MURRAY STATE UNIVERSITY

Murray State's Digital Commons

Murray State Theses and Dissertations | Graduate School

2020

Cannabidiol in the horse: pharmacokinetics and effects of a pelleted supplement on reactivity and movement

Anna L. Draeger
Murray State University

Follow this and additional works at: https://digitalcommons.murraystate.edu/etd

Part of the Animal Sciences Commons, Plant Sciences Commons, and the Veterinary Medicine Commons

Recommended Citation

Draeger, Anna L., "Cannabidiol in the horse: pharmacokinetics and effects of a pelleted supplement on reactivity and movement" (2020). *Murray State Theses and Dissertations*. 189.
https://digitalcommons.murraystate.edu/etd/189

This Thesis is brought to you for free and open access by the Graduate School at Murray State's Digital Commons. It has been accepted for inclusion in Murray State Theses and Dissertations by an authorized administrator of Murray State's Digital Commons. For more information, please contact msu.digitalcommons@murraystate.edu.

Cannabidiol in the horse: pharmacokinetics and effects of a pelleted supplement on reactivity and movement

A thesis
Presented to
the Faculty of the Hutson School of Agriculture of Animal/Equine Science
Murray State University
Murray, KY

In Partial Fulfillment
of the Requirements for the Degree
of Master of Science in Agriculture

Anna Draeger
Fall 2020

CANNABIDIOL IN THE HORSE: PHARMACOKINETICS AND EFFECTS OF A PELLETED SUPPLEMENT ON REACTIVITY AND MOVEMENT

DATE APPROVED: _____

_____

Shea Porr, PhD, Thesis Advisor

_____

Amanda Davis, PhD, Member, Thesis Committee

_____

Laura Hoffman, DVM, Member, Thesis Committee

_____

Tony Hicks, DVM, Member, Thesis Committee

_____

Patricia Godwin, MS, Member, Thesis Committee

Kristie Guffey, PhD, Hutson School of Agriculture Graduate Coordinator

_____

Tony Brannon, PhD, Dean of the Hutson School of Agriculture

_____

Robert Pervine, PhD, Associate Provost for Graduate Education and Research

_____

Tim Todd, PhD, Provost/vice President for Academic Affairs

_____

Acknowledgements

I would like to extend my gratitude to the many individuals that came together to ensure this project's success. It would not have been possible without the amazing team members, volunteers, and mentors that surrounded me. Thank you to Patricia Godwin, Dr. Laura Ken Hoffman, and Dr. Amanda Davis for your consistent encouragement and support as committee members. Whether that meant staying up for a midnight blood draw, spending hours crunching statistics, or standing in the rain to scare a horse with an umbrella rather than use it for yourself, your presence was deeply appreciated. Thanks also go to Dr. Tony Hicks and Equine Veterinary Services Pharm, LLC for supplying the CBD pelleted product and for the generous donation of time. Your participation as a committee member and assistance throughout the project was critical for success and sincerely appreciated.

I would also like to thank Evan Thomas, Kiara Jones, Destiny P'Pool, and Joy Sparks. From spinning blood at 1 am to helping to carry out CBD feedings for 8 weeks, the assistance of each fellow student was particularly appreciated. The staff and students of the Murray State University Equine Center as a whole were essential to helping this project run smoothly. Thank you all for your donation of time and energy towards completing each project phase, and for the use of university horses.

The support offered from my husband Kevin Draeger, along with his willingness to volunteer whenever our team could use an extra hand assisted in keeping each data collection organized and efficient. Thank you for your constant positive energy and encouragement.

I was extremely fortunate for the expertise and support offered from multiple labs and industry professionals. Thank you to Dr. Travis Mays of Texas A&M VDML, Dr. Daniel Gustafson of the Colorado State Pharmacology Shared Resource Veterinary Medical Center and Dr. Robert Silver of Folium Biosciences for sharing your knowledge of CBD and assisting in the development of various phases in this study.

Finally, I cannot thank my faculty mentor, Dr. Shea Porr, enough for her unwavering encouragement, feedback, and guidance during this research study. I am inspired by your determination, motivation, and enthusiasm evident in the high-quality work and guidance you offer. Thank you for endless hours of your time in project development, edits, and genuine advice applicable beyond the classroom. I truly could not have done this without you and am extremely grateful for the opportunity to work as your graduate assistant.

Abstract

A multitude of claims exist regarding therapeutic benefits of cannabidiol (CBD) in human and animal medicine. Though supportive evidence of CBD as a nutraceutical option exists, lack of regulation means that product safety, consistency, and efficacy cannot be guaranteed. Trials for specific conditions and species are needed. The objective of this study was to evaluate CBD safety and use effects on reactivity and movement in the horse. Project 1 examined the bioavailability of a single 50 mg dose of an oil and pelleted CBD product. One of 2 Quarter Horse geldings received the oil product. The second received the pelleted product. Blood samples for serum cannabinoid concentration occurred at 1 h and 2 h post administration. Both products were below LLOQ at 1 h and detectable at 2 h post administration (PEL= 0.163 ng/ml; OIL 0.11 ng/ml). Project 2 examined pharmacokinetics of a single feeding of pelleted CBD at 50 mg (TXT1), 100 mg (TXT2) and 250 mg (TXT3) in 18 stock-type geldings. Blood was collected at 0 (pre-treatment), 0.5, 1, 2, 4, and 12 h post treatment for serum CBD concentration. Safety was monitored via serum chemistry and complete blood count. Statistical analysis was completed on serum chemistry values through the PROC MIXED procedure of SAS. Though CBC and serum chemistry results were within reference ranges, treatment differences were observed for creatinine (TXT1=1.41, TXT2=1.22, TXT3=1.49; $P \leq 0.01$) and blood urea nitrogen (BUN; TXT1=15.5, TXT2=16.52, TXT3=18.61; $P \leq 0.03$). Peak serum CBD concentrations were observed at 2 h post TXT. The results demonstrated relative safety of a single CBD dose up to 250 mg in the horse, providing foundational knowledge concerning equine dosing. Project 3 evaluated pelleted CBD fed once daily over 6 wk to 24 university riding horses. Pre- and post-TXT evaluations were completed

on movement parameters and reactivity. Movement analysis examined stride length, and duration of stance and swing phase. Reactivity was observed through a novel object test (NOT). Reactivity scores were documented via live and video evaluators. Heart rate (HR) monitors collected HR data at NOT test points: start, stimulus, and stop. Instructors completed surveys to evaluate movement and behavior patterns of horses during classes. The population was reduced to stock-type geldings (n=17) for NOT and movement statistical analysis. The population was further reduced (n=15) for survey data to only evaluate stock type geldings observed in duplicate (before and after supplementation). Main effects included heart rate (HR), time on stride length (SL), and duration of stance or swing phase. Data was analyzed using the PROC MIXED procedure of SAS and survey data were evaluated using Chi Square for the effect of TXT and age on reactivity scores from the novel object test (NOT). Fisher's Exact Test was implemented if fewer than 5 responses were observed per observation parameter. No differences were observed in NOT HR values. Low reactivity scores were more frequently observed in TXT horses after 6 wk. During walk, TXT horses spent more time in stance phase (TXT=0.57 sec, CON=0.51 sec; $P<0.01$) and swing phase (TXT=0.38 sec, CON=0.36 sec; $P<0.01$). In both groups, walk stance phase duration increased over time (Pre=0.37 sec, Post=0.71 sec; $P<0.01$), while duration of trot stance (Pre=0.30 sec, Post= 0.26 sec; $P<0.01$) and swing phase (Pre=0.37 sec, Post= 0.33 sec; $P<0.01$) decreased. Trot SL shortened by 6 wk (Pre=1.68 m, Post=1.55 m; $P=0.03$). Survey results indicated a higher instance of positive behaviors when tied and during tack up in TXT horses. Both TXT and CON were best represented in the high suppleness category. Control horses were more

frequently rated high for suppleness on a circle and ability to track up. Movement analysis revealed no other significant parameters.

Table of Contents

Acknowledgements iii
Abstract v
Table of Contents viii
List of Tables x
Table of Figures xi
1. Introduction 12
Background and Setting 12
Statement of the Problem 14
Purpose of the Study 16
Research Questions/Hypotheses 16
Significance of the Study 17
2. Review of Related Literature 18
Introduction 18
Cannabis Overview and Regulations 19
Cannabis Taxonomy and Cannabidiol Processing 21
Carbon Dioxide Extraction 23
Steam Distillation 23
Solvent Extraction 24
The Endocannabinoid System 25
Endocannabinoid Receptors 26
Primary Endocannabinoids 29
Primary Phytocannabinoids 32
Synthetic Cannabinoids 36
Cannabinoid Detection 37
Current Clinical Studies 38
Monitoring Movement Influencers in Horses 41
CBD and Behavioral Modification 43
Summary 45
3. Methodology 46
Project 1 46
Horse Selection and Management 46
Treatments and Data Collection Procedures 46
Project 2 48
Horse Selection and Management 48
Treatments and Data Collections 49
Statistical Analysis 50
Project 3 51
Horse Selection and Management 51
Treatments and Data Collection Procedures 52
Movement Methods 53
Behavior 56

Statistical Analysis 58
Product Sampling 59

4. Results 61
Project 1 61
Project 2 62
Project 3 63
NOT Heart Rate 64
NOT Reactivity Scores 64
Movement Parameters 65
Instructor Survey 65
Product Sampling 68

5. Discussion 69
Project 1 69
Project 2 70
Project 3 74
NOT Heart rate and behavior scores 74
Movement Parameters 76
Product Sampling 80
Summary 80

References 82

Appendix A 96
IACUC Approval 96

Appendix B 97
Instructor Surveys 97

Appendix C 99
Definition of Terms 99

Appendix D 101
Limitations &Assumptions 101

Appendix E 103
Budget 103

Appendix F 104
Time Schedule 104

List of Tables

Table 1: *Horse Demographics Project 3* 52

Table 2: *Lameness category explanations during veterinary examination of subsample* 54

Table 3: *Rubric used to evaluate horse reactivity during the novel object test* 56

Table 4: *Enzyme concentration differences based on treatment* 63

Table 5. *Enzyme concentration differences over time* 63

Table 6. *Summary of heart rate observations through the novel object test based on treatment* 64

Table 7. *Summary of heart rate observations throughout the novel object test based on age* 64

Table 8. *Frequency and percent of novel object test scores based on treatment group* ... 65

Table 9. *Results of the Chi-Square Analysis and Fisher's Exact test on survey responses for stock type geldings observed in duplicate* 66

Table 10. *Cannabidiol potency degradation throughout the study* 68

Table of Figures

Figure 1: *Diagram of movement design* 55

Figure 2: *Diagram of behavior startle test procedures*. 58

Figure 3: *Serum detection comparison of pellet (PEL) and oil (OIL)* 61

Figure 4: *Serum CBD concentrations above LLOQ (ng/ml)* 62

Figure 5: *Comparison of concentration maximum (Cmax) using the geometric mean in horses based on treatment type.* 71

Figure 6*: Comparison of time of maximum concentration and time of terminal half-life among species from various studies at the same dose rate (2mg/kg)* 72

Figure 7: *Comparison across species for a 2 mg/kg oral CBD dose*. 72

Chapter 1

Introduction

Background and Setting

Despite recent promotion of the therapeutic abilities of hemp-derived cannabinoids, claims to the medicinal potential of the plant *Cannabis sativa L.* have been prevalent for centuries. In Ayurvedic medicine, it was deemed to be one of the five most sacred plants, characterized for offering "freedom from distress" (Hartsel et al., 2016). Its uses extend beyond medicine to other industries, including fiber, oil, and food (Zuardi, 2006; Russo, 2007). Within the United States, cannabis popularity started to decline in 1906, initiated by fairly stringent restrictions. First came the 1906 Pure Food and Drug Act, followed by taxation in the Marihuana Tax Act of 1937 and the implementation of its ban in 1970. However, in 1996 the state of California was the first to legalize the use of medical marijuana (Proposition 215) despite the federal governments opposing stance. Despite restrictions and a tense public perception, researchers managed to continue the exploration of various hemp constituents for therapeutic potential (Malfait et al., 2000; Kogan et al., 2004; Wade et al., 2004). The 2014 and 2018 Farm Bill Acts helped to return hemp to the forefront of agricultural discussions. As a result, research analyzing production methods and the crop as an agricultural commodity became a particular focus. Part of this process was clearly defining the terms of legally compliant hemp as compared

to marijuana. Upon removal from the Federal List of Controlled Substances, hemp was categorized as any part or derivative of the Cannabis sativa plant with a concentration less than 0.3% of delta-9 tetrahydrocannabinol (Sec. 297A Federal Farm Bill Act, 2018). All hemp related research and production is required to go through an application and licensing process with the United States Department of Agriculture (USDA), in addition to meeting current state specific regulations.

Alongside legal changes and shifting societal perceptions, hemp research was permitted to expand on therapeutic components of the plant (Jeong et al., 2014; Hammell et al., 2016; Philpott et al., 2017). There are two primary phytocannabinoids that interact with the naturally occurring mammalian endocannabinoid system. These are identified as cannabidiol (CBD) and delta-9 tetrahydrocannabinol (Δ^9-THC). Though CBD and Δ^9-THC are the most referenced, numerous other cannabinoids have been identified (Pertwee, 2006; Palazzoli et al., 2018). Claims have emerged regarding the abilities of cannabinoids, including CBD, to have a plethora of healing effects on both humans and domestic animals. Proposed therapies range from physical healing to behavioral modification. Conditions CBD might influence include but are not limited to: epilepsy (Devinsky et al., 2018), multiple sclerosis (Sastre-Garriga et al., 2011), anxiety (Crippa et al., 2010), rheumatoid (Blake et al., 2006) and osteoarthritis (Gamble et al., 2018), and pain nociception (Ellis and Contino, 2019). Despite current claims, the Food and Drug Administration (FDA) as well as the Association of American Feed Control Organization (AAFCO) have encouraged research for use validation and the creation of dose standards for consumer safety. Though officially classified as a nutraceutical, the pharmaceutical

nature of some claims suggests a need for extensive research trials of all labeled uses to achieve FDA approval.

A theory referred to as the entourage effect suggests a more inclusive representation of CBD, Δ^9–THC, and other cannabinoids forms a synergistic action, ultimately heightening the comprehensive medicinal result (Russo, 2011). While CBD may have therapeutic benefits, the component most commonly associated with unwanted psychoactive effects is Δ^9–THC (Pertwee, 2004). Current research examines the potential of CBD, various other cannabinoids, and a compliant concentration of Δ^9–THC as a medicinal option without negative consequence (Malfait et al., 2000; Lodzki et al., 2003; Jeong et al., 2014; Hammell et al., 2016)

Statement of the Problem

Cannabidiol has demonstrated neuroprotective action and influence on inflammatory and behavioral responses (Fusar-Poli et al., 2009; Giacoppo et al., 2014; Philpott et al., 2017; Ellis and Contino, 2019). These methods of action promote CBD as a viable candidate for managing cases of unsoundness, chronic neurodegenerative disorders, and high stress environments. Causes of unsoundness, such as osteoarthritis, have been a longstanding management issue within the horse industry (Rossdale et al., 1985). Current treatment options targeting pain reduction and inflammation are available, however these are often costly, require prolonged use, and carry inherent risks (King and Mansmann, 1997; Frisbie et al., 2015). Despite limited availability of studies specifically on the anti-arthritic properties of CBD, those present demonstrate positive findings of pain and inflammation reduction with minimal side effects (Philpott et al., 2017; Gamble

et al., 2018). Therapeutic studies on CBD have primarily been completed in murine models, dogs, and humans (Malfait et al., 2000; Hammel et al., 2003; Lodzki et al., 2003; Blake et al., 2006; Jeong et al., 2014; Devinsky et al., 2018). Despite progress, future research should be directed towards species-specific dose specifications and a dose response relationship for medicinal claims. Such studies are necessary as certain species may demonstrate lower tolerance or unique effects from a pharmaceutical or nutraceutical product (as reviewed in Hartsel et al., 2019). Determination of potential positive and negative consequences, if any, resulting from CBD medicinal use is a necessary step for consideration as an alternative form of therapy within any physical or behavioral condition. Research is particularly needed regarding the effects of oral and transdermal dosages within horses. Additionally, careful examination is required to determine the actual cause for observed changes. For example, it is possible that other components within CBD or hemp based products, such as a better-balanced omega 3:omega 6 fatty acid ratio, could be the true catalyst for improved health (Simopoulos et al., 2002). In the absence of diligent research, consumers lack an understanding of appropriate conditions to be treated with CBD and what those efficacious concentrations would be. This research is what provides a foundation for regulatory bodies to base their standards from and ultimately ensure a safe, appropriate product accompanied with sound dosing recommendations.

