009a; Schell, 2009). Thus, the present study was undertaken to determine the effectiveness of dietary curcumin as an anti-inflammatory supplement in the senior horse.

No appreciable differences were seen in various inflammatory markers for senior horses administered curcumin compared to placebo in this study. Specifically, horses supplemented with curcumin were expected to have decreased lymphocyte-mediated pro-inflammatory cytokine expression and production compared to horses receiving placebo; however no differences between groups were found. Anti-inflammatory cytokines were also expected to potentially increase, as curcumin has many anti-inflammatory mechanisms of action, but again, no differences in treatment groups were present. Gene expression of inflammatory cytokine IL-6 exhibited an overall treatment group effect (p<0.05); however, there was no interaction between treatment group and time (p>0.05). Additionally, the difference in IL-6 between treatment groups appeared to be present prior to the beginning of the supplementation study; thus, the overall difference in treatment group is likely not due to curcumin supplementation, especially since the difference of means at the culmination of the study on Day 90 is numerically less than at Day 0 (0.06 and 0.11, respectively).

Curcumin is known to selectively inhibit pro-inflammatory COX-2 (Goel et al., 2001), thus the curcumin-supplemented group was expected to decrease COX-2 gene expression compared to the placebo group without inhibiting COX-1 expression. As expected, there were no differences in COX-1 between treatment groups. In regard to COX-2, overall differences in treatment group were present; however, no interaction effect
of treatment group over time was present for COX-2. Since there was no treatment by time point interaction, and since COX-2 in placebo-treated horses was numerically greater (0.16) prior to the beginning of the study, the overall significant difference in COX-2 between treatment groups is likely not due to treatment with curcumin. Additionally, horses in the two groups appear to have equalized COX-2 expression by Day 90, (with the placebo group even having a slightly lower mean of 2.34 as compared to the curcumin group mean of 2.40). Thus, COX-2 in the curcumin-supplemented group did not decrease in relation to the placebo group, which was unexpected.

Furthermore, pro-inflammatory PGE2 in serum also did not exhibit significant differences in regards to treatment group or over time. Again, this was not originally anticipated, as the horses supplemented with curcumin were expected to have decreased levels of inflammatory PGE2 when compared to the placebo group. However, since no appreciable differences were seen in the interaction of treatment group over time for COX-2, which is upstream of PGE2 in the pro-inflammatory signaling cascade as a catalyst for the conversion of arachidonic acid to PGE2 (Chizzolini and Brembilla, 2009; Park and Conteas, 2010), the lack of a treatment effect for the horses supplemented with curcumin is not surprising.

In previous studies, many of the anti-inflammatory effects of curcumin were demonstrated in vitro, where the known hurdle of bioavailability is not an issue. However, due to the enhanced bioavailability of Longvida® curcumin (Gota et al., 2010), the effectiveness of curcumin as an in vivo anti-inflammatory was not expected to be impeded due to bioavailability. Furthermore, several in vivo studies with curcumin-containing products with enhanced bioavailability including Longvida® have demonstrated biological
effects (Cox et al., 2015; Ma et al., 2013). However, few dietary Longvida® curcumin studies have focused on anti-inflammatory effects of curcumin. One study found lower exercise-induced inflammation (TNF-α and IL-8) in subjects receiving Longvida® curcumin in comparison to placebo (McFarlin et al., 2016). However, another study found serum inflammatory cytokine levels were below detection limits for most parameters analyzed, and C-reactive protein levels did not differ in Longvida® curcumin-receiving subjects (Cox et al., 2015).