Purpose of the Study

The purpose of this study was multifaceted. The primary intent was to evaluate the properties of oral CBD products in horses to better understand product availability, CBD pharmacokinetics in the horse, and the consequences of extended treatment. Due to the scarcity of published studies on recommended dosage and administration methods in horses, pilot studies were implemented to better understand absorption and palatability. Dosages were derived from a combination of manufacturer recommendations and published research in other species. Comparative lab analysis through accredited labs was incorporated to validate CBD content in the products and blood concentrations. Pharmacokinetics were evaluated to determine the patterns of absorption and elimination from a single dose. Dose safety was also monitored through a serum chemistry and complete blood count. Equine reaction response observation was included with extended treatment to determine the validity of CBD use for stress related issues in horses. Finally, long term feeding effects of CBD on movement in horses was evaluated. Ultimately, this project intended to provide framework for further advancement in equine CBD dosage and use recommendations for the safety and welfare of the animal.

Research Questions/Hypotheses

The following questions were addressed during this study:

1. Do the current recommended equine oral dose rates of CBD result in detectable concentrations in equine serum?
2. What are the pharmacological actions of CBD in the equine circulatory system following absorption?

3. Are there negative side effects in the horse from oral supplementation of CBD at certain dose rates?
4. Are there observable changes in behavior or movement of horses fed CBD pellets over an extended period of time?

Significance of the Study

Currently, formulations of cannabidiol in animal products remain in an unregulated market. This is due to the absence of approval from the FDA alongside a lack of AAFCO recommendations. Lack of regulation allows for product inconsistency and poor clarity of appropriate conditions for prescription. Approval from regulatory organizations requires rigorous testing to demonstrate both safety and efficacy for the labeled uses. The pilot study was intended to serve as foundational material in the pursuit of CBD related dosing recommendations for the horse. The primary study incorporated these findings into a clinical use investigation, exploring use recommendations. An overarching goal of this project was to fill the gap in equine specific CBD research.

Chapter 2

Review of Related Literature

Introduction

Lameness in the equine athlete encompasses a range of ailments, observable as pain, restricted range of motion, or gait abnormality (King and Mansmann, 1997). Primary treatments for lameness include variations of non-steroidal anti-inflammatory drugs (NSAIDS) such as Banamine ® (flunixin meglumine) and phenylbutazone. Despite existing treatment options, the limitations, side effects, and inherent administration risks provide opportunity for the exploration of other therapeutic methods for the equine athlete and aging companion (Schlueter and Orth, 2004; Koenig et al., 2014; Frisbie et al., 2015). Current subjective claims regarding CBD focus on its potential to serve as an overarching form of treatment for a variety of ailments. Studies that demonstrate medicinal abilities are becoming more prevalent, however significant gaps remain (Blessing et al., 2015; Landa et al., 2016; as reviewed in Hartsel et al., 2019). The following review considers the current understanding of cannabinoids and the cannabinoid system to examine the efficacy and safety of cannabis-based equine feed supplementation as an alternative therapy for chronic pain, inflammation and anxiety related issues.

Cannabis Overview and Regulations

Indigenous to Central Asia, cannabis is one of the earliest plants cultivated by man. However, it was not until the isolation and characterization of Δ^9-THC in the mid-1900s that official pharmacological studies of cannabinoids began to advance. Following the 1987 National Institute on Drug Abuse meeting, two discoveries significantly contributed to the foundational understanding of the endocannabinoid system processes. One was the discovery of the G-protein coupled receptors with which cannabinoids were interacting, CB1 and CB2 (as reviewed in Pertwee, 2006). This coincided with the isolation of 2-Arachidonyl glycerol (2-AG) and Arachidonoyl ethanolamide (Anandamide) as endocannabinoids naturally occurring in mammalian tissues, capable of activating CB1 and CB2 and recreating biological effects characteristic of Δ^9-THC (as reviewed in Pertwee, 2008). These findings serve as the foundation of our current understanding concerning the interactive pathways associated with this system.

Though now legal under the 2014 and 2018 Farm Bill Acts, those involved in hemp research and production must participate under the collective guidance of an application and licensing process. State laws are permitted to be at a higher standard than federal, given all minimum federal regulations are met. While hemp production and research may be monitored through documents such as the University and College Affiliation Application Packet and the Hemp Production Plan, regulations regarding product testing remain less clearly defined. The principal authority for feed approval lies with the FDA. The other organization affiliated is the AAFCO. Although lacking official regulatory powers, this organization is responsible for providing state specific guidelines

regarding safety and effectiveness for the labeled benefits of animal products. The FDA has encouraged careful and thorough research regarding the potential therapeutic benefits of hemp components in animal and human diets. The drug-like claims surrounding CBD requires the FDA to investigate associated products as a drug, with special attention to misbranding (Glenn, 2017). The components of *C. sativa* are more commonly referred to as nutraceuticals (as reviewed in Hartsel et al., 2019). A nutraceutical encompasses herbal substances with physiological benefits specifically concerning chronic diseases (Nasri et al., 2014). Currently, CBD products are not legal in livestock feeds. Equine organizations such as the United States Equestrian Federation (USEF), Fédération Equestre Internationale (FEI) and American Paint Horse Association (APHA) have explicitly prohibited CBD and other cannabinoid products in competition as of 2019. In a public statement, the US Equestrian Communications Department announced the ban, reasoning that CBD contains the potential to influence behavior and performance, alongside concerns of product consistency and safety[1].

As CBD currently exists in an unregulated market, mandated testing standard for other food and medicinal products are not yet enforced. Apart from managing the consistency of product potency, contaminant testing of agricultural products is also important for the health of the consumer. This helps protect consumers from exposure to detrimental pesticides, heavy metals, molds, bacteria, and aflatoxins that could have been introduced to the product at any point of production, from farm practices to extraction solvents (Romano and Hazekamp, 2013). Though current analytical labs can reliably

[1] US Equestrian Communications Department (2019, May 14) *USEF Announces Positive Tests of Cannabinoids (CBD) Will Result in GR4 Violations as of September 1, 2019.* https://www.usef.org/media/press-releases/usef-announces-positive-tests-of-cannabinoids

detect such contaminants in other agricultural products, without regulatory testing these could go unnoted during the extraction process and manifest in a concentrated form within the final product (Hazekamp, 2018).

The FDA has approved one pharmaceutical form of CBD, Epidiolex® in the United States for humans. Following extensive studies, this oral product is now a treatment option for two rare forms of epilepsy and is the first ever offered treatment for Gavet Syndrome, a type of epilepsy entailing life-long presence of frequent and potentially prolonged seizures, typically starting in the first year of life (Divensky et al., 2018). While this demonstrates progress for CBD treatments within specific epilepsy cases, the numerous health issues targeted for treatment by CBD requires more research. Investigation of products should be specific to the disorders and ailments it is advertised to assist with. A vital distinction in the products of focus, such as Epidiolex®, is that they are highly purified forms of plant-derived CBD. Research conducted on Epidiolex® did reveal potential long-term detrimental effects on the liver (Divensky et al., 2018). However, the positive outcomes of this particular product toward the conditions it was being tested for were determined to outweigh the risk.

Cannabis Taxonomy and Cannabidiol Processing

There are three primary subspecies of cannabis within the *Cannabaceae* family following the monotypic view (Small et al., 1976; Beutler and Marderosian, 1978). A European strain, known as *C. sativa sativa* was the first to be identified by Carl Linnaeus in 1737. This version tends to grow taller and has historical uses for fiber and seed. Jean-Baptiste Lamarck differentiated between *C. sativa sativa*, and the Asian variety, *C. sativa*

indica. *C. indica* is primarily grown for the production of hemp and drug varieties. The third strain, *C. sativa ruderalis,* was defined as a Russian strain of cannabis by Soviet botanist Janishevsky. This subspecies tends to be the shortest of the three and naturally exhibits lower concentrations of Δ^9-THC and higher concentrations of CBD. Although its short stature reduces its appeal as a cash crop within the fiber and textile industries, this strain has been used for crossbreeding for its hardiness and autoflowering ability.

Higher concentrations of Δ^9-THC in *C. indica* from Southeast Asia seem to be a result of natural evolution in the plant (as reviewed in Hartsel et al., 2016). Current industry interest in cannabinoid-based therapy has led to selective breeding for maximal CBD content, while maintaining Δ^9-THC concentrations within the United States 0.3% legal limit. Though different strains of cannabis can naturally vary in cannabinoid concentration, it is characteristic that individual strains also fluctuate, having an inverse relationship of CBD and THC content ratios (Aizpurua-Olaizola et al., 2016). Sample storage also plays an important role in cannabinoid content, as aging of a sample through exposure to light, heat and oxygen can accelerate the process of converting carboxylic acid-containing precursors usually initiated by heat (Lindholst, 2010). Therefore, even if a crop is harvested in compliance, the final product may have an incompliant Δ^9-THC concentration if product storage is inadequate.

The purified full-spectrum nutraceutical products under investigation in this paper are independent from livestock feed studies examining the nutritional quality of hempseed cakes, meals, and oils, derived from the hemp seeds (Silversides and Lefrançois, 2005; Hessle et al., 2008; Gakhar et al., 2012). Cannabinoid and terpene production occurs within the glandular trichomes of the plant (Hartsel et al., 2016). This

would consist of the bracts in the female and the anther of the male plant. Cannabidiol is extracted primarily from the floral component of female plant. The method of phytocannabinoid extraction from the hemp plant to be used in a tincture, or other edible version can vary. Three primary methods of extraction include carbon dioxide extraction, steam distillation, and solvent extraction.

Carbon Dioxide Extraction

This method may also be referred to as supercritical fluid extraction, due to the use of CO_2 that contains properties of both a liquid and a gas for extraction. A series of three pressurized chambers are used. The first contains the hemp plant while the second contains pressurized CO_2. Supercritical CO_2 filtered into the chamber containing the hemp plant forces the oil to separate from the plant. Pressure ratios and solvent amounts can be adjusted for specific CBD concentrations. Both the CO_2 and oil are then pumped into a third chamber where the gas evaporates, leaving behind a pure CBD oil. This is considered a high efficiency method of oil extraction as compared to alternative methods (Gomez et al., 1996).

Steam Distillation

As suggested in the name, this method uses steam as opposed to supercritical CO_2 to separate CBD from the plant material. A flask with an entrance and exit outlet contains the hemp plant. A glass flask containing boiling water is attached to the other side of the entrance outlet. Steam from the boiling water moves to the second flask, which separates the CBD containing oil vapors. On the other side of the exit chamber, a condenser

condenses the vapors into oil and water. Distillation is the final step, causing separation of CBD oil from the water. Because cannabinoids and terpenes can be very sensitive to significant changes in temperature, using this method carries the risk of altering the chemical components from the original crop and can make it more difficult to extract exact concentrations as compared to CO_2 extraction (Anitescue, 1997).

Solvent Extraction

Working in a similar context as steam distillation, solvent extraction uses a solvent in place of steam to extract the desired concentration of CBD. Depending on what solvent is being used, this method can carry risk of toxicity and cancer if the solvent is not completely eliminated during the evaporation step (Romano and Hazekamp, 2013). Though natural solvents such as olive oil or ethanol can be used in place of petroleum or propane to avoid health risks, natural solvents can occasionally extract chlorophyll during this process. The presence of chlorophyll is associated with reduced palatability in oral supplements. (Romano and Hazekamp, 2013)

Understanding the earliest stages of formulating a CBD nutraceutical product helps to clarify inherent risks and concerns to the public. Evident from each of the options discussed, is that even when CBD is extracted from one hemp strain with precise methods, opportunities remain within extraction methods for cannabinoid concentration to fluctuate. This could make it difficult for nutraceutical companies to ensure consistency of product content. Additionally, unregulated solvent testing could result in inadvertent consumer exposure to dangerous solvent concentrations during consumption.

The Endocannabinoid System

Cannabinoids include a multitude of bioactive molecules classified into three primary groups: endocannabinoids, phytocannabinoids, and synthetics (Scuderi et al., 2009). Endocannabinoids represent molecules that naturally exist within the mammalian body. Phytocannabinoids encompass a class of over 86 currently known components within *C. sativa*, while synthetic cannabinoids are those engineered for increased potency. Both phytocannabinoids and synthetic cannabinoids mimic natural endocannabinoid effects (Landa et al., 2016). Following bodily absorption, these play a role in overall cannabinoid interaction with receptors via pathways of agonism, partial agonism and antagonism. Work from Gaoni and Mechoulam provides foundational pharmacological research regarding synthetic cannabinoids in medicine (1964).

The endocannabinoid system exists within all vertebrates and primarily serves as the master regulator of homeostasis for the body (McPartland et al., 2005; De Laurentiis, 2014; Sallaberry and Astern, 2018; as reviewed in Hartsel et al., 2019). In work by Alger, it is described as "a bridge between body and mind" (2013). Functioning through a negative feedback loop, it is reliant on the interaction of cannabinoids, the enzymes that regulate them, and the appropriate receptors they bind to (Pertwee, 2006; Mackie, 2008). Therefore, when the endocannabinoid system is activated by a stimulus it will react by attempting to restore equilibrium through reduction of a relevant function or output. Research supports this systems role in a wide range of central nervous system and endocrine functions, such as: immune system and inflammatory responses, influence on blood pressure, inhibition of tumor cell growth, nociception modulation, and influence on reproductive function (Di Marzo et al, 1998; Kogan et al., 2004).

Endocannabinoid Receptors

Endocannabinoid receptors exist in the brain, nervous system, skin, immune system, kidneys, and gastrointestinal tract (Mackie, 2008; Scuderi et al., 2009). Considered to be the most diverse group within vertebrates, G-protein coupled receptors are one of the more prominent receptors involved within cannabinoid physiological responses (Rosenbaum et al., 2009). There are two primary G-protein coupled receptors (GPRs) in mammals related to cannabinoids, known as CB1 and CB2. The CB1 receptors are predominantly associated with the central nervous system and mediating the restriction of transmitter release, while CB2 receptors are affiliated with the immune system and managing cytokine release (Howlett et al., 2002; Pertwee, 2006; Scuderi et al., 2009). Activation of one or both receptors appears to terminate neuropathic and inflammatory pain (Pertwee, 2006; Philpott et al., 2017). Therefore, cannabinoids primarily function through interference with signaling pathways. In accordance with other GPRs, CB1 and CB2 are susceptible to the same pharmacological influence regarding partial agonism, functional selectivity and inverse agonism in their cellular response to specific cannabinoid receptor ligands (Mackie, 2008).

An important discovery in relation to prevalence of CB1 receptors in humans is that despite being highly present in areas of the brain such as the cortex, hippocampus, basal ganglia and cerebellum, they do not appear to be located in the medulla, which responsible for controlling autonomic features such as breathing and heartbeat (as reviewed in Hartsel et al., 2019). Such an observation provides explanation for the diminutive overdose risk of cannabinoids in humans (as reviewed in Hartsel et al., 2019).

However, an important distinction is that minimal CB1 receptor presence in the medulla has not been proven true for all species. Heart rate could still be affected by the endocannabinoids system through other pathways. Aside from the strong prevalence of CB1 in various areas of the brain, these receptors may also be found in the heart, blood vessels, liver, lungs, digestive system, fat cells and sperm cells (as reviewed in Hartsel et al., 2016). Therefore, product safety should be acquired on a by-species basis. However, receptor prevalence throughout multiple bodily systems demonstrates how CBD might achieve influence on a wide range of health conditions. Thus far, cannabinoid receptor locations in the horse brain have been verified in the sensory neurons and satellite glial cells of the dorsal root ganglia (Chiocchetti et al., 2020). The dorsal root ganglia's role of housing nerves that relay sensory information to the spinal cord supports the investigation of CBD for pain management.

The influence of CB1 receptors in the central nervous system (CNS) and peripheral tissues has also demonstrated a role in energy balance regulation (Cota, 2007). Agonists of CB1 are capable of increasing appetite and consumption, while antagonists can suppress appetite (Wiley et al., 2005; Jamshidi and Taylor, 2009). Beyond the roles of the hypothalamus in controlling eating habits for energy regulation, the involvement of the endocannabinoid system with the release of neurotransmitters such as serotonin and dopamine suggest appetite influence could also be related to a sense of reward or satisfaction (Gardner, 2005). This information could potentially assist in appetite stimulation for horses refusing feed because of stress or discomfort.

Though CB2 receptors are usually present in relatively low concentrations in the CNS and brain, they will rise in abnormal conditions such as cancer, inflammation, or

neurodegenerative disease (Hartsel et al., 2016). This demonstrates reactivity of the endocannabinoid system to periods of imbalance in the body. Cannabinoid interaction with CB2 receptors results in the inhibition of adenylyl cyclase, which decreases the second messenger cyclic adenosine monophosphate levels and results in the reduction of response to immune challenge upon receptor activation (as reviewed in Hartsel et al., 2019). Such actions would suggest that administration of CBD could calm a hyper-reactive immune response. Due to the location of CB2 receptors primarily being in peripheral tissues and involved with immune response rather than the CNS, the activation of these receptors lack psychotropic side effects commonly associated with CB1 receptor activation (Grotenhermen and Muller-Vahl, 2012).

There are numerous enzymes coupled to G proteins that result in the functionality of receptors within the signaling process. These include adenylate cyclase, protein kinase, potassium channels, and calcium channels (Howlett et al., 2002; Pertwee, 2006; Mackie, 2008). Other receptors believed to be involved with the endocannabinoid system include GPR 55 and GPR 119 (Brown et al., 2011). As these are also G-proteins, they are structurally similar to CB1 and CB2 receptors (Brown et al., 2011; Alexander et al., 2013). The protein transient receptor potential cation channel subfamily V member 1 (TRPV1) has also demonstrated activation from endocannabinoids and their metabolites (Pertwee, 2006). Further investigation is required regarding TRPV1, as there is potential that cannabinoids are not acting directly on the receptor, but through an indirect sequence resulting in subsequent activation (van der Stelt et al., 2005; Thomas et al., 2007).

Primary Endocannabinoids

The most prominent endocannabinoids include arachidonoyl glycerol (anandamide), 2-arachidonoyl glycerol (2-AG), O-arachidonoyl (virodhamine), and 2-arachidonoyl glycerol ether (noladin ether) (Ivanov, I., Borchert, P., Hinz B. 2014). Unique to these molecules is their on-demand formation. This is in comparison to being presynthesized and stored in synaptic vessels (Mackie, 2006). The on-demand action associated with endocannabinoids is the result of intense central nervous system activity, which can spur the necessary release of calcium from membrane phospholipid cleavage (Mackie, 2006; Blessing et al., 2015). Therefore, this system is reliant on being reactive and quick acting to achieve system balance.

A popular observation is that simple phospholipids are not just structural components of the cellular membrane, but also serve as precursors for transmembrane signaling within the CNS (Freund et al., 2003). Various classes of communication are included within CNS cellular communication (Freund et al., 2003; Contos et al., 2000). Endocannabinoids most similarly align with eicosanoids, as they are lipid-signaling molecules that bind to and activate receptors (Freund, Katona, and Piomelli, 2003). The two are distinguished by endocannabinoids not functioning through oxidative metabolism (Gerdeman and Lovinger, 2003). However, the close alignment of endocannabinoids with eicosanoids supports consideration for system manipulation through phytocannabinoids, particularly in regard to influence on pro- and anti-inflammatory agents, pain intensity and duration, and blood pressure.

The two endocannabinoids with the greatest current understanding are anandamide and 2-AG. Anandamide is part of the family of fatty acid amides,

specifically amide of arachidonic acid and thanolamine (Ivanov et al., 2014). Capable of interacting with cannabinoid receptors, these anandamide precursor fatty acid ethanolamides have demonstrated potential biological effects, such as anti-inflammatory properties (Freund, Katona, and Piomelli, 2003). The local, on-demand action of the endocannabinoid system provides support for a potentially quick acting method to influence conditions such as arthritis or obesity. Anandamide is also known for influencing a number of other processes. The involvement of anandamide in feeding behaviors, motivation, and pleasure makes it a capable influencer of appetite (Jamshidi and Taylor, 2009). It can also be found within reproductive processes, with concentrations influencing embryo implantation (Liu et al., 2002). When stress causes the reduction of fatty acid amide hydrolase (FAAH) activity, anandamide concentrations are also reduced and may cause an increase in anxiety type behaviors (Mayo et al., 2020). The other targeted endocannabinoid is 2-AG of the glycerol esters. Anandamide and 2-AG tend to be found at opposite ends of the signal channel, with anandamide being post-synaptic and 2-AG at pre-synaptic locations (Gulyas et al., 2004; Mackie, 2008). As is characteristic of other neurotransmitters, uptake into nerve endings and glia is the most common method of lipid messenger uptake (Piomelli et al., 2003). Due to the fact that Anandamide and 2-AG compete with Δ^9-THC for CB1 and CB2, they are capable of producing biological effects characteristic of Δ^9-THC (Hartsel et al., 2016). However, unlike phytocannabinoids, the effects of endocannabinoids are short lived because they are quickly deactivated. Continued benefit requires continued production. Concentrations of both endocannabinoids are regulated though FAAH and monoacylglycerol (MAGL). Endocannabinoid presence, or tone, has demonstrated a role in anxiety responses and

influence on brain reward circuits (Arnone et al., 1997; Arévalo et al., 2001). When rats were injected with a known CB1 receptor antagonist that blocked endogenous cannabinoids from binding to the CB1 receptor, results demonstrated both withdrawal and anxiety-like responses (Arévalo et al., 2001). This observation provides a key demonstration of the consequences related to endocannabinoid system imbalance. If the interruption of equilibrium were able to elicit a notable behavioral response, anxiety related issues could be aided by phytocannabinoids or synthetics as a means of rebalancing the system.

Overall, the effects of cannabinoids are attributed to influence on the receptors, inhibition of neurotransmitter release, inhibition of associated enzymes, and cannabinoid transmembrane transport (Mackie, 2006; Fišar, 2009). Despite the need for further investigation, current evidence of endocannabinoid mediating effects exists within short term and long-term plasticity (Gerdeman and Lovinger, 2003; Mackie, 2006). Neuronal plasticity refers to the effectiveness of neuron communication. The synaptic strength between neurons can be effective short-term over a matter of seconds, or long-term for minutes to years (Kandel et al., 2013). Long-term plasticity is considered the primary method for memory creation and storage in the brain (Kandel et al., 2013). Synaptic plasticity can alter the neurotransmitter release amount or the number of post-synaptic receptors available. In the cannabinoid system, short-term plasticity occurs through processes of depolarization-induced suppression of inhibition and depolarization-induced suppression of excitation, meaning that postsynaptic terminals actively release endocannabinoids and presynaptic terminal receptors are activated (Mackie, 2006). This form of plasticity explains how the system can respond promptly to short-term

imbalances. However, activation of endocannabinoids through short-term plasticity would not allow for lasting benefits. Long-term plasticity follows four different circuits, categorized into the retrograde messenger sector of long-term depression (Mackie, 2006). These circuits are divided into excitatory and inhibitory pathways within the brain including areas of the cortex, dorsal striatum, prefrontal cortex, nucleus accumbens, hippocampus and amygdala (Mackie, 2006). Categorization into long-term depression offers a potential explanation for system functionality in observations of chronic pain alleviation with consistent CBD treatment despite a reduction in the dosage amount (Ellis and Contino, 2019). Ultimately, understanding the interworking of naturally occurring endocannabinoid system can assist in the appropriate application of phytocannabinoids and synthetic cannabinoids for desired therapeutic benefits.

Primary Phytocannabinoids

Thus far, the two primary phytocannabinoids that have been identified from cannabis include Δ^9-THC and CBD. These components are two of many, which have gained attention primarily from their potential therapeutic benefits. Commonly accepted as the primary psychoactive cannabinoid, Δ^9-THC is found within the resin on the leaves and flowers of a female cannabis plant (Freund et al., 2003). Negative effects associated with Δ^9-THC use include hypothermia and reflex tachycardia (Freund et al., 2003). Thus far, CBD has lacked these negative effects while maintaining therapeutic properties (Pertwee, 2004; Blake et al., 2006; Devinsky et al., 2018). Negative effects are largely believed to be a result of Δ^9-THCs high binding capacity for CB1 receptors. The strong bond created when THC interacts with CB1 and CB2 receptors is also the hypothesized

catalyst for withdrawal symptoms. In contrast, CBD has been noted for its low binding affinity for both CB1 and CB2, behaving as an inverse agonist to CB2 receptors and a complete antagonist towards CB1 receptors (Pertwee, 2008; Thomas et al., 2009; Hill et al., 2012; Philpot et al., 2017). While agonists are responsible for increasing receptor activity, inverse agonists are capable of binding to the same receptor and reducing the response of an endogenous agonist, such as anandamide or 2-AG. Inverse agonists will reduce overall receptor activity. Antagonists will bind to, but entirely block a receptors action. The implications of such a finding suggest that the presence and effects of CBD actually inhibits reactions initiated by Δ^9-THC. This was demonstrated in trials where, despite Δ^9-THC presence in the product, negative effects often associated with the compound were absent (Blake et al., 2006; Gamble et al., 2018). The important interplay of multiple cannabinoids is also an explanation for the dangerous potency of certain synthetic cannabinoid compounds, particularly synthetic versions of Δ^9-THC. When consumed from plant-based options, other cannabinoids that act as antagonists or inverse agonists are present and are therefore capable of softening or blocking the response of the body to Δ^9-THC. However, synthetics are highly purified versions lacking the natural balance that can offer consumer protection from negative or dangerous side effects. Furthermore, claims of the entourage effect promote the concept that therapeutic efficiency increases when multiple cannabinoids are present (Russo, 2011).

Cannabidiol has demonstrated strong anti-inflammatory properties. One primary contributor to inflammation is reactive oxygen species (ROS). Reactive oxygen species can interact with and damage cell components, ultimately resulting in inflammation. Cannabidiol works to reduce inflammatory response through neutralizing ROS (Hartsel et

al., 2016). An additional method of action includes immune cell migration inhibition, observed in murine models treated with CBD (Pertwee, 2008; Hill et al., 2012). Particularly important is the association of reduced pro-inflammatory cytokine release with CBD administration (Malfait et al., 2000). In a study by Costa and colleagues, edema in the paws of rats treated with a single dose of cannabidiol was reduced within 3 hours of treatment in all test subjects treated at 7.5, 10, 20 or 40 mg/kg (2004). In a subsequent study also on rats, transdermal application demonstrated significant reduction of joint inflammation down to a 6 mg/d dose (Hammell et al., 2016). The transdermal application demonstrated increased efficiency of CBD absorption by avoiding the first pass effect at the liver. Involvement of TPRV1 in inflammation reduction is a result of this receptor functioning as the trigger of myeloid-derived suppressor cell (MDSC) release, which serves as a primary regulatory cell within the immune system. Activation of TRPV1 through administration of CBD causes the subsequent release of MDSCs at the site of inflammation, ultimately suppressing T cell functions that tend to contribute to inflammation (Hegde et al., 2011). Despite the advances, further research is needed to fully comprehend the role of TRPV1 in this process (Burstein, 2015).

Additional evidence of CBD stimulated inflammation reduction has been observed through dose dependent reduction of inflammatory biomarkers cyclooxygenase (COX) and prostaglandin E_2, as well as the production of nitric oxide (Burstein, 2015). Targeting a reduction of COX is relatable to the processes associated with NSAID use. Though NSAIDs should not be used in high doses for a long period of time due to resulting liver damage, the processes by which they work has demonstrated effectiveness at pain alleviation (Clegg and Booth, 2000). Therefore, if CBD is working through a

similar process and is more suitable for long-term use, it could serve as a substitute for chronic cases.

The primary explanation for diminished pain perception associated with CBD use concerns the interaction of CBD with G protein-coupled receptor 55 (GPR55). This encounter results in the inaction of pronociceptive signaling (Ryberg et al., 2007; Staton et al., 2008; Godlewski et al., 2009). Therefore, if CBD binds to GPR55, pain signals should be interrupted before they would be perceived. The available literature supports the role of CBD in pain and inflammation reduction responses (Lodzki et al., 2003; Burstein, 2015; Hammell et al., 2016; Gamble et al., 2018; Ellis and Contino, 2019). Further promoting its potential to serve in place of other painkillers, CBD appears to lack induced tolerance (Malfait et al., 2000).

Other phytocannabinoids included within feed and blood analysis includes cannabigerol (CBG), cannabinol (CBN), and cannabichromene. Associated with immune function, and containing potential for central nervous system support, CBG is also partially responsible for the reuptake of the endocannabinoid anandamide (Freund et al., 2003). Serving as a metabolite of Δ^{9}-THC, CBN is the converted form obtained following degradation. A higher affinity is held for the CB2 receptor, in accordance with effects typically seen within immune cells. Cannabichromene serves as an influencer of inflammatory response and is thought to promote healthy digestive motility (Wirth et al., 1980; Romano, et al., 2013). Due to its influence, this compound could be particularly important to include within cannabinoid product formulations. Each neutral compound contains precursors or homologs, as this is how they are present within the cannabis

plant. Those for reference within the data include: CBD*a*, CBDV, CBDVA, THCA, THCV, THCVA, CBNA, CBGA, CBCA.

Synthetic Cannabinoids

Advancement in the synthetic class of cannabinoids started in the 1960's with the isolation, characterization, and synthesis of Δ^9-THC in attempt to gain a better understanding of the endogenous endocannabinoid system (Davis, 2019). Though structurally similar, synthetics are considered new compounds produced for a more direct and potent effect (Hartsel et al., 2016). The work of Eli Lilly led to the first synthetic cannabinoid, Nabilone. This derivative was first approved in Canada in 1982, under the name of Cesamet™ for the reduction of chemotherapy associated nausea and pain (Slatkin, 2007; as reviewed in Giacoppo et al., 2014). It is now approved in the United States. Nabilone is understood to be the more potent version of the closely related, Dronabinol (Marinol®). First approved for use in the U.S. by the FDA in 1986, this synthetic compound was also intended to reduce the effects of chemotherapy as well as being applied to anorexia associated with HIV/AIDS patients (Lutge et al., 2013). While the previous two are synthetic derivatives of Δ^9-THC, the recently developed oral-mucosal spray Sativex ® is a combination of purified CBD and Δ^9-THC. Inclusion of CBD has helped to block unwanted negative effects associated with Δ^9-THC. The whole plant medicinal extract has demonstrated success in the reduction of muscle spasticity in adult multiple sclerosis patients and pain for rheumatoid arthritis patients (Wade et al., 2004; Blake et al., 2006). Cannabinoids falling under the synthetic class have demonstrated a significantly stronger binding affinity for CB1 and CB2 receptors than

both endo- and phytocannabinoids. Despite modern advances, focus on developing synthetic medications has diminished due to a limited pharmacological understanding and increasing evidence of safer therapeutic action from combinations of less potent phytocannabinoids (Hartsel et al., 2016). According to the Center for Disease Control, illegal synthetics such as spice or K2 can produce effects uncharacteristic of plant-derived versions and are known for being unpredictable and potentially toxic[2].

Cannabinoid Detection

Cannabinoid detection in blood samples is still an evolving technology. One of the more common methods of serum cannabinoid detection is using liquid chromatography-mass spectrometry (LC/MS) (Gamble et al., 2018; Davis, 2019; Deabold et al., 2019). This highly sensitive method includes physical separation through dissolving the compound in a solution, followed by the analysis of mass. For LC-MS/MS instruments, there is a second mass spectrometry detector attached. Some of the most sensitive methods are reading serum cannabinoid concentrations down to a 0.05 ng/mL lower limit of quantification (LLOQ), though concentration LLOQs used for data reports are typically above 1 ng/mL (Davis, 2019). Product analysis may occur through both LC-MS/MS and high performance liquid chromatography diode-array detectors (HPLC-DAD). Following physical separation, DAD allows researchers to examine samples for organic compounds based on wavelengths.

---

[2] Center for Disease Control (2018, April 24) *Health Studies-Understanding Chemical and Radiation Exposure: Synthetic Cannabinoids.* www.cdc.gov/nceh/hsb/chemicals/sc/default/html.

Current Clinical Studies

The nutritional composition of hempseed has contributed to a heightened interest towards animal feeds, particularly laying hens, sheep, cattle, and farm fish. The protein content can range from 20-25%, with reasonable percentages of carbohydrates (20-30%), oil (25-35%), fiber (10-33%), and minerals (Callaway, 2004; Kriese et al., 2004; Latif and Anwar, 2009; Snider, 2020). Additionally, omega 6 and omega 3 fatty acids have been observed in a 3:1 ratio in the raw product (Leizer et al., 2000). Increasing evidence exists that balancing this ratio assists in the management of chronic conditions, cardiovascular disease, cancer, inflammatory, and autoimmune diseases (Simopoulos et al., 2002). Hempseed has been found comparable to flax in the yield of linoleic acid, although both are considered less efficient than that of sunflower seed (Kriese et al., 2004). Nutritional studies have demonstrated an improvement of omega fatty acid profile in the eggs of hempseed fed chickens (Silversides, 2002), as well as serving as a natural source of rumen-undegraded protein in cattle and sheep (Mustafa et al., 1999). Various components of the plant may be employed to contribute to the overall economic potential of hemp. The nutritional profile of hempseed makes it an interesting topic of discussion for livestock production, while specific cannabinoids within the plant have gained attention for behavioral and physical therapeutic potential (Pertwee, 2004; Thomas et al., 2007; Latif et al., 2009; Mechoulam et al., 2017; Gamble et al., 2018).