In addition to determining effects of curcumin supplementation on inflammation, this study examined the effects of curcumin supplementation on endocrine function, specifically regarding basal insulin, basal ACTH, and ACTH response to TRH stimulation. Horses experience various endocrine disorders, with two of the most common being equine metabolic syndrome (EMS) and pituitary pars intermedia dysfunction (PPID). EMS is similar to metabolic syndrome in humans in that it involves regional adiposity, insulin resistance (which may include hyperinsulinemia), and sometimes heightened levels of triglycerides (Frank et al., 2010b). EMS is particularly problematic due to its associated predisposition to laminitis, an inflammatory condition of the hoof that can be fatal to horses (Frank et al., 2010b; Johnson et al., 2012). Curcumin has been shown to decrease triglycerides and insulin resistance (Ghorbani et al., 2014; Panahi et al., 2014; Song et al., 2015b), thus it was thought that curcumin may be able to mitigate EMS in the horse. PPID is common in the senior horse, caused by dopaminergic neurodegeneration of the pars intermedia lobe of the pituitary and frequently resulting in hypertrichosis, polydipsia, polyuria, hyperhidrosis, laminitis, muscle atrophy, and abnormal fat distribution (McFarlane, 2011). Endocrinologically, this frequently results in increased levels of basal
circulating adrenocorticotropin hormone (ACTH), while also resulting in decreased ability of horses to return to resting levels of ACTH after intravenous injection of thyrotropin releasing hormone (TRH) (Beech et al., 2007). Curcumin has been shown to exhibit anti-tumor activity, including in the pituitary (Bangaru et al., 2010; Curic et al., 2013; Schaaf et al., 2009; Shan et al., 2012), specifically decreasing hormone levels associated with pituitary tumors (Miller et al., 2008a; Schaaf et al., 2009). Thus, it was expected that senior horses supplemented with curcumin would have improved endocrine function, both increasing insulin sensitivity and decreasing excessive production of ACTH in response to TRH stimulation. However, no differences in endocrine function were found for horses supplemented with curcumin in comparison to placebo.

In addition to examining endocrine dysfunctions in the horse, plasma Aβ was also examined. Amyloid-β production and deposition is one of the two primary pathologies associated with Alzheimer’s disease (Murphy and LeVine, 2010). Since Longvida® curcumin has previously decreased various Alzheimer’s-associated pathologies (Begum et al., 2008; Ma et al., 2013), and since both the PPID horse and Alzheimer’s patients experience age-related neurodegeneration, examining plasma Aβ was of interest. However, no differences in plasma Aβ were seen between treatment groups in this study. A trend was present in the interaction between time point and treatment group for Aβ, but no significant differences between treatment groups occurred. There was, however, a significant overall time effect. Variations in Aβ with season have not yet been investigated, although in this study, it appears that season or some other condition experienced by this group of senior horses in general seems to influence levels of circulating Aβ. Although horses would most likely be more apt to exhibit seasonal changes than humans due to their outdoor housing,
it may bear investigation to determine how easily plasma Aβ may be influenced by conditions aside from the presence of Alzheimer’s disease.

Many inflammatory and metabolic parameters increased over the course of the study for horses regardless of treatment group, which may be due to seasonal effects. Insulin has previously been shown to vary seasonally in equids, particularly for those grazing on pasture, as insulin was correlated with grass carbohydrate composition (Borer-Weir et al., 2013; Frank et al., 2010a). Likewise, ACTH has known seasonal rhythms in the horse as well (Cordero et al., 2012; Frank et al., 2010a). Inflammatory markers have exhibited seasonal rhythms in other species (Bilbo et al., 2002; Bowden et al., 2007; McFarlane et al., 2012), though work in the horse regarding seasonal inflammatory parameters is limited thus far with only a few studies showing seasonal fluctuations in unhealthy animals (Beadle et al., 2002; Hamza et al., 2007). Although this study was conducted in spring/summer (from late March to late June) to control for changes in season as much as possible, the results of this study show an overall increase in many inflammatory and metabolic parameters, suggesting that similarly to other species, seasonal variation of inflammatory markers may occur in the healthy horse as well. Additionally, these increases in inflammatory markers over the course of the study may at least in part be due to the increased body condition scores, which indicate an increase in body fat. And, increased body fat in the horse specifically has been associated with increased PBMC-mediated inflammation (Adams et al., 2009).

Regarding the lack of curcumin and curcumin metabolites in blood fractions for all but two of the horses supplemented with curcumin, several factors may contribute. First, the curcumin may truly not have reached the bloodstream, in which case it would be
expected that curcumin glucuronide and curcumin sulfonate would be present (Kumar et al., 2010); however, this does not appear to be the case. Second, the timing of the blood collection may not have been ideal to detect curcumin and metabolites for most of the horses despite the fact that quantifiable levels were present for two of the horses. Studies have shown that some curcumin formulations are rapidly absorbed into tissues and spend very little time in the bloodstream, yielding bioactive effects even at undetectable plasma levels (Pawar et al., 2012). This is entirely possible however does not explain the lack of biological effects seen in this study. Third, the dose and/or formulation of curcumin was not effective to reach peak levels in the blood for most horses and thereby have biological effects in the horse.