Despite an initial focus on murine models (Blessing et al., 2015), trials have materialized in recent years on humans and other animals (Devinsky et al., 2018; Gamble et al., 2018; Deabold et al., 2019; Ellis and Contino, 2019). Methods of administration include intraperitoneal, oral, and transdermal applications (Malfait et al., 2000; Lodzki,

2003; Jeong et al., 2014; Hammel et al., 2016). Further research is needed to accurately assess pharmacokinetics, bioavailability, efficacy, molecular stability, and drug interactions within veterinary and equine medicine (Ellis and Contino, 2019; as reviewed in Hartsel et al., 2019). Long-term dosing effects and efficacy in chronic cases are particularly lacking (Blessing et al., 2015).

An initial issue encountered in oral treatment options was to overcome the highly hydrophobic nature of CBD (Lodzki et al., 2003). Oil mixtures have shown increased effective uptake in clinical settings, however, bioavailability of cannabinoids remains dependent on the vehicle and status of encapsulation (Gamble et al., 2018). The lipophilic nature of CBD and first pass effect at the liver directed some researchers toward alternative methods of administration such as transdermal application (Hammel et al., 2003; Lodzki et al., 2003). Lodzki and colleagues reported a product that delivered consistent systemic drug levels to a targeted area and was absent of psychoactive effects, through a 3% CBD and 20% ethanol carbomer gel (2003). A subsequent study tested various concentrations of CBD in an application gel (Hammel et al., 2003). This product demonstrated a reduction in numerous inflammatory measures, including joint swelling, limb posture scores, immune cell infiltration, and thickening of the synovial membrane (Hammel et al., 2003). Not only did it demonstrate success in inflammation alleviation, but motor control measures remained unchanged throughout the study demonstrating little to no effect on higher brain function (Hammel et al., 2003).

Oral and oral-mucosal therapeutic treatments have been one of the primary routes tested. Successful uptake has been achieved in humans, dogs, and mice (Malfait et al., 2000; Blake et al., 2006; Crippa et al., 2011). Cannabidiol safety is supported through

existing research, however, the lack of regulation on product content carries risks for both animals and handlers (Crippa et al., 2011; as reviewed in Hartsel et al., 2019). Despite current efforts, the issue is that CBD pharmacology is so poorly defined that it is difficult to truly understand the safety and efficacy, especially for chronic conditions and long term use (Gustafson, personal communication[3]). Existing studies demonstrate a particular affinity of CBD towards chronic, neurological, and autoimmune diseases (Blake et al., 2006; Jeong et al., 2014; La Porta, 2014; Philpott et al., 2014; Devinsky et al., 2018). Positive outcomes were demonstrated in a human study treating rheumatoid arthritis through an oral mucosal spray (Blake et al., 2006). Despite being a THC positive product, side effects were minimal and withdrawal effects absent. The pharmacokinetics available in other species demonstrated a blood concentration maximum to be between 1-2 hours, and a half-life of 4 to 4.5 hours (Crippa et al., 2011; Gamble et al., 2018).

There is a case study available on a single horse treated with a pure crystalline oral CBD product over an extended time frame (Ellis and Contino, 2019). The patient was administered a dosage formulated loosely from human recommendations, equivalent to about 0.5 mg/kg twice daily for initial treatment. Positive results were observed in less than 48 hr. Although the product could not be completely removed without symptom recurrence, it was successfully dropped over a 2 mo period to a maintenance dose of 0.33 mg/kg once daily. This case emphasizes the ability of CBD to work as a pain reduction agent, but not as a product capable of addressing and healing the source of the problem. Despite success regarding the alleviation of nociception and neuropathic pain in this

[3] Daniel L. Gustafson, PhD., Colorado State University, October 30, 2019

study, continued research was encouraged for the development of dosage standards, safety guidelines, and an improved understanding of drug interactions in the horse.

Monitoring Movement Influencers in Horses

Forms of unsoundness or lameness may be a result of conformational defects, improper shoeing, injury, workload or age (Schlueter and Orth, 2003). Pain is typically the primary concern; however, even when pain is relieved movement restriction can persist (King and Mansmann, 1997). Current treatment options for associated pain and inflammation include nonsteroidal anti-inflammatory drugs (NSAIDS) and corticosteroids. Despite effective reduction of inflammation and pain these products are associated with detrimental health effects, particularly with prolonged use, as their function interferes with protective mechanisms in the body (Clegg and Booth, 2000). Such issues can include increased risk of ulcers, colitis and potentially toxicity.

Study of synovial fluid samples, gait analysis, and diagnostic imaging are considered ideal methods for identification and evaluation of osteoarthritis progression (Tulamo et al., 1989; as reviewed in Kawcak, 2001; Bertone et al., 2001). While imaging provides observation of visual changes, it does not quantify inflammatory changes. Synovial samples used to monitor joint degradation can carry the risk of introducing infection, potentially worsening the condition (Seidman and Limaiem, 2019).

Investigation of inflammatory biomarkers could be one method of testing CBD efficacy for inflammation reduction. Currently, a specific blood test does not exist to monitor osteoarthritis progression (KER, 2016). Therefore, research must rely on alternative tests to track inflammatory biomarkers. In the assessment of rheumatoid

arthritis patients, there is a widely accepted blood test known as the disease activity score 28 (Karimafer et al., 2012). This test uses an erythrocyte sedimentation rate test and a C-reactive protein test to evaluate inflammation levels. Although not specific to the joint affected, the test creates a relatively accurate representation of inflammation presence. Other inflammatory biomarkers of significance include metalloproteinase and cytokine tumor necrosis alpha levels (TNFa) (Hammell et al., 2016). Dependent on zinc and calcium, metalloproteinase is an enzyme linked with inflammatory cytokine levels. This could play an important role in cartilage destruction. The TNFa is a cytokine produced during periods of acute inflammation subject to a variety of cell signaling pathways that eventually lead to cell death. Examination of the enzyme lactate dehydrogenase (LDH) in blood samples could also serve as an inflammatory measure (Drent et al., 1996). Located in the cell cytoplasm, LDH only rises in serum in the event of cell death or damage caused by various systemic stressors (Drent et al., 1996). Although not ailment specific, LDH presence is directly correlated to physical stress.

In addition to pharmacological analysis, researcher evaluation of joint rigidity scores through a 4-tiered scale, ranging from normal to severe arthritic changes, served as a method of evaluating disease progression and CBD efficacy in a murine model (Malfait et al., 2000). In horses, there is a lameness test that follows similar philosophy, but observes the horse in motion for systemic soundness and mobility evaluation by licensed veterinarians (Keegan, 2012). The American Association of Equine Practitioners recognizes this as the standard for equine lameness evaluation. There is concern regarding test subjectivity among veterinarians. However, using it strictly among licensed

veterinarians allows the test to be maintained as a mobile, affordable, and immediate method of evaluation.

CBD and Behavioral Modification

Informal observations of pharmacological effects have promoted CBD products as a method of behavioral modification. Equine behavior issues as the result of stress, boredom, or frustration may be expressed in the form of weaving, cribbing, or having a generally poor attitude (Houpt and McDonnell, 1993; as reviewed in Sarrafchi and Blokhuis, 2013). Such behaviors can be a catalyst for weight loss, injury and ultimately reduced performance ability. Methods of addressing behavioral issues include alteration of animal husbandry practices, providing physical restraint, or taking pharmacological action (as reviewed in Sarrafchi and Blokhuis, 2013). Calming products are most commonly used in association with horse shows (Joss and Roberts, 2018). Two issues exist with current therapeutic agents. Primarily, there are products advertised as calming agents, such as tryptophan, that demonstrate bioavailability but lack consistent efficacy in horses (Noble et al., 2008). Despite such evidence from research trials, up to 84% of owners report administering a form of supplement to at least one horse in their care, with 47% believing these supplements to be helpful with behavioral issues (Swirsley et al., 2017). While equine owners appear extremely open to administering supplementation, anecdotal success rates could be a result of the placebo effect. The mild sedative acepromazine maleate has reduced animal response rate to external stimuli, however, it can be associated with negative side effects (Griffith, 2006). Perhaps the most comparable product also used as an anxiolytic nutraceutical in horses is magnesium

supplementation. While demonstrating some ability to reduce heart rate and reaction speed of horse, studies have found the nutraceutical inconsistent and highly variable in regard to anxiolytic capabilities (Dodd et al., 2015; Pearson and MacNicol, 2017). The proposed use of CBD offers an approach to stress reduction absent of health risks and potentially calming the horse without risk to performance ability.

Investigation of CBD for its anxiolytic components exists in both human and animal trials (Crippa et al., 2004; Blessing, et al., 2015). A variety of neuropsychiatric disorders are incorporated, including those specifically related to fear and anxiety (Blessing et al., 2015). Research suggests CBD may act through anxiolytic mechanisms within the limbic and paralimbic processes in the brain (Crippa et al., 2004; Fusar-Poli et al., 2009; Crippa et al., 2011). The limbic system has been accepted as an area involved in emotion, memory, and behavior processes with the specific ability to regulate autonomic or endocrine function in response to emotional stimuli and assist in behavior reinforcement (Morgane, Galler, and Mokler, 2005). Relative structures associated include the amygdala, hippocampus, hypothalamus, and limbic cortex (Crippa et al., 2011). Given that CBD receptors have been confirmed in many such areas of the brain, it is unsurprising that the systems would be linked. Evidence of CBD involvement in endocrine function through the limbic system supports the ability of appropriate products to affect hormone release. Impact on the signaling pathways that control hormone release would permit a waterfall effect, creating influence on a multitude of functional processes. These could include metabolism, development, reproduction, sleep and mood. Though currently banned by USEF and FEI as an under investigated, potential performance

enhancement drug, a deeper understanding of the functions and safety of CBD mechanisms would allow for educated decisions on its therapeutic use.

Summary

While the current understanding of cannabinoids and the system they operate within is growing, many questions remain to be answered. Current literature demonstrates reasonable cause for the investigation of CBD to serve as treatment candidate for inflammation, pain and anxiety. There is evidence of interaction within neuronal signaling pathways that result in positive changes within chronic diseases and anxiety disorders. Current treatment options for horses with pain and inflammatory related conditions rely on drugs that are effective but often contain long-term use health risks. Given that CBD appears to lack the degree of negative side effects in short term therapeutic studies, natural progression would be to examine the safety of the product over an extended treatment period to determine its ability as an alternative therapeutic approach within specific equine conditions. With numerous products being marketed and used for a variety of ailments it is pertinent that proper research is executed to ensure animal safety and welfare.

Chapter 3

Methodology

Subjects for all projects were derived from the Murray State University equine population. Standard management practices and use patterns were maintained throughout each project. Medications and management changes were documented throughout. All procedures were approved through the Institutional Animal Care and Use Committee (Appendix A).

Project 1

Horse Selection and Management

Project 1 was a one-shot experimental case study, serving as a pilot to the subsequent projects. Two Quarter Horse geldings of similar age and use were selected for this project. Both horses belonged to a University riding program, however, neither subject was in active work at the time of the study.

Treatments and Data Collection Procedures

Horses were randomly assigned one of two forms of CBD treatment. Treatments consisted of a pellet (PEL) containing 25 mg of cannabinoids per serving (10 g/tbsp) and an oil (OIL) containing 25 mg of cannabinoids per ml (Equine Veterinary Services

Pharm, Paducah, KY, USA). Treatments were administered based on the manufacturer high dose recommendations of 50 mg. Both products were labeled as 0 THC.

Horses were brought from pasture into stall confinement in order to feed the PEL and OIL supplements. Horse 1 was fed 0.1 kg of Kalm'N EZ ® (Tribute Equine Nutrition ®, Upper Sandusky, OH, USA), to increase palatability, plus PEL in an elevated feed pan to avoid spillage and monitored to ensure complete consumption. Horse 2 was administered the CBD OIL via an oral syringe. Upon administration the horse's head was held up to prevent lack of consumption. Subsequent blood collections were taken via jugular venipuncture at 1 h and 2 h post treatment. Samples were collected in four 10 mL serum vacutainers (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ), transported within 20 min to a university laboratory and centrifuged at 3500 rpm (LWS-Combo-V24 Centrifuge LW Scientific, Atlanta, GA). A minimum of 5 cc of serum was pipetted into 5 ml Eppendorf storage tubes (Eppendorf Tubes® 0030119452, online-shop.eppendorf.us, USA) and immediately placed into a refrigerator. Once collections were complete, samples were stored on ice, and shipped overnight to Texas A&M University Veterinary Medical Diagnostic Laboratory (College Station, TX, USA) for analysis. Samples were analyzed using LC-MS technology with positive and negative controls. The remaining serum from both samples was comparatively analyzed using LC-MS/MS technology by the Pharmacology Shared Resource (University of Colorado Cancer Center) housed at Colorado State University (P30 CA046934). Separation began by an Agilent© 1200 HPLC system (Agilent Technologies, Inc. Santa Clara, CA) being coupled with an Applied Biosystems® Q3200 MSMS (Applied Biosystems, Inc. Foster City, CA) using Analyst 1.7.1 software for the analysis. Samples were received knowing

that CBD content was low; therefore, traditional methods were adjusted to enhance the likelihood of detection. A C8 Sunfire column was used with Q3 resolution adjusted to low yielding maximum sensitivity. Approximately 500 mL of serum was extracted two times with cyclohexane in order to detect CBD concentrations as low as 50 pg/mL. Samples were vortexed for 5 min, and then centrifuged at 13,300 relative centrifugal force (rcf) for 10 min.

To determine validity of the blood serum readings a comparative lab analysis was completed on the same samples from Project 1, using two different labs. The Colorado State Pharmacology Shared Resource Veterinary Medical Center was chosen due to involvement in similar studies (Gamble et al., 2018; Deabold et al., 2019). The pelleted product was also evaluated for cannabinoid concentration stability throughout the three-phase project. Manufacturer claims ensure a minimum of 25 mg phytocannabinoids per 10 g (1 tbsp) scoop. Following the original internal analysis, four samples were tested through two separate labs, the Murray State University Analytical Chemistry Department as well as Botanacor ™ (Botanacor, www.botanacor.com, Denver, CO). All labs used for blood and product analysis were accredited.

Project 2

Horse Selection and Management

Project 2 included 18 Quarter Horse geldings, (avg age =15± 4.2 yrs, avg BW = 555.2 ±40.8 kg). Horses were randomly allocated to one of three treatments based on age category (≤14 yrs = young, ≥15 yrs = older), level of use (low = 1-2 hr/wk, moderate = 3 hr/wk, or intense (≥4 hr/wk; National Research Council, 2007), and housing (pasture or

stalled). Horses known to have adverse reactions to stall confinement were excluded from the study. One older horse used for this study also received daily long term Vit E supplementation; however no other horses received any additional supplementations except their respective treatment.

During the feeding trial, horses were confined to stalls, fed concentrates (including their respective treatment) and recommended amounts of Bermuda grass hay with ad libitum access to water. Concentrate consisted of either Kalm'N EZ ® (Tribute Equine Nutrition ®, Upper Sandusky, OH, USA), (n=3), or HSS Reliable 12% protein, 6% fat bulk horse pellets (Southern States ®, Cadiz, KY), (n=15). Rations were dependent on the horse's individual demands as follows: 2 q (n=1), 4 q (n=15), or 6 q (n=2). Horses were hand walked two times throughout the study during stall cleanings.

Treatments and Data Collections

All horses were fitted with an indwelling catheter to reduce stress associated with blood collections. A licensed veterinarian inserted catheters within 2 h of horses being brought into stall confinement. Jugular blood was collected immediately before supplemental feeding began allowing each horse to serve as their own control. Blood collection consisted of four 10 ml serum Vacutainers for cannabinoid analysis, one 10 ml EDTA Vacutainer for complete blood count (CBC) analysis, and one additional 10 ml serum Vacutainer for a liver and kidney serum chemistry (SC) analysis (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ).

Treatment consisted of the same PEL CBD supplement used in Project 1. A single administration was given in one of three dosage rates (50 mg = TXT1, n=6; 100 mg =

TXT2, n=6; 250 mg = TXT3, n=6; Equine Veterinary Services Pharm, Paducah, KY, USA). Subsequent blood collections occurred for: cannabinoid analysis at 0.5, 1, 2, 4, and 12 h post treatment; CBC analysis at 12 h post treatment; and SC collections to evaluate liver and kidney function at 4 h and 4 d post treatment.