The dose of curcumin certainly may be the primary reason that supplementation did not demonstrate biological anti-inflammatory effects. In many previous studies where oral curcumin has been used to correct pathophysiology, the dose was often larger in relation to mg/kg body weight (Kumar et al., 2011; Maithilikarpagaselvi et al., 2016; Venkatesan and Chandrakasan, 1995). Additionally, previous work in our lab showing in vitro anti-inflammatory effects of curcumin on PBMC from senior horses indicated a much higher concentration of curcumin (without enhanced bioavailability) needed to achieve biological effects (Siard et al., 2016). However, the dose used in the current study was originally thought to be sufficient due to: 1) the enhanced bioavailability of Longvida® curcumin (Gota et al., 2010), 2) the length of study (90 days, whereas several other studies were shorter term), and 3) the quantity of curcumin already being given to horses, even at a lower mg/kg body weight dose.
Another potential reason anti-inflammatory effects of curcumin were not seen in this study may be that naturally-occurring basal inflamm-aging alone was not the best model in which to evaluate the effectiveness of curcumin as an anti-inflammatory supplement. Perhaps inflamm-aging of the senior horse was not as readily responsive to anti-inflammatory supplements as experimental models. Most studies showing curcumin to have effects on inflammation, metabolism, oxidative stress, senescence, etc. involve induced models in experimental animals (Kinney et al., 2015; Kumar et al., 2011; Ma et al., 2013; Maithilikarpagaselvi et al., 2016; Nanji et al., 2003; Venkatesan and Chandrakasan, 1995). For example, D-galactose-induced senescence in mice was attenuated by oral treatment with curcumin, improving memory dysfunction and oxidative damage (Kumar et al., 2011). However, perhaps because senescence was induced, curcumin may not have the same degree of effectiveness in naturally-occurring senescence. Additionally, some models of inflammation that have shown the effectiveness of curcumin and particularly Longvida® involve some type of physiological challenge, such as exercise, in which the degree of inflammation and muscle damage was modulated in comparison to a group receiving placebo (McFarlin et al., 2016).

While the expected anti-inflammatory effects of curcumin were not exhibited in this study at the dose given, negative side effects were not present either, which were monitored by clinical examination and CBC with differential and chemistry panels prior to and post treatment. This aligns with a multitude of previous work, showing that curcumin in generally regarded as safe even at much higher dosages than those given in the present study (Cox et al., 2015; Nanji et al., 2003).
In conclusion, senior horses (≥20 years) supplemented with curcumin compared to placebo did not differ in regard to modulation of inflamm-aging. Treatment with curcumin also did not appear to affect endocrine function. Numerous parameters showed overall time effects, but these did not appear to be due to treatment. Since a low dose of curcumin supplementation was unable to mitigate age-associated inflammation, potentially increasing the dose of curcumin may be able to mediate anti-inflammatory effects.

Acknowledgements

The authors would like to thank Dr. Sally Frautschy of UCLA for her expertise regarding collection of blood samples for curcumin analysis as well as for facilitating the connection of the University of Kentucky with Verdure Sciences in order to perform this curcumin study. The authors would also like to thank Verdure Sciences for partial funding of this project as well as for providing the Longvida® and placebo to administer as supplement to the horses.
Table 6.1. RT-PCR gene expression of horses receiving curcumin and placebo for 90 days.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Treatment</th>
<th>Time point</th>
<th>Mixed Procedure P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 0</td>
<td>Day 15</td>
</tr>
<tr>
<td>Log (COX-1)</td>
<td>Curcumin</td>
<td>0.81 ± 0.13</td>
<td>0.94 ± 0.12</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td>0.86 ± 0.11</td>
<td>0.90 ± 0.11</td>
</tr>
<tr>
<td>Log (COX-2)</td>
<td>Curcumin</td>
<td>2.39 ± 0.09</td>
<td>2.41 ± 0.09</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td>2.55 ± 0.09</td>
<td>2.60 ± 0.09</td>
</tr>
<tr>
<td>GATA3</td>
<td>Curcumin</td>
<td>5.51 ± 0.85</td>
<td>8.45 ± 0.82</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td>6.74 ± 0.82</td>
<td>6.99 ± 0.82</td>
</tr>
<tr>
<td>Log (IFN-γ)</td>
<td>Curcumin</td>
<td>3.47 ± 0.19</td>
<td>4.05 ± 0.19</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td>3.53 ± 0.19</td>
<td>3.66 ± 0.19</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Curcumin</td>
<td>494.92 ± 396.08</td>
<td>1714.11 ± 379.50</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td>995.37 ± 379.50</td>
<td>1219.70 ± 379.50</td>
</tr>
<tr>
<td>IL-4</td>
<td>Curcumin</td>
<td>26.59 ± 15.32</td>
<td>80.88 ± 14.68</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td>38.41 ± 14.68</td>
<td>56.55 ± 14.68</td>
</tr>
<tr>
<td>Log (IL-6)</td>
<td>Curcumin</td>
<td>1.88 ± 0.08</td>
<td>1.91 ± 0.08</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td>1.99 ± 0.08</td>
<td>2.07 ± 0.08</td>
</tr>
<tr>
<td>Log (IL-10)</td>
<td>Curcumin</td>
<td>2.14 ± 0.14</td>
<td>2.48 ± 0.13</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td>2.13 ± 0.13</td>
<td>2.08 ± 0.14</td>
</tr>
<tr>
<td>Log (IL-12)</td>
<td>Curcumin</td>
<td>2.07 ± 0.08</td>
<td>2.10 ± 0.07</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td>2.26 ± 0.07</td>
<td>2.19 ± 0.07</td>
</tr>
<tr>
<td>Log (Tbet)</td>
<td>Curcumin</td>
<td>1.07 ± 0.10</td>
<td>1.23 ± 0.10</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td>1.06 ± 0.10</td>
<td>1.14 ± 0.10</td>
</tr>
</tbody>
</table>
Table 6.1. (cont.) RT-PCR gene expression of horses receiving curcumin and placebo for 90 days.

Gene expression data are reported as mean RQ values ± SEM for horses in each treatment group (n=15 curcumin and n=15 placebo) at each time point (Days 0, 15, 30, 60, 90). Mixed procedure p-values are also reported for each PCR variable in regards to overall treatment effect, time point effect, and interaction between treatment and time point. Data considered statistically significant (p<0.05) are denoted with an asterisk (*).