Blood samples were allowed to clot and serum extracted as described previously. Serum samples for SC and CBC analysis were immediately transported on ice to the Murray State University Breathitt Veterinary Center (Hopkinsville, KY, USA). The SC panels were analyzed using a Beckman AU480 instrument (Beckman Coulter, Inc.™, Miami FL, USA). Complete blood count concentrations were evaluated through an XT-2000iv blood panel (Sysmex◦, Norderstedt, Germany). Serum samples designated for CBD analysts were stored in a -20° C freezer until shipped on dry ice and analyzed by the Pharmacology Shared Resource (University of Colorado Cancer Center) housed at Colorado State University (P30 CA046934). Catheters were removed after the final blood collection. Horses remained stalled and monitored for signs of adverse reactions for 12 h before being returned to standard housing.

Statistical Analysis

Complete blood cell count variables evaluated were reported within normal ranges and were not included in statistical analysis. Due to cannabinoid concentrations being lower than LLOQ, statistical analysis could not be performed.

Statistical analysis was performed for effects of treatment and time upon SC variables using the MIXED procedure of SAS (SAS, Cary, NC, USA). Serum chemistry variables included: blood urea nitrogen (BUN), albumin, creatinine, and alkaline

phosphatase (ALP). Significance was reported if P≤0.05 with tendencies reported as P≤0.09. Experimental unit was the horse with each horse serving as its own control.

Project 3

Horse Selection and Management

Project 3 followed the format of a pretest-posttest control group design to evaluate extended CBD treatment. Horses used for this project (n=17) were randomly selected from the same University population. All horses were maintained in their normal housing, consisting of either a stall with pasture turnout or pasture only. Diet consisted of Bermuda hay and concentrate with free access water. Two forms of concentrate were fed based on standard rations for individual needs: Kalm'N EZ ® (14% protein, 6% fat; n=2; Tribute Equine Nutrition ®, Upper Sandusky, OH) or HSS Reliable (12% protein, 6% fat; n=15; Southern States ®, Cadiz, KY). The majority of horses (n=14) received 2.2 kg of concentrate/d. Other rations fed include 1 kg/d (n=1) and 3 kg/d (n=2). Weights were monitored with a digital livestock scale through a pre (wk 0), midpoint (wk 4) and final (wk 6) collection. All medications administered to subjects were documented throughout the study. One TXT horse received Previcox® (firocoxib) throughout the clinical study. Management changes, weight, and workload were tracked for the study duration. A summary of the population used for statistical analysis (n=17) is represented in Table 1.

Table 1

Horse Demographics for entire population used in Project 3

		Weight (lb)					
Group	Age	1	2	3	Location	Use	Grain (scoop)
T	≥15	1340	1329	1343	P	Moderate	1 HSS
T	≥15	1088	1102	1128	P	Light	1 HSS
C	≤14	1361	1380	1360	P	Light	1 HSS
C	≤14	1233	1215	1222	Stall	Light	0.5 HSS
T	≥15	1242	1199	1212	P	Light	1 Tribute
T	≥15	1290	1288	1292	Stall	Light	1 Tribute
C	≤14	1195	1163	1172	Stall	Light	1 HSS
C	≤14	1188	1169	1125	P	Light	1 HSS
C	≥15	1187	1154	1193	P	Moderate	1 HSS
T	≥15	1178	1150	1174	Stall	Light	1.5 HSS
T	≤14	1155	1148	1185	P	Light	1 HSS
C	≤14	1365	1341	1368	Stall	Moderate	1 HSS
T	≤14	1166	1165	1185	Stall	Moderate	1.5 HSS
T	≥15	1235	1247	1270	P	Moderate	1 HSS
C	≥15	1205	1175	1196	P	Light	1 HSS
C	≤14	1147	1124	1103	P	Light	1 HSS
T	≥15	1205	1160	1152	P	Moderate	1 HSS

Note. Treatment group (T), control group (C), pasture (P), light (1-2 hr/wk), moderate (3 hr/wk), heavy (≥4 hr/wk), young (≤14 yr), aged (≥15 yr), Kalm N' Easy ® (Tribute), Southern States (HSS), one 4 q scoop =2.5 lb.

Treatments and Data Collection Procedures

Seventeen Quarter Horse geldings were randomly assigned to two groups for an 8 wk feeding study evaluating horse behavior and movement parameters. Horses were randomly assigned to treatments based on the same parameters as in Project 2. Control horses (CON; n = 8) received no CBD supplement while treatment horses (TXT; n = 9) received a once daily dose of 100 mg CBD PEL (Equine Veterinary Services Pharm,

Paducah, KY, USA). Treatment dosage rate was determined based on Project 2 results and committee consultation. Though the CBD supplement was fed for a total of 8 wk, behavior and movement parameters were evaluated immediately before initial feeding of the CBD supplement began and again at conclusion of the study in wk 6. Blood samples for SC were collected every 2 wk from TXT horses during the entire 8 wk feeding to monitor product safety via liver enzymes.

Movement Methods

Movement analysis was completed through multiple methods. During week one, a random subsample of TXT (n=6) and control horses (n=6) received a soundness evaluation by a veterinarian. This consisted of a standard flexion test, and a grade from the AAEP lameness scale when warranted (Back et al., 2007). Horses were first lunged at the walk and trot on a 15 m circle, both directions in an indoor arena. The veterinarian, blind to treatment groups, then observed the horse being hand jogged on a 9.14 m straight, flat concrete aisle. Flexion tests were performed for the upper and lower limb for 45 sec, with the horse immediately jogging away from the veterinarian for observation. Upper forelimb flexion examined the carpus, while lower limb flexion analyzed the fetlock, pastern and coffin. Hind limb flexion examined primarily the stifle, and hock in upper flexion, and the fetlock, pastern and coffin during lower limb flexion. Based on this exam, each horse was assigned a lameness category (Table 2) for the upper and lower section of each limb. Positive scores were given based on the presence of unsoundness over the length of the jog with categories of mild, moderate, or severe. If appropriate, a subsequent score from the AAEP Lameness Scale was then assigned.

Table 2

Lameness category explanations during veterinary examination of subsample

Category	Explanation
Negative	Evidence of unsoundness absent for the length of the jog
Mild Positive	Unsoundness present no more than ½ of the jog
Moderate Positive	Unsoundness present the majority of the jog, might be slight
Severe Positive	Extremely obvious unsoundness present the entire jog

There have been concerns regarding the subjectivity of the AAEP flexion test (Keegan et al., 2010). However, the AAEP lameness scale and flexion test remain in use for basic equine soundness examination (Björnsdóttir, Árnason, and Lord, 2003).

All horses were examined through Dartfish 360 (DARTFISH, www.dartfish.com Fribourg, Switzerland) video analysis with a Canon EOS Rebel T6 (Canon U.S.A., Inc., usa.canon.com, Melville, NY). Video recordings were taken in an indoor arena. The Dartfish program has been used as an effective method of human stride analysis (Eltoukhy et al., 2012), while video observations have been implemented in equine stride analysis (Licka et al., 2004). Four experienced equine handlers were used consistently throughout the project for movement and behavior tests. As lameness can demonstrate inconsistencies based on rider presence, horses were hand jogged to reduce variability (Licka et al., 2004). Each horse was first walked and then trotted past a series of 6 cones (Figure 1), with the recorded distance between cones 3 and 4 being 9.14 m. Handlers were asked to allow the horse to move freely, without lead rope tension. When necessary, a volunteer was asked to drive the horses from behind. The video camera was placed on a stand 14.63 m from the center of the trot stretch. Horses were asked to be in the

appropriate gait by cone 2 and to continue steadily until cone 6. Three clean, consecutive strides from the 9.14 m path were used for frame-by-frame video analysis. Cones 3 and 4 served as a 1 m calibration mark for Dartfish analysis. Stride length was measured for individual limbs from the toe during stance phase to the subsequent stance phase. Time spent in stance and swing phase were measured from the same strides in hundredths of a second with frame-by-frame analysis.

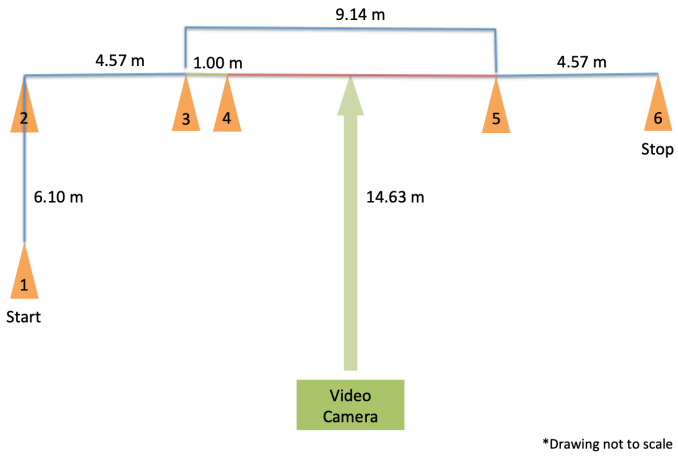

Figure 1. *Diagram of movement design*

A survey was developed to represent instructor interpretation of horse cadence over time. Questions were based primarily on a 5-grade scale (Appendix B). The survey was presented separately to University riding instructors who remained blind to TXT groups, within a week of the movement and behavior data collections.

Behavior

Behavior data was collected at the start of the study during wk 0 (PRE) and wk 6 (POST). A novel object test (NOT) was implemented for reactivity evaluation. The NOT exposed the horse to a startle stimulus to elicit a reactive response. The testing method and rubric were modeled from previous equine behavior research (Table 3; Holland et al., 1996). Observations were completed in triplicate by one live evaluator and two other evaluators via video playback. Evaluators were asked to rate each horse from the rubric based on the expressed reactivity to the stimulus.

Table 3

Rubric used to evaluate reactivity during the novel object test (Holland et al., 1996)

Score	Description
1	Horse shows no reaction or interest in the stimulus.
2	Horse looks in the direction of the stimulus but has no other reaction.
3	Horse jumps when stimulus is applied but does not try to run away.
4	Horse jumps away from the stimulus and tries to leave.
5	Horse completely loses control and tries to flee or refuses to move from the spot.

In addition to the behavior rubric heart rate monitors were attached to the horse throughout the NOT (Polar Electro USA, Equine V800, Bethpage, NY, USA), and marked “A” and “B” to ensure that transmitters and receivers remained paired appropriately. After the withers and heart girth were dampened with water, electrodes were secured along with the transmitter through the assistance of a saddle pad and surcingle. The positive electrode was placed against the withers and the negative on the heart girth. Once heart rate was detected on the wireless receiver, the heart rate recording was started and the horse stood for one minute in the aisle of a relatively quiet barn. At

the 1 min mark, the heart rate indicated on the receiver was documented. The horse was immediately walked on a slack line down a barn aisle to the location of the NOT. At the stimulus point of the NOT, the operator opened a polka dotted umbrella as soon as the horse's head was visible around the barn corner. The umbrella remained opened as the horse was allowed to react and process. Handlers were not to offer reassurance or attempt to force the horse to move in any direction. Once the horse passed the object, the umbrella was closed and the horse continued to the side entrance of the barn. A series of cones outlined the path each horse should follow (Figure 2). A cone was placed to ensure the umbrella operator would consistently open the umbrella approximately 3.05 m from the horse. The horse handler observed heart rate at stimulus exposure from the wireless receiver and reported this number for documentation upon return to the barn. Video recording started as soon as the horse was visible from the barn aisle exit, and continued until the horse passed the object. Once the horse returned to the barn, a 1 min timer was started. The horse was asked to stand and relax during this time, after which the final heart rate was documented and the monitor recording halted.

Jugular blood samples were collected one day after behavior data was collected PRE and POST to determine cannabinoid potency, CBC, and SC analysis. Blood collections were performed prior to hand jogging for evaluation of movement parameters. Blood was collected in four 10 ml serum Vacutainers and one 10 ml EDTA Vacutainer. Serum cannabinoid potency was analyzed by the Pharmacology Shared Resource (University of Colorado Cancer Center) that is housed at Colorado State University (P30 CA046934) using LC-MS/MS technology. Additional blood samples

were collected in serum Vacutainers on wk 2 and 4 to evaluate long-term product safety through SC.

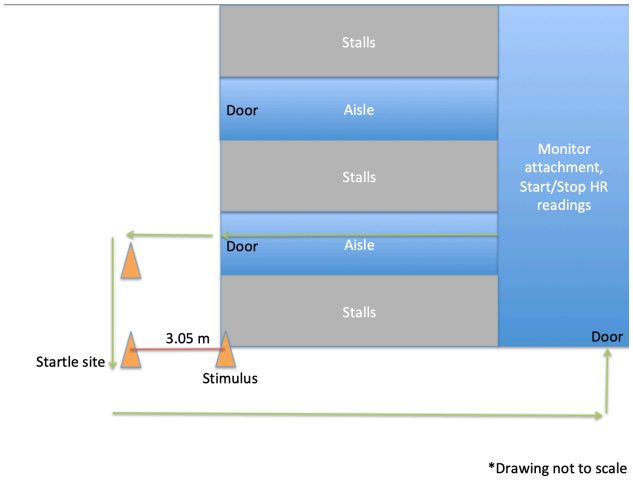

Figure 2. *Diagram of behavior startle test procedures. Green lines indicate path of the horse.*

Statistical Analysis

Blood samples for cannabinoid concentration, SC, and CBC were not analyzed, and have been stored in a -20° C freezer for future analysis as funding becomes available.

Statistical analysis was performed using SAS (SAS, Cary, NC, USA). The PROC MIXED Procedure was used to evaluate movement and behavior parameters. The effects of treatment and wk were evaluated for movement parameters including: stride length at walk and trot, time spent in stance phase in walk and trot, and time spent in swing phase in walk and trot. Overall stride length, stance phase and swing phase values for each horse was based on the average of the four limb scores recorded. For behavior parameters, effects of treatment and age were evaluated upon stimulus heart rate, start heart rate, and stop heart rate.

Chi-square analysis was performed using the PROC FREQ procedure to determine: 1) the relationship between CBD treatment and reactivity scores in horses and 2) effects of treatment on survey responses. Behavior scores were completed in triplicate and averaged. To simplify statistical analysis, the 1-5 scoring system was modified so that scores of 1 and 2 were considered "low", a score of 3 was considered "moderate", and scores of 4 and 5 were considered as a "high" degree of reactivity. Fisher's Exact Test was used when fewer than 5 observations occurred per column.

Product Sampling

Two batches of CBD pellets were used throughout the entire 8 wk feeding of Project 3, COHP19-03 (EVS Pharm) and COHP19-01 (Folium Biosciences). Both were produced through Folium Biosciences (Folium Biosciences, Colorado Spring, CO). However, only the batch from EVS Pharm was fed during the 6 wk trial, pharmacokinetic study, and pilot project. Feeding was continued an additional 2 wk with the intent of monitoring SC and CBC with extended treatment. It was during the final 2 wk that the batch directly from Folium was used. An original cannabinoid concentration at the point of original packaging was obtained from an in house lab for each batch. Subsequent analysis through two independent labs occurred throughout the study to monitor any changes in CBD concentration.

Manufacturer claims for COHP19-03 ensured a minimum of 25 mg phytocannabinoids per tbsp scoop. Other components of the product included 20% plant protein, 30% insoluble fiber, 50% complex carbohydrates, a flavoring agent (Apple Ade) and an FDA approved mold inhibitor (Myo Curb ®). Including the distributing company's original internal analysis, four subsequent samples were tested through two

separate accredited labs, including the Murray State University Analytical Chemistry Department (MSUAC) and Botanacor ™ (Botanacor Laboratories, Denver, CO). Analysis completed by the MSUAC was completed in triplicate, using ultrasonication for analysis. The CBD pellet samples were first ground. Subsequently, ~0.5g was extracted and analyzed for CBD and THC content using a validated LC-MS/MS method. Concentrations from Botanacor™ were achieved through Agilent HPLC-DAD instrumentation compliant with Good Laboratory Practices and current Good Manufacturing Practices requirements.

Chapter 4

Results

Project 1

Concentrations of both administration forms were below the standard lower limit of quantification (LLOQ) for Lab A. Detection methods were adjusted by Lab B by lowering the LLOQ to 0.05 ng/mL. Concentrations were at or above this concentration at hours 1 and 2 post administration in the PEL (0.0595 ng/ml, 0.163 ng/ml, respectively) and at 2 hours post for the OIL (0.11 ng/ml). Both sampling times demonstrated slightly higher concentrations of CBD in the PEL form (Figure 3).