COX = Cyclooxygenase; IFN = interferon; IL = interleukin; TNF = tumor necrosis factor; GATA3 and Tbet are transcription factors.
Figure 6.1. Peripheral blood mononuclear cell (PBMC)-mediated production of interferon(IFN)-γ and tumor necrosis factor(TNF)-α from n=15 curcumin and n=15 placebo-receiving senior horses (≥20 years). PBMC were stimulated with PMA and analyzed via intracellular staining and flow cytometry to determine both percent of lymphocytes producing (A) IFN-γ and (C) TNF-α and mean fluorescence intensity (MFI) for each of these cytokines (B&D). P-values for significant (p<0.05) data are denoted on the figures. Ptime refers to the p-value for overall effects of time.
**Figure 6.2.** Serum Prostaglandin E2 (PGE2) production in curcumin and placebo supplemented senior horses. Serum from n=12 curcumin and n=11 placebo-receiving horses was analyzed via ELISA to determine pro-inflammatory PGE2 production.
Figure 6.3. PBMC-mediated inflammatory gene expression of curcumin (n=15) and placebo (n=15)-receiving senior horses (≥20 years). Gene expression is reported as the log of relative quantity (RQ) values of (A) cyclooxygenase (COX)-2 and (B) interleukin (IL)-6. P-values for significant (p<0.05) data are denoted on the figures. P_{trt} refers to the p-value for treatment group overall, while P_{time} refers to the p-value for overall time effects.
Figure 6.4. Thyrotropin releasing hormone (TRH) stimulation testing in senior horses (≥20 years) pre and post 90-day supplementation with curcumin (n=15) or placebo (n=15). Plasma was analyzed both for (A) basal ACTH and for (B) ACTH 10 minutes post intravenous injection with TRH, as measures of pituitary function. Data with p<0.05 were considered significant, while those with p<0.10 were considered trends. P-values for significant and trending data are denoted on the figures. $P_{\text{time}}$ refers to the p-value for overall effects of time (pre vs. post study).
Figure 6.5. Basal insulin pre vs. post study for curcumin (n=15) vs. placebo-receiving (n=15) horses. P-values for significant (p<0.05) data are denoted on the figure. P_{time} refers to the p-value for overall effects of time (pre vs. post study).
Figure 6.6. Plasma Aβ pre vs. post study for curcumin (n=15) and placebo-receiving (n=15) senior horses (≥ 20 yrs). Data with p<0.05 were considered significant, while those with p<0.10 were considered trends. P-values for significant and trending data are denoted on the figures. $P_{\text{time}}$ refers to the p-value for overall effects of time (pre vs. post study), while $P_{\text{trt x time}}$ refers to the p-value for the interaction between treatment group and time point. Bars with different lettering significantly differ (p<0.05) from each other.
Figure 6.7. Blood chemistry panel parameters with significant (p<0.05) or trending (p<0.10) interactions between time point and treatment group (n=15 curcumin and n=15 placebo-treated horses). P-values for significant and trending data are denoted on the figures. P_time refers to the p-value for overall time effects (pre vs. post study), while P_trt x time refers to the p-value for the interaction between treatment group and time point. Bars with different lettering significantly differ (p<0.05) from each other.
**Figure 6.8.** Body weights and body condition scores (BCS) for senior (≥20 yrs) horses supplemented with curcumin (n=15) or placebo (n=15) for 90 days. Body weights (A) and BCS (B) were monitored approximately every 30 days throughout the duration of the study. P-values for significant (p<0.05) data are denoted on the figures. \( P_{\text{time}} \) refers to the p-value for overall time effects.
CHAPTER 7

CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

Inflamm-aging, the chronic low-grade inflammation occurring systemically with advanced age, is well-characterized in the horse. Lymphocyte-mediated inflamm-aging has specifically been demonstrated in the horse, which is not surprising given that a vast body of literature in various species has shown lymphocytes to be particularly affected by advanced aging. In humans, inflamm-aging is associated with various age-related diseases as well as morbidity and mortality; however, clearly defined clinical effects in the horse have not yet been determined. Given that senior horses (≥20 years) comprise a substantial component of the equine population, with many continuing to be used for competition and recreation into their senior years, understanding the implications of inflamm-aging for the horse is key to promote healthspan as well as lifespan.

In this research, senior horses exhibiting inflamm-aging and adult horses exhibited seasonal variations in inflammatory cytokine production. Specifically, lymphocyte-mediated production of pro-inflammatory cytokines was decreased in winter and elevated during spring and summer. Similarly, PBMC-mediated gene expression of TNF-α was elevated in summer, while IL-6 expression was actually decreased in summer. Some of these seasonal inflammatory changes appeared to be associated with body weight and may be associated with temperature and/or photoperiod, but likely a complex array of neuro-endocrine-immune signaling pathways is involved. Future research should focus on understanding what these seasonal changes may mean for the health of the senior horse, already immune-compromised due to immunosenescence and inflamm-aging.
Inflamm-aging was also examined in relation to the common age-associated endocrinopathy, pituitary pars intermedia dysfunction (PPID), which has been associated with immunosuppression and increased susceptibility to opportunistic infections. Senior PPID horses and age-matched non-PPID horses do not appear to differentially experience lymphocyte-mediated inflamm-aging. Specifically, no differences were found between PPID and non-PPID horses regarding lymphocyte-mediated inflammatory cytokine production or PBMC-mediated inflammatory cytokine gene expression. Likely, the aspects of PPID impacting immunity are primarily mediated by neutrophils, as some previous research has demonstrated, and not lymphocytes. Future research should examine what stimulates the initiation of the dopaminergic neurodegeneration associated with PPID, particularly examining any relationships with inflammation and/or the immune system.