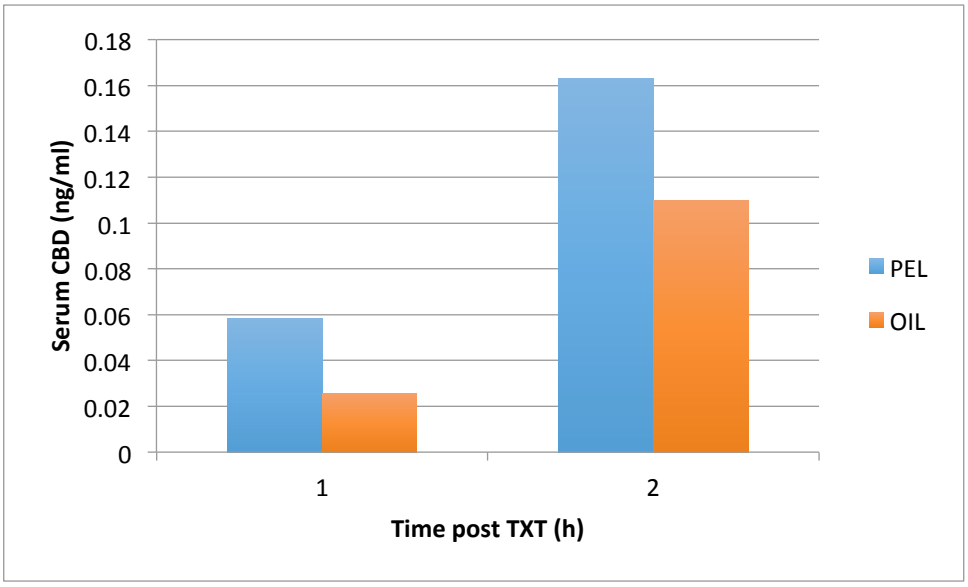

Figure 3. *Serum detection comparison of pellet (PEL) and oil (OIL)*

Project 2

Data from project 2 suggests a time of CBD concentration maximum in the equine species to be 2 h. Except for one horse from the 100 mg group, only horses administered the 250 mg dose showed detectable CBD concentrations above the standard 0.1 ng/ml LLOQ. Out of 6 horses on the 250 mg dose, one did not reach LLOQ detection levels. This horse only consumed 120 mg of the assigned 250 mg dose. Figure 4 represents changes in serum concentration for those subjects above standard LLOQ throughout the 12 h sampling period.

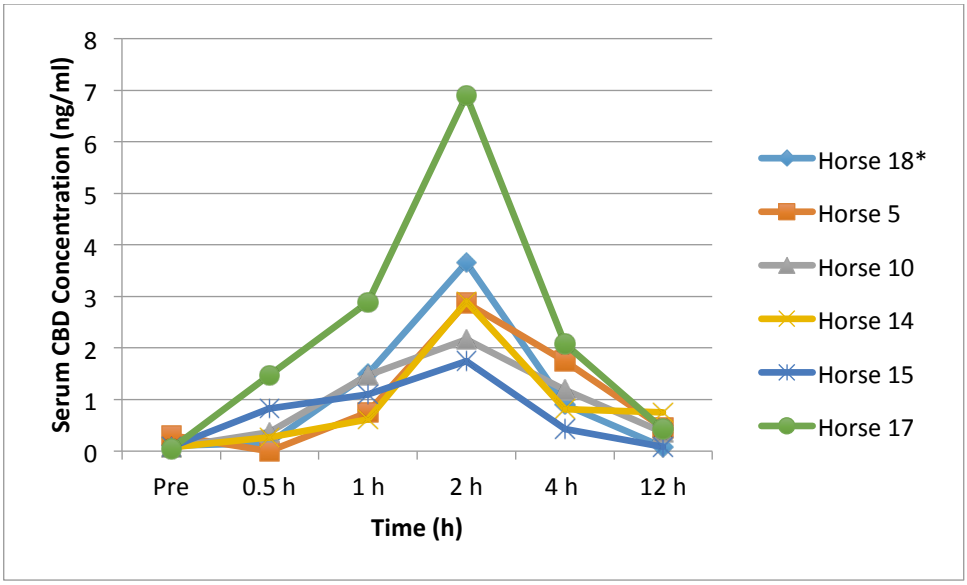

Figure 4. *Serum CBD concentrations above LLOQ (ng/ml). Asterisk (*) indicates subject from 100 mg TXT group. Horses without an asterisk belonged to the 250 mg TXT group.*

Results from the complete blood count returned within normal parameters. Though serum chemistry concentrations remained within reference ranges, changes within ranges were observed in relation to TXT (Table 4) and time (Table 5).

Table 4

Enzyme concentration differences based on treatment

Enzyme	50mg	100mg	250mg	SEM	P value
BUN	15.50^{b}	16.52^{b}	18.61^{a}	0.66	≤0.03
Creatinine	$1.41^{b\dagger}$	1.22^{a}	1.49^{b*}	0.03	≤0.01
ALP	101.56	100.44	114.22	5.20	0.12
Albumin	3.41^{a}	3.35	3.24^{b}	0.05	0.01

Note. Different letters within the same row differ by p≤0.05. Different symbols within the same row indicate tendencies that differ by p>0.05, and p≤0.09.

Table 5

Enzyme concentration differences over time

Enzyme	Pre	4 hours	4 days	SEM	P value
BUN	$15.89^{b\dagger}$	16.00^{b*}	18.74^{a}	0.66	<0.01
Creatinine	$1.39^{\dagger}$	1.46^{a*}	1.34^{b}	0.03	≤0.01
ALP	104.22	107.06	104.94	5.20	0.92
Albumin	3.28	3.39	3.34	0.05	0.27

Note. Different letters within the same row differ by p≤0.05. Different symbols within the same row indicate tendencies that differ by p>0.05, and p≤0.09.

Project 3

Initial Veterinary Examination

Lameness scores were assigned to a portion of the observed subsample (CON=4, TXT=3). The highest score was a 3, given to a control horse following right hind upper limb flexion. Scores were seen in both groups of a mild (CON=5, TXT=5) and moderate (CON=4, TXT=2) rating. No severe ratings were given.

NOT Heart Rate

Statistical significance was not observed for start, stop, or stimulus HR during the NOT based on TXT (Table 6) or time (Table 7).

Table 6

Summary of heart rate (HR) observations through the novel object test based on treatment

Parameter	Treatment	Control	SEM	P value
Start HR	40.22	44.29	2.91	0.3468
Stimulus HR	93.36	112.64	8.34	0.1253
Stop HR	45.39	47.94	3.14	0.5852

Table 7

Summary of heart rate (HR) observations throughout the novel object test based on age

Parameter	Young	Aged	SEM	P value
Start HR	42.84	41.67	2.92	0.7875
Stimulus HR	103.94	102.06	8.37	0.8804
Stop HR	47.67	45.66	3.16	0.6705

NOT Behavior Scores

Treatment horses demonstrated a lower frequency of moderate to high reactivity scores than control horses (TXT=24, CON=37; p=0.0018). A relationship summary is found in Table 8.

Table 8

Frequency and percent of novel object test scores based on treatment group

Group	High	Moderate	Low	Total
Control	13 (12.87)	24 (23.76)	10 (9.90)	47 (46.53)
Treatment	10 (9.90)	14 (13.86)	30 (29.7)	54 (53.47)
Total Scores	23 (22.77)	38 (37.62)	40 (39.60)	101 (100.00)

Note. *f* (%). P=0.0018.

Movement Parameters

The effects of both TXT and time were evaluated. Treatment horses spent slightly longer in stance phase during walk (TXT=0.57 sec, CON=0.51 sec; P<0.01) and swing phase during walk (TXT=0.38 sec, CON=0.36 sec; P<0.01) than control horses.

Time spent in stance phase during walk increased over time (Pre=0.37 sec, Post=0.71 sec; P<0.01). When in trot, time spent in both stance (Pre=0.30 sec, Post= 0.26 sec; P<0.01) and swing phase (Pre=0.37 sec, Post= 0.33 sec; P<0.01) decreased. Additionally, stride length at trot shortened by the study conclusion (Pre=1.68 m, Post=1.55 m; P=0.03).

No other parameters were significant.

Instructor Survey

Survey responses (Table 9) for the behavior focus indicated a tendency for more TXT horses to demonstrate a positive attitude during tack up as compared to CON (TXT=25, CON=20; P=0.0878). Positive behavior also tended to be observed more

frequently when TXT horses were tied as compared to CON horses (TXT=25, CON=23; P=0.0757).

Within the movement parameters, both TXT and CON groups were best represented in the category of high suppleness category for circle left and circle right (Table 8; MVM1, MVM2), with a higher frequency of CON horses receiving a high suppleness rating than TXT (MVM1, P=0.0084; MVM2, P=0.0032). Responses provided in the open ended question suggested aspects of movement or behavior unaddressed by the survey included: previous unsoundness, physical ability of the horse to carry riders of heavier weight, anticipation of upward transitions during ridden work, and lack of reactivity to unintended rider cues.

Table 9

Results of the Chi-Square Analysis and Fisher's Exact test on survey responses for stock type geldings observed in duplicate

Focus			Response *f*(%)			
	Question	Group	Quiet	Moderate	Poor	P-Value
Behavior	BQ1	C	20(33.9)	3(5.08)	1(1.69)	0.0878
		Trt	25(42.37)	2(3.39)	8(13.56)	
	BQ2	C	21(35.59)	3(5.08)	0(0.00)	0.4738
		Trt	26(44.07)	7(11.86)	2(3.39)	
	BQ3	C	23(38.98)	1(1.69)	0(0.00)	0.0757
		Trt	25(42.37)	6(10.17)	4(6.78)	
	BQ4	C	18(32.14)	3(5.36)	1(1.79)	1
		Trt	27(48.21)	5(8.93)	2(3.57)	

	BQ5	C	19(32.20)	4(6.78)	1(1.69)	0.1219
		Trt	28(47.46)	5(8.47)	2(3.39)	
	BQ6	C	19(32.20)	1(1.69)	4(6.78)	0.473
		Trt	31(52.54)	2(3.39)	2(3.39)	
	BQ7	C	1(5.88)	2(11.76)	2(11.76)	0.7964
		Trt	1(5.88)	4(23.53)	7(41.18)	
			Low	Moderate	High	
Variable Influencers	VI1	C	14(24.56)	7(12.28)	3(5.26)	0.4014
		Trt	19(33.33)	6(10.53)	8(14.04)	
	VI2	C	3(5.26)	6(10.53)	15(26.32)	0.3066
		Trt	2(3.51)	15(26.32)	16(28.07)	
Movement	MVM1	C	0(0.00)	4(7.14)	20(35.71)	0.0084
		Trt	7(12.50)	10(17.86)	15(26.79)	
	MVM2	C	0(0.00)	4(7.02)	20(35.09)	0.0032
		Trt	8(14.04)	11(19.30)	14(24.56)	
	MVM3	C	1(1.75)	9(15.79)	14(24.56)	0.0495
		Trt	5(8.77)	19(33.33)	9(15.79)	
	MVM4	C	1(1.75)	0(0.00)	23(40.35)	0.4466
		Trt	2(3.51)	3(5.26)	28(49.12)	
	MVM5	C	1(1.75)	0(0.00)	23(40.35)	0.7541
		Trt	1(1.75)	2(3.51)	30(52.63)	
	MVM6	C	2(3.51)	2(3.51)	20(35.09)	0.2984
		Trt	0(0.00)	3(5.26)	30(52.63)	

Note. Values were significant at $P \leq 0.05$, tendencies were noted as $P \leq 0.09$, $P > 0.05$.

Product Sampling

Generally, CBD content decreased for both batches from initial analysis (0.69%) to final analysis (0.28%). However, there was discrepancy between labs on the final content reading (Table 10).

Table 10

Cannabidiol potency degradation throughout the study by month

Lab	Batch	02-19	04-19	10-19	12-19	02-20	03-20
Folium	1	9.9	--	--	--	--	--
	3	--	6.9	--	--	--	--
Botanacor	1	--	--	--	--	--	10.4
	3	--	--	4.7	--	--	7.6
MSU	1	--	--	--	--	--	3.2
	3	--	--	--	2.6	2.7	2.8

Note. Concentrations presented in mg CBD/g product. MSU=Murray State University Analytical Chemistry Department.

Chapter 5

Discussion

Project 1

Lab B was able to detect CBD presence above the 0.05 LLOQ at 2 hr, however concentrations for both the pellet and the oil were still extremely low (0.163 ng/mL, 0.11 ng/mL, respectively). Given that traditional methods of analysis had to be adjusted to achieve cannabinoid detection, it is likely that current dose rates in the horse are too low for consistent serum detection required for detailed pharmacokinetic study. This project demonstrated evidence of absorption for both forms of the product in the horse, however PEL did consistently appear higher at hours 1 and 2, respectively (PEL=0.0585 ng/ml, 0.163 ng/ml; OIL=0.0258 ng/ml, 0.11 ng/ml). While precautions were taken to ensure maximum ingestion of both products, it is possible that a portion of the OIL was not properly ingested. Otherwise, the PEL product could offer superior availability. Ultimately, a greater sample size would be required to determine the significance of absorption efficiency based on product type. The time of maximum concentration is at least 2 hr, though further research is required to determine the exact peak. Results from this pilot study demonstrate that CBD in the pelleted product was available to horses, providing justification and framework for future CBD pharmacokinetics trials.

Project 2

Despite serum chemistry concentration fluctuations, all observations for the single dose remained within standard ranges for a healthy animal. Although changes occurred, those exhibited were not representative of immediate danger to the horse. Changes characteristic of kidney dysfunction would typically demonstrate a simultaneous rise of BUN and creatinine. Based on the observed changes, BUN consistently increased with TXT concentration and progression of time, but creatinine did not. Though CBD related physiological impacts could exist and cause fluctuations, the changes observed did not reach levels of concern for this dose rate and time period. Outside factors such as dehydration or a high protein diet can also cause these parameters to shift (Hosten, 1990). Such effects are unlikely to have played a role, given that diets consisted primarily of Bermuda hay, free choice water, and grain amounts consistent of standard feedings. The observed results suggest relative safety of a single dose up to the 250 mg/horse/d in horses weighing 555 kg on average. However, observations from this study and others (Gamble et al., 2018; Deabold et al., 2019) supports attention to parameters such as BUN, creatinine, and ALP; especially with repetitive dosing or increased concentrations.

Responses in horses appear to be relatively comparable to existing research, having a 2 hr CBD concentration maximum. However, some equine research has documented Tmax extending past 2 hr (2.9 hr; Davis, 2019). A potential explanation for the discrepancy would be the relevant time gaps that existed within this project. While CBD concentration ultimately dropped between 2 hr and 4 hr, it is impossible to state when exactly the drop was initiated. Concentration could have continued to rise without the opportunity for detection. However, results from this study were reasonably close to

current research on Tmax across species where ranges are consistently ~1.5 hr to 3 hr (Gamble et al., 2018; Deabold et al., 2019; Davis, 2019). Method of administration could also be instrumental in CBD bioavailability. Though measures were taken to ensure ingestion, it is possible that more product is wasted when given in a pellet as compared to oil. In a CBD oil based equine study (Davis, 2019), horses were administered a lower dose rate (0.10 mg/kg) than the maximum dose (0.22 mg/kg) in this study (Figure 4). While this trial demonstrated a quicker Tmax, the Cmax geometric mean within Davis' study was higher (27.2 ng/mL versus 3.04 ng/mL, respectively). Although the oil took slightly longer to absorb, it required less product to achieve a final maximum concentration greater than the pelleted version.

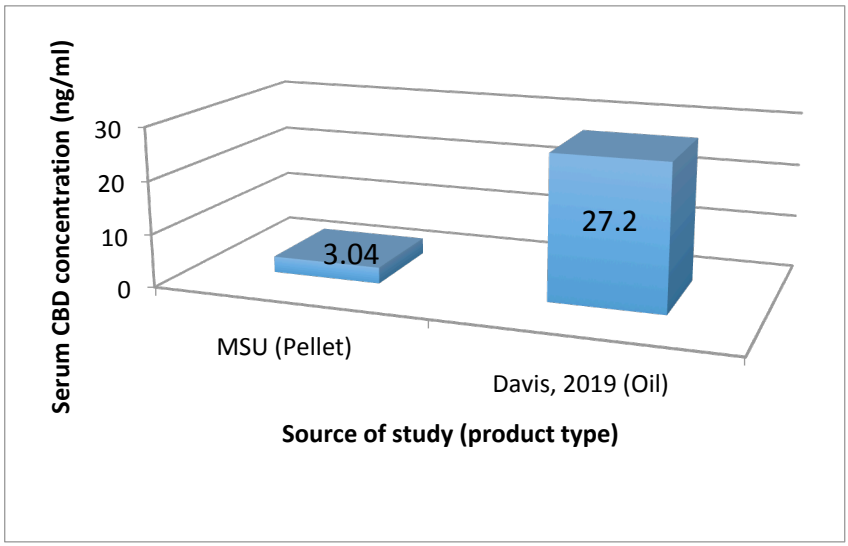

Figure 5. *Comparison of concentration maximum (Cmax) using the geometric mean in horses based on treatment type.*

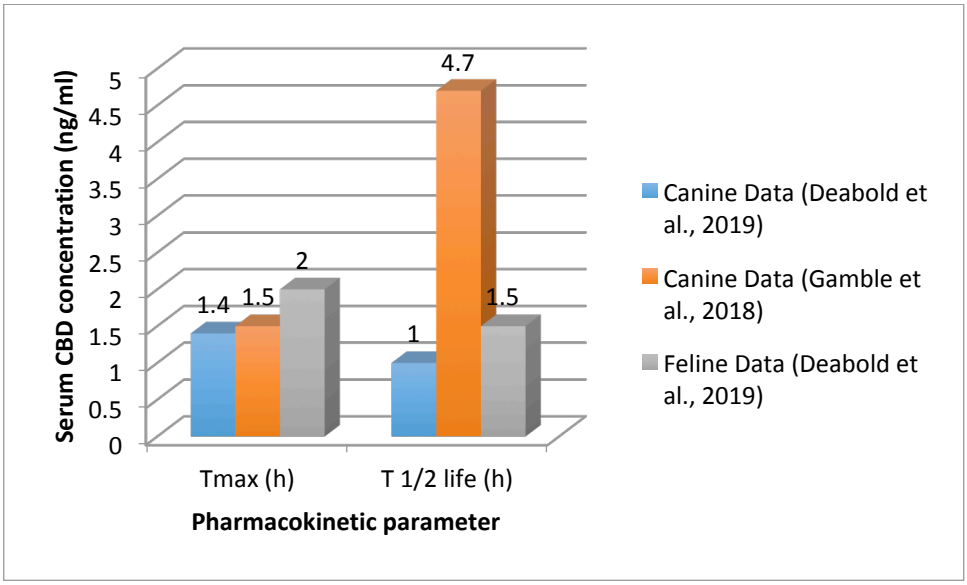

Figure 6. *Comparison of time of maximum concentration (Tmax) and time of terminal half-life (T1/2 life) among species from various studies at the same dose rate (2mg/kg). In Deabold et al., 2019 cats were given an oil capsule, dogs were given a soft chew. In Gamble et al.,2018 dogs received oil.*

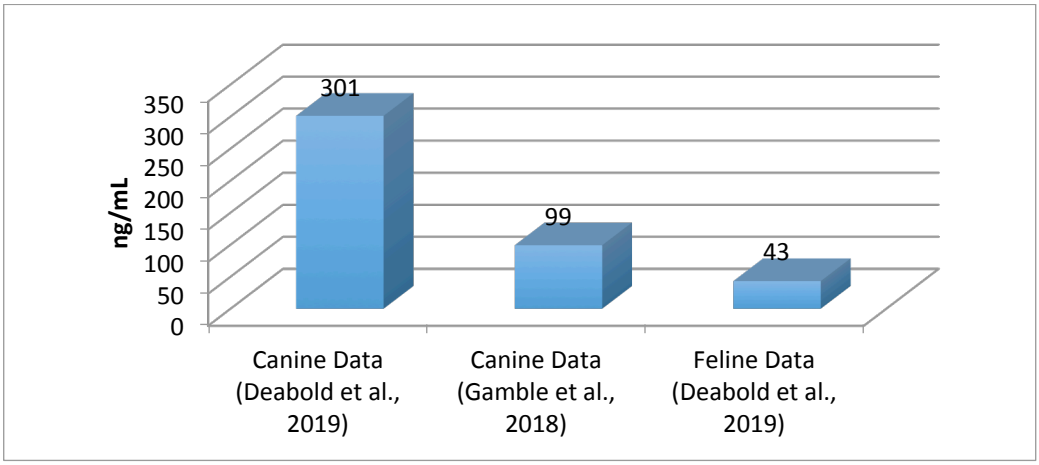

Figure 7. *Comparison of concentration maximum across species for a 2 mg/kg oral CBD dose. In Deabold et al., 2019 cats were given an oil capsule, dogs were given a soft chew. In Gamble et al., 2018 dogs received oil.*

Congruent with our observations in this trial, additional research in canine and feline trials also demonstrates a Tmax relatively close to 2 hr (Figure 6). Further demonstrating the potential role of administration type in absorption efficiency this comparison introduces a soft chew, administered to dogs (Deabold et al., 2019) as compared to the oil type (Gamble et al., 2018; Deabold; 2019) all at a higher dose rate of 2 mg/kg (Figure 7). No negative side effects were reported in either of these studies despite the higher dose rate as compared to those represented in this study or Davis et al. (2019). While oil seems more readily absorbed in the horse as compared to a pellet, there was a much higher absorption rate in dogs that were given a soft chew than dogs given oil. It is possible that this method allows more precise dosing with increased assurance that the entire dose is consumed. In both canine and feline species, CBD administered through an oil form appeared to remain in the system longer than a soft chew product (Figure 6). However, despite maintaining a longer half-life the maximum absorption rate reached for oil was lower (Figure 7). This suggests that an oil product could require less frequent dosing. Aside from the effect of product type on variability, Figure 7 also highlights species associated CBD absorption rate variability, further supporting species-specific research. Fairly different concentration readings were observed between cats and dogs administered the same dose rate of the oil (Feline= 43 ng/ml; Canine= 99 ng/ml). Furthermore, Davis' oil dose saw fairly impressive absorption rates as compared to the feline trial, despite having a much lower dose rate (Equine=0.22 mg/kg; Feline=2 mg/kg).

Ultimately, Project 2 served to better understand pharmacological actions of CBD products in the horse. Except for one horse, only those in the 250 mg TXT group achieved the standard LLOQ. Despite this, all readings were relatively low. Future trials

should consider using higher doses for consistent CBD detection, or implementing a more readily absorbed form. For more precise research evaluation, use of a CBD oil or CBD treat, similar to the canine soft chew previously mentioned (Deabold et al., 2019), might be more appropriate. Additionally, trials should work to fill the time gaps existent within this trial to better represent the rate of absorption and elimination in the horse.

Project 3

NOT Heart rate and behavior scores

Though heart rate was not significantly impacted by CBD dosage, results from the behavior scores showed a higher percentage of TXT horses earning a low reactivity score as compared to a high reactivity score (29.7%, 9.9% respectively). Additionally, control horses were more frequently in the high to moderate reactivity levels as compared to TXT horses (36.63%, 23.76%). Characteristic of the release of stress related hormones, epinephrine and norepinephrine, is the rise of heart rate and blood pressure. Under the assumption that cannabinoids work through the endocrine system, it would be assumed that heart rate would have exhibited a physiological difference reflective of the observed behavioral responses (Leiner and Fendt, 2011). However, while the reaction scores of TXT horses were reduced, their heart rate was not significantly affected. Habituation to the stimulus by all horses might have been an explanation to lack of heart rate difference between control and TXT groups, except that rater scores only showed a significant difference between observed reactions of TXT horses. One fault of the reactivity-scoring chart alone is that it relies on a subjective method of evaluation versus the objectivity

offered though physiological parameters like HR. Though raters remained blind to TXT horses and observations were completed in triplicate, it is possible that this method is less accurate than physiological measures. However, variations of startle response tests have been used in other studies to accurately measure reactivity in horses (Holland et al., 1996; Borstel et al., 2011; Leiner and Fendt, 2011). The reduction of reactivity seen in TXT horses is consistent with another study that found stress behaviors in CBD treated mice to be reduced as compared to control (Resstel et al., 2009). Additionally, it is important to note that HR does not consistently adjust linearly with behavior reactions (Munsters et al., 2013; Pearson and MacNicol, 2017). The fitness level or even the level of training or experiences with unexpected stimuli could have impacted the varying results seen between stimulus HR and reaction scores.

Another possibility is that dose rate was not high enough to serve as the catalyst for detectable physiological responses to CBD, despite having an effect on behavioral actions. Dose rate has been discussed as a limiting agent of efficacy in another nutraceutical product (Dodd et al., 2015). The average by body weight dose rate used in Project 3 was 0.18 mg/kg for a target of 100 mg per day. It may not be economically feasible for owners to dose horses at efficacious concentrations seen of CBD success in other animal models, as some administered up to a total dose of 300 μg (1.71 mg/kg) in mice (Philpott et al., 2017), 8 mg/kg in dogs (Gamble et al., 2018) and 50 mg/kg/day in humans (Devinsky et al., 2018) due to equine body weight. In this case it may be useful to look towards more potent cannabinoid options, such as synthetics, rather than plant extractions, such as the product used in this study. Many structures are known to be involved in the functionality of the cannabinoid system (Crippa et al., 2004; Fusar-Poli et

al., 2009; Crippa et al., 2011). Lack of physiological response during the reactivity trial is characteristic of other trials in multiple species (Resstel et al., 2009; as reviewed in Bergamaschi et al., 2011), suggesting CBD consistently lacks an effect on HR.

Movement Parameters

While significant changes were found with TXT and time, the practical implications are limited. A horse changing its pattern of motion as a result of unsoundness would shorten one phase of the stride and lengthen another as they attempt to avoid bearing weight on the affected limb. In regard to TXT differences based on stride phases, both the stance and swing phase of the stride were shorter in control horses during walk (TXT=0.57 sec, 0.38 sec; CON=0.51 sec, 0.36 sec respectively). Though the control group demonstrated an overall shorter phase completion of stride, the difference observed cannot be directly attributable to CBD. One reason is that despite quicker phase completion, there was not a significant difference in stride length at walk between the two groups (TXT=1.34; CON=1.34). This could be because CON horses naturally had quicker stride completion than TXT animals. Significant differences of time spent during each stride phase based on TXT group were only observed at walk, not in trot. Typically, stride limitations due to unsoundness or stiffness are exaggerated in trot due the symmetrical, diagonal motion (King and Mansmann, 1997). As inconsistencies between the groups seemed to disappear during trot, it is more likely that the TXT group naturally tended to travel with a quicker stride.

When evaluating the effect of time on stride parameters, both groups of horses spent longer in stance phase of walk by the conclusion of the study (Pre=0.37 sec,

Post=0.71 sec; P<0.01). In trot, stride length (Pre=1.68 m, Post=1.55 m; P=0.03) and time spent in both stance (Pre=0.30 sec, Post=0.26 sec; P<0.01) phase and swing phase (Pre= 0.37 sec, Post=0.33 sec; P<0.01) decreased with time. With all examined parameters of stride in trot showing the same pattern, it is likely that an outside factor attributed to stride change. Just before the pre-treatment collections, horses were just returning to stalls and work as most are maintained in pasture during the academic winter break. It is possible that increased time in stalls combined with increased work for the 6-week trial period could have promoted the observed trend. Another possibility is that horses were already at their maximum stride ability and without soundness issues, therefore making it impossible for them to increase stride length. Observation of CBD effects on horses with confirmed and observable soundness issues would be an interesting direction for future study.

Given that CBD tends to slow or block signaling pathways, it would be reasonable to anticipate horses administered the product would move slower. Aside from the dangers and welfare concerns associated with administering a pain-blocking agent to an athlete during competition, it is more likely that the TXT would slow reaction responses to stimuli and cues, and ultimately reduce their performance ability. This is confirmed when examining the NOT data, where reaction responses were more frequently a lower rating in TXT than CON. The movement data also supports this idea, as CON horses demonstrated a quicker stride in walk than TXT. However, when evaluated for the effects of time, both groups spent more time in stance phase of walk and moved with shorter, quicker strides by study conclusion. Because both groups

demonstrated this trend over time, it is inaccurate to specifically associate differences with CBD treatment.

Primarily, it is likely the dose rate was inefficient for clinical changes regarding movement. Clinical doses demonstrating positive results for epilepsy in humans reported CBD dose rate at 25 mg/kg/day (Devinsky et al., 2018) as compared to the maximum dose in Project 3 of 0.23 mg/kg/day. While pharmaceutical products do not always have a linear dose-efficacy relationship among species, the gap presented is fairly substantial and worth considering.

Survey Responses

Overall, TXT horses tended to demonstrate more positive behaviors than CON horses when tied and during tack up (Table 7; BQ1, BQ3). Negative stable behaviors such as pulling back, weaving, biting, or kicking can be the result of physical sensitivity, activity anticipation, or undetermined hierarchy of nearby horses (Houpt, 1981; Ellis and Contino, 2019). Although the survey alone cannot explain why more positive behaviors occurred in TXT horses, the responses do demonstrate potential anxiolytic and nociceptive properties of CBD treatment suggested in other studies (as reviewed in Scuderi et al., 2009; as reviewed in Blessing et al, 2015; Ellis and Contino, 2019). Stress that can lead to negative or dangerous behaviors has been observed in horses during competition (Schmidt et al., 2010) and transport (Górecka-Bruzda et al., 2015). Cannabidiol could assist in stress alleviation to improve the health and performance of the animal. However, surveys are a more subjective measure not immune to forms of

bias. Therefore, more thorough research is required to confirm if CBD is the specific catalyst for this difference.

Questions examining movement parameters showed a higher percentage of control horses with high ability to track up (TXT=24.56%, CON=15.79%; P=0.0495) and for suppleness on a circle left (TXT=26.79%, CON=35.71%; P=0.0084) and circle right (TXT=24.56%, CON=35.09%; P=0.0032). However, when individual groups were examined on each category of low, moderate, and high ability respectively, both TXT and control groups were best represented in the category of either moderate or high ability to track up (TXT=15.15%, 57.58, 27.27; CON=4.17%, 37.50%, 58.33%), circle left (TXT= 21.88%, 31.25%, 46.88%; CON=0.00%, 16.67%, 83.33%) and circle right (TXT=24.24%, 33.33%, 42.42%; CON=0.00%, 16.67%, 83.33%). Therefore, while representation of control horses was more substantial within the relevant high suppleness and ability to track up parameters, this suggests that the entire population naturally had an ability to excel in each of the appropriate question points. The effect of time would need to be examined to determine if there was a change in suppleness or ability to track-up over the 6 wk period. Other studies have documented improved movement abilities and reduced pain from CBD treatment (Gamble et al., 2018; Ellis and Contino, 2019). Unlike such studies, horses included within this trial did not begin the trial with a confirmed, specific source of pain, though low to moderate grade positive flexion scores were determined from a subsample at the start of Project 3. Thus, CBD may not have been able to achieve a notable improvement on natural movement abilities if the horse's abilities were maximal from the start.

Product Sampling

One concern from the product and blood testing is that, despite being advertised as a 0 THC product, LC-MS/MS technology did report readings of Δ^9-THC presence. However, such readings were below standard LLOQ (0.0014 ± 0.0003%) in the product and highly variable in the equine serum (maximum: 55.6 ng/mL; minimum: 0.00873 ng/mL). In regard to product sampling, generally readings below LLOQ do not incur significance. Given the product contained no more than 0.0014 ± 0.0003% Δ9-THC, serum detections as high as 55.6 ng/mL suggest there was a potential misreading or contamination of the detection system for the compound. This highlights the concern surrounding a lack of regulatory action and testing standards. Though current federal legislation grants products with a Δ^9-THC presence of <0.3% on a dry weight basis, current production practices of CBD do not have defined standards for cannabinoid concentrations or consistent methods for testing. The definition of what is a safe and efficacious concentration for the horse currently stands in an undefined area. A pelleted supplement from one company could contain 50 mg of CBD/g product or it could contain 1 mg CBD/g product. In addition, consumers are relying on manufacturers to monitor the content of other potential health risks such as heavy metals, pesticides, residual solvents and microbials that may have accumulated during the growth or manufacturing processes.