Relationships of inflamm-aging with overall health of the senior horse were also examined, particularly in regard to nutrition status, body composition, hematology/biochemistry, and PPID status. Inflamm-aging appears to be associated with increased levels of serum docosadienoic acid (C22:2n6c), docosapentaenoic acid (C22:5n3c), and folate, which may be due to a compensatory effect, given that these nutrients are generally considered to be anti-inflammatory. No relationships between inflamm-aging and markers of body composition were found. However, within the group of senior horses, age itself was inversely associated with multiple markers of body composition, indicating the incidence of decreased muscle, body condition, and weight with advanced age. Further research should focus on elucidating implications of the relationships found, with the goal of promoting the health and longevity of the senior horse.
The ability of phytonutrient polyphenols to mitigate lymphocyte-mediated inflamm-aging of the senior horse was also examined *in vitro*. Curcumin, resveratrol, quercetin, pterostilbene, and hydroxypterostilbene all significantly reduced lymphocyte-mediated inflamm-aging of the senior horse *in vitro*. These anti-inflammatory results were achieved at concentrations that were not cytotoxic. Additionally, the anti-inflammatory capacity of polyphenols was compared to common NSAIDs, and polyphenols were found to significantly reduce inflamm-aging similarly. In fact, curcumin decreased inflammation at a concentration lower than the NSAIDs or any of the other polyphenols, demonstrating its potent anti-inflammatory capacity. Thus, polyphenols were found to mitigate inflamm-aging of the senior horse *in vitro*.

As a follow-up to our *in vitro* polyphenol work, curcumin was evaluated *in vivo* as a potential anti-inflammatory supplement for the senior horse exhibiting inflamm-aging. Senior horses receiving enhanced-bioavailability curcumin and placebo did not significantly differ in regard to degree of inflamm-aging after being supplemented for 90 days. Specifically, no differences were found between curcumin and placebo-receiving horses in regards to lymphocyte-mediated inflammatory cytokine production or PBMC-mediated inflammatory gene expression. This lack of anti-inflammatory response in horses supplemented with curcumin may be due to an inadequate dose, as only two of the fifteen horses administered curcumin showed quantifiable levels of curcumin and/or metabolites in blood, or a poor response of the senior horse to the specific formulation. Future research should be conducted to determine whether a higher dose or different formulations of curcumin delivery systems may enable curcumin to function as an effective anti-inflammatory agent in the senior horse. Furthermore, some previous research suggests that
curcumin in combination with other polyphenols may act synergistically, which would be of interest to test in the inflamm-aging horse \textit{in vivo}.

As a whole, this body of research demonstrates that various factors are associated with lymphocyte-mediated inflamm-aging of the senior horse. Furthermore, this inflamm-aging may potentially be modulated by anti-inflammatory polyphenols; however, further research is necessarily to determine dosage and formulations effective as anti-inflammatory supplements in the horse. Additionally, future research to better understand the implications of inflamm-aging on overall health of the senior horse should be undertaken in an effort to promote healthspan in addition to longevity of horse.
REFERENCES


Cuomo, J., Appendino, G., Dern, A.S., Schneider, E., McKinnon, T.P., Brown, M.J.,
Togni, S., Dixon, B.M., 2011. Comparative absorption of a standardized curcuminoid
Curic, S., Wu, Y., Shan, B., Schaaf, C., Upatadel, D., Lange, M., Kuhlen, D., Perone, M.J.,
Arzt, E., Stalla, G.K., Renner, U., 2013. Curcumin acts anti-proliferative and pro-apoptotic
Das, N., Sikder, K., Bhattacharjee, S., Majumdar, S.B., Ghosh, S., Majumdar, S., Dey, S.,
2013. Quercetin alleviates inflammation after short-term treatment in high-fat-fed mice.
Food & function 4, 889-898.
effectiveness of Abeta1-42 immunization in APP transgenic mice with significant amyloid
Dasilva, G., Pazos, M., Garcia-Egido, E., Perez-Jimenez, J., Torres, J.L., Giralt, M.,
Nogues, M.R., Medina, I., 2016. Lipidomics to analyze the influence of diets with different
EPA:DHA ratios in the progression of Metabolic Syndrome using SHROB rats as a model.
Food Chem 205, 196-203.
Dato, S., Bellizzi, D., Rose, G., Passarino, G., 2016. The impact of nutrients on the aging
P.R., 2006. Cartilage-derived biomarkers and lipid mediators of inflammation in horses
with osteochondritis dissecans of the distal intermediate ridge of the tibia. Am J Vet Res
67, 1156-1162.
de Grauw, J.C., van de Lest, C.H., van Weeren, P.R., 2009. Inflammatory mediators and
cartilage biomarkers in synovial fluid after a single inflammatory insult: a longitudinal
de van der Schueren, M.A., Lonterman-Monasch, S., van der Flier, W.M., Kramer, M.H.,
Maier, A.B., Muller, M., 2016. Malnutrition and Risk of Structural Brain Changes Seen on
Derochette, S., Franck, T., Mouithys-Mickalad, A., Ceusters, J., Deby-Dupont, G.,
Lejeune, J.P., Neven, P., Serteyn, D., 2013. Curcumin and resveratrol act by different ways
on NADPH oxidase activity and reactive oxygen species produced by equine neutrophils.
Comparative evaluation of the pain-relieving properties of a lecithinized formulation of
curcumin (Meriva((R))), nimesulide, and acetaminophen. Journal of pain research 6, 201-
205.
Influence of feeding status, time of the day, and season on baseline adrenocorticotropic
hormone and the response to thyrotropin releasing hormone-stimulation test in healthy
horses. Domest Anim Endocrinol 48, 77-83.
Doucet, M.Y., Bertone, A.L., Hendrickson, D., Hughes, F., Macallister, C., McClure, S.,
Reinemeyer, C., Rossier, Y., Sifferman, R., Vrins, A.A., White, G., Kunkle, B., Alva, R.,
Romano, D., Hanson, P.D., 2008. Comparison of efficacy and safety of paste formulations
of firocoxib and phenylbutazone in horses with naturally occurring osteoarthritis. Journal
of the American Veterinary Medical Association 232, 91-97.


Miller, M., Chen, S., Woodliff, J., Kansra, S., 2008a. Curcumin (diferuloylmethane) inhibits cell proliferation, induces apoptosis, and decreases hormone levels and secretion in pituitary tumor cells. Endocrinology 149, 4158-4167.


VITA
Melissa H. Siard

EDUCATION

<table>
<thead>
<tr>
<th>INSTITUTION AND LOCATION</th>
<th>DEGREE</th>
<th>YEAR CONFERRED</th>
<th>FIELD OF STUDY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asbury University</td>
<td>BA</td>
<td>2012</td>
<td>Chemistry</td>
</tr>
<tr>
<td>Wilmore, KY</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CURRENT GPA: 3.865

PROFESSIONAL EXPERIENCE

08/2012 – present  Graduate Student/Research Assistant, Gluck Equine Research Center, University of Kentucky, Lexington, Kentucky

08/2014 – 12/2014  Teaching Assistant for Equine Management, Department of Animal Science, University of Kentucky, Lexington, Kentucky

03/2011 – 08/2012  Student Worker (Undergraduate)/ Horse Caretaker, Gluck Equine Research Center, University of Kentucky, Lexington, Kentucky

05/2012 – 07/2012  Horse Groom, Paige Farms, Knoxville, Tennessee

09/2010 – 12/2010  College Peer Tutor for Biology for Non-Science Majors and Physics, Asbury University, Wilmore, Kentucky

02/2009 – 05/2009  College Peer Tutor for Biology for Non-Science Majors and French, Asbury University, Wilmore, Kentucky

PEER REVIEWED PUBLICATIONS


HONORS AND AWARDS

3rd Place Graduate Student Award, Nutrition Section, 2013 Equine Science Society symposium, Mescalero, NM – May 2013

Hughes Foundation Scholarship Fellow (departmental coverage of full tuition and stipend), University of Kentucky, Lexington, KY – Fall 2013—present

Graduated Magna Cum Laude, Asbury University, Wilmore, KY – May 2012

Hughes Scholarship (full tuition), Asbury University, Wilmore, KY – August 2008 – May 2012

Dean’s List, Asbury University, Wilmore, KY – August 2008 – May 2012