Summary

Cannabidiol did demonstrate bioavailability to the horse, with basic pharmacokinetic characteristics similar to those in other species. However, current dose rates in the horse are not adequate for consistent serum detection that would allow for

more thorough and informative pharmacokinetic trials. In association, when concentrations are not consistently detected in the serum it is difficult to formulate dosing recommendations for clinical efficacy trials. Though monitoring blood parameters though serum chemistry and CBC have demonstrated single dose safety up to a 250 mg dose, evidence of BUN and creatinine concentration shifts support the need for safety trials on extended exposure. Results from this study support further investigation of the effects of CBD on behavior and reactivity responses in horses. Effects of CBD on movement abilities will likely require higher dosing and more specific conditions to adequately evaluate improvement. Safety should remain the primary priority as dose rates increase. Existing trials with positive results concerning CBD treatment for osteoarthritis in canine and murine models could provide direction for future study. The primary difficulty in dosing livestock species with CBD products surrounds economic feasibility, specifically within consumer practicality. Evidence thus far shows that continued observable benefits from CBD require continued treatment. To reach comparable doses of successful clinical trials completed in other mammals would require quite a bit more product due to the weight of the average equine compared to a dog, cat or even human. Finally, detection methods are still somewhat inconsistent depending on the methods used, even amongst accredited labs. Adequate regulation through product and blood testing requires testing methods to be consistent and dependable. Therefore, if regulation is to be enforced, it is important that a standard protocol is developed for testing labs for comparable results.

References

Aizpurua-Olaizola, O., Soydaner, U., Öztürk, E., Schibano, D., Simsir, Y., Navarro, P., Etxebarria, N., Usobiaga, A. 2016. Evolution of the Cannabinoid and Terpen Content during the Growth of Cannabis sativa Plants from Different Chemotypes. *J Nat Prod* 79(2): 324-331. DOI: 10.1021/acs.jnatprod.5b00949

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, Peters JA, Harmar AJ, CGTP Collaborators. 2013. The concise guide to pharmacology: G protein-coupled receptors. *Br J of Pharmacol* 170: 1459–1581. DOI: 10.1111/bph.12444/full

Alger, B.E. 2013. Getting High on the Endocannabinoid System. *Cerebrum*. 14. Retrieved from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3997295/

Anitescu, G., Doneanu, C., Radulescu, V. 1997. Isolation of Coriander Oil: Comparison Between Steam Distillation and Supercritical CO_2 Extraction. *Flavour Frag J* 12:173-176. DOI: 10.1002/(SICI)1099-1026

Arévalo, C., Miguel, R., Herández-Tristán, R. 2001. Cannabinoid effects on anxiety-related behaviors and hypothalamic neurotransmitters. *Pharmacol Biochem Behav* 70(1): 123-131. DOI: 10.1016/S0091-3057(01)00578-0

Arnone, M., Maruani, J., Chaperon, F., Hélène, M., Poncelet, T.M., Soubrié, P., Le Fur, G. 1997. Selective inhibition of sucrose and ethanol intake by SR 141716, an antagonist of central cannabinoid (CB1) receptors. *Psychopharmacology* 132: 104-106. Retrieved from: https://link.springer.com/content/pdf/10.1007/s002130050326.pdf

Back, W., MacAllister, C.G., Pollmeier, M., Hanson, P.D. 2017. Vertical Frontlimb Ground Reaction Forces of Sound and Lame Warmbloods Differ From Those in Quarter Horses. *J Equine Vet Sci* 27(3): 123-129. DOI: 10.1016/j.jevs.2007.01.007

Bertone, A.L., Palmer, J.L., Jones, J. 2001. Synovial Fluid Cytokines and Eicosanoids as Markers of Joint Disease in Horses. *Vet Surg* 30: 528-538. DOI: 10.1053/jvet.2001.28430

Beutler, J.A., Der Marderosian, A.H. 1978. Chemotaxonomy of Cannabis I. Crossbreeding between Cannabis sativa and C. ruderalis with analysis of Cannabinoid Content. *Econ Bot* 32(4): 387-394. DOI: 63.150.211.93

Björnsdóttir, S., T. Árnason, P. Lord. 2003. Culling Rate of Icelandic Horses due to Bone Spavin. *Acta Vet Scand* 44(161). DOI:10.1186/1751-0147-44-161

Blake, D.R., Robson, P., Ho. M., Jubb, R.W., McCabe, C.S. 2006. Preliminary assessment of the efficacy, tolerability and safety of a cannabis-based medicine (Sativex) in the treatment of pain caused by rheumatoid arthritis. *Rheumatology* 45: 50-52. DOI: 10.1093/rheumatology/kei183

Blessing, E.M., Steenkamp, M.M., Manzanares, J., Marmar, C.R. 2015. Cannabidiol as a Potential Treatment for Anxiety Disorders. *Neuropathics* 12(4): 825-836. DOI:10.1007/s13311-015-0387-1

Borstel, U.K., Euent, S., Graf, P., König, S., Gauly, M. 2011. Equine behaviour and heart rate in temperament tests with or without rider or handler. 2011. *Physiol Behav* 104(3): 454-463. DOI: 10.1016/j.physbeh.2011.05.010

Brown, AJ., Daniels, DA., Kassim, M., Brown, S., Haslam, C.P., Terrell, V.R., Brown, J., Nichols, P.L., Staton, P.C., Wise, A., Dowell, S.J. 2011. Pharmacology of GPR55 in yeast and identification of GSK494581A as a mixed-activity glycine transporter subtype 1 inhibitor and GPR55 agonist. *J Pharmacol Exp Ther* 337: 236-246. DOI:10.1124/jpet.110.172650

Bergamaschi, M.M., Queiroz, R.H., Crippa, J.A., Zuardi, A.W. 2011. Safety and Side Effects of Cannabidiol, a Cannabis sativa Constituent. *Curr Drug Saf* 6(4): 237-49. DOI: 10.2174/157488611798280924

Burstein, S. 2015. Cannabidiol (CBD) and its analogs: a review of their effects on inflammation. *Bioorg Med Chem* 23:1377-1385. DOI: 10.1016/j.bmc.2015.01.059

Callaway, J. 2004. Hempseed as a nutritional resource: an overview. *Euphytica* 140: 65-72. Retrieved from: https://link.springer.com/content/pdf/10.1007/s10681-004-4811-6.pdf

Chiocchetti, R., Rinnovati, R., Tagliavia, C., Stanzani, A., Galiazzo, G., Giancola, F., De Silva, M., Capodanno, Y., Spadari, A. 2020. Localisation of cannabinoid and cannabinoid-related receptors in the equine dorsal root ganglia. *Equine Vet J* 00:1-9. DOI: 10.1111/evj.13305

Clegg, P., T. Booth. 2000. Drugs used to treat osteoarthritis in the horse. *In Practice* 22(10): 594-603. DOI:10.1136/inpract.22.10.594

Conti, S., Costa, B., Colleoni, M., Parolaro, D., Giagnoni, G. 2002. Antiinflammatory action of endocannabinoid palmitoylethanolamide and the synthetic cannabinoid nabilone in a model of acute inflammation in the rat. *Br J of Pharmacol* 135 (1): 181-187. DOI: 10.1038/sj.bjp.0704466.

Contos, J.J.A., Ishii, I., Chun, J. 2000. Lysophosphatidic Acid Receptors. *Mol Pharmacol* 58(6): 1188-1196. DOI:10.1124/mol.58.6.1188

Costa, B., Colleoni, M., Conti, S., Parolaro, D., Franke, C., Trovato, A.E., Giagoni, G. 2004. Oral anti-inflammatory activity of cannabidiol, a non-psychoactive constituent of cannabis, in acute carrageenan-induced inflammation in the rat paw. Naunyn-Schmiedeberg's *Arch Pharmacol* 369: 294-299. DOI: 10.1007/s00210-004-0871-3

Cota, D. 2007. CB1 receptors: emerging evidence for central and peripheral mechanisms that regulate energy, balance, metabolism, and cardiovascular health. *Diabetes Metab Res Rev* 23:507-517. DOI:10.1002/dmrr.764

Crippa, J.A., Zuardi, A.W., Garrido, G.E., Wichert-Ana, L., Guarnieri, R., Ferrari, L., Azevedo-Marques, P.M., Hallak, J.E., McGuire, P.K., Busatto, G.F. 2004. Effects of Cannabidiol (CBD) on Regional Cerebral Flow. *Neuropsychopharmacology* 29: 417-426

Crippa, J.A., Derenusson, G.N., Ferrari, T.B., Wichert-Ana, L., Duran, F.L., Martin-Santos, R., Simões, M.V., Bhattacharyya, S., Fusar-Poli, P., Atakan, Z., Filho, A.S., Freitas-Ferrari, M.C., McGuire, P.K., Zuardi, A.W., Busatto, G.F., Hallak, J.E. 2011. Neural basis of anxiolytic effects of cannabidiol (CBD) in generalized social anxiety disorder: a preliminary report. *J Psychopharmacol* 25(1): 121-130. DOI: 10.1177/0269881110379283

Davis, H.A. 2019. Novel Analgesics and the Impact of Route of Administration in the Horse (doctoral dissertation). Auburn University, Auburn, AL.

De Laurentiis A., Araujo, H.A., Rettori, V. 2014. Role of the endocannabinoid system in the neuroendocrine responses to inflammation. *Curr Pharm Des.* 20(29): 4697-4706. Retrieved from: https://anandaenterprises.com.au/wp-content/uploads/2019/12/Role-of-the-Endocannabinoid-System-in-the-Neuroendocrine-Responses-to-Inflammation-1.pdf-1.pdf

Deabold, K.A., W.S. Schwark, L. Wolf, J.J. Wakshlag. 2019. Single-Dose Pharmacokinetics and Preliminary Safety Assessment with Use of CBD-Rich Hemp Nutraceutical in Healthy Dogs and Cats. *Animals* 832 (9) DOI: 10.3390/ani9100832

Devane, W.A. Hanus, L., Breuer A., Pertwee, R.G., Stevenson, L.A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., Mechoulam, R. 1992. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 258: 1946-1949. DOI: 10.1126/science.1470910

Devinsky, O., Verducci, C., Thiele, E.A., Laux, L.C., Patel, A.D., Filloux, F., Szaflarski, J.P., Wilfcong, A., Clark, G.D., Park, Y.D., Seltzer, L.E., Bevin, E.M., Flamini, R., Wechsler, R.T., Friedman, D. 2018. Open-label use of highly purified CBD (Epidiolex) in patients with CDKL5 deficiency disorder and Aicardi, Dup15q, and Doose syndromes. *Epilepsy Behav* 86:131-137. DOI:10.1016/j.yebeh.2018.05.013

Di Marzo, V., Melck, D., Bisogno, T., De Petrocellis, L. 1998. Endocannabinoids: endogenous cannabinoid receptor ligands with neuromodulatory action. *Trends Neurosci* 21(12): 521-528. Retrieved from: http://www.medicalcannabis.com/wp-content/uploads/di_marzo_1998_endocannabinoids.pdf

Dodd, J.A., Doran, G., Harris, P., Noble, G.K. 2015. Magnesium aspartate supplementation and reaction speed response in horses. *J Equine Vet Sci* 35(5):401-402. Retrieved from: file:///Users/kevindraeger/Downloads/conference_poster_ICEEP_2018_Final.pdf

Drent, M., Cobben, N.A.M., Henderson, R.F., Wouters, E.F.M., van Dieijen-Visser, M. 1996. Usefulness of lactate dehydrogenase and its isoenzymes as indicators of lung damage or inflammation. *Eur Respir J* 9: 1736-1742. DOI: 10.1183/09031936.9609081736

Ellis, K.L., Contino, E.K. 2019. Treatment using cannabidiol in a horse with mechanical allodynia. *Equine Vet Educ* DOI:10.1111/eve.13168

Eltoukhy, M., Asfour, S., Thompson, C., Latta, L. 2012. Evaluation of the Performance of Digital Video Analysis of Human Motion: Dartfish Tracking System. *Int J of Eng Res* 3(3). Retrieved from: http://www.ijser.org

Fišar, Z. 2009. Phytocannabinoids and Endocannabinoids. *Curr Drug Abuse Rev* 2: 51-75. Retrieved from:https://www.researchgate.net/profile/Zdenek_Fisar/publication/260952192_Phytocannabinoids_and_Endocannabinoids/links/54e5da150cf2bff5a4f1c9b9/Phytocannabinoids-and-Endocannabinoids.pdf

Freund, T.F., Katona, I. Piomelli, D. 2003. Role of Endogenous Cannabinoids in Synaptic Signalling. *Physiol Rev* 83:1017-1066. DOI:10.1152/physrev.00004.2003

Frisbie, D.D., Mcllwraith, C.W., Kawcak, C.E., Werpy, N.M. 2015. Efficacy of intravenous administration of hyaluonan, sodium chondroitin sulfate, and *N*-acetyl-D-glucosamine for prevention or treatment of osteoarthritis in horses. *AJVR* 77(10): 1064-1070

Fusar-Poli, P., Crippa, J.A., Bhattacharyya, S. 2009. Distinct Effect of Δ9-Tetrahydrocannibinol and Cannabidiol on Neural Activation and Emotional Processing. *Arch Gen Psychiatry* 66(1): 95-105. DOI: 10.1001/archgenpsychiatry.2008.519

Gakhar, N., Goldberg, E., Jing, M., Gibson, M., House, J.D. 2012. Effect of feeding hemp seed and hemp seed oil on laying hen performance and egg yolk fatty acid content: Evidence of their safety and efficacy for laying hen diets. *Poult Sci J* 9193: 701-711

Gamble, L.J., Boesch, J.M., Frye, C.W., Schwark, W.S., Mann, S., Wolfe, L., Brown, H., Berthelsen, E.S., Wakshlag, J.J. 2018. Pharmacokinetics, Safety, and Clinical Efficacy of Cannbidiol Treatment in Osteoarthritic Dogs. *Front Vet Sci.* 5:165. DOI:10.3389/fvets.2018.00165

Gaoni, Y., Mechoulam, R. 1964. Isolation, structure, and partial synthesis of an active constituent of hashish. *J Am Chem Soc* 86: 1646-1647

Gardner, E.L. 2005. Endocannabinoid signaling system and brain reward: Emphasis on dopamine. *Pharmacol Biochem Be* 81: 263-284. DOI: 10.1016/j.pbb.2005.01.032

Gerdeman, G.L., Lovinger, D.M. 2003. Emerging roles for endocannabinoids in long-term synaptic plasticity. *Br J of Pharmacol* 140: 781-789. DOI:10.1038/sj.bjp.0705466

Giacoppo, S., Mandolino, G., Galuppo, M., Bramanti, P., Mazzon, E. 2014. Cannabinoids: New Promising Agents in the Treatment of Neurological Diseases. *Molecules* 19:18781-18816. DOI: 10.3390/molecules191118781

Godlewski, G., Offertáler, L., Wagner, J.A., Kunos, G. 2009. Receptors for acylethanolamides-GPR55 and GPR119. *Prostaglandins Other Lipid Mediat.* 89(3-4): 105-111. DOI:10.1016/j.prostalandins.2009.07.001

Gómez, A.M., López, C.P., Ossa, E.M. 1994. Recovery of grape seed oil by liquid and supercritical carbon dioxide extraction: comparison with conventional solvent extraction. *Chem Eng* 61(3): 227-231. DOI: 10.1016/0923-0467(95)03040-9

Górecka-Bruzda, A., Kosińka, I., Jaworski, Z., Jezierski, T., Murphy, J. 2015. Conflict behavior in elite show juming and dressage horses. *J Bet Behav* 10(2): 137-146. DOI: 10.1016/j.jveb.2014.10.004

Griffith, S.C. 2006. Can a Horse Learn While Under the Influence of a Tranquilizer (Acepromazine Maleate)? (masters thesis) Auburn University, Auburn, AL

Grotenhermen F, Muller-Vahl K. 2012). The therapeutic potential of cannabis and cannabinoids. *Dtsch Arztebl Int* 109: 495–501. DOI:10.3238/arztebl.2012.0495

Gulyas, A.I., Cravatt, B.F., Bracey, M.H. Dinh, T.P., Piomelli, D., Boscia, F., Freund, T.F. 2004. Segregation of two endocannabinoid-hydrolyzing enzymes into pre- and postsynaptic compartments in the rat hippocampus, cerebellum and amygdala. *Eur J Neurosci* 20: 441-458. DOI:10.1111/j.1460-9568.2004.03428.x

Hall, C., N. Huws, C. White, E. Tayloy, H. Owen, P. McGreevy. 2006. Assessment of Ridden Horse Behavior. *J Vet Behav* 8(2): 62-73. DOI: 10.1016/j.jveb.2012.05.005

Hammell, D.C., Zhang, L.P., Ma, F., Abshire, S.M., Mcllwrath, S.L., Stinchcomb, A.L., Westlund, K.N. 2016. Transdermal cannabidiol reduces inflammation and pain related behaviours in a rat model of arthritis. *Eur J Pain* 20(6): 936-948. DOI: 10.1016/j.jveb.2012.05.005

Hartsel, J.A., Boyar, K., Pham, A., Silver, R.J., Makriyannis, A. 2019. Cannabis in Veterinary Medicine: Cannabinoid Therapies for Animals. *Nutraceuticals in Veterinary Medicine*. 121-155. DOI:10.1007/978-3-030-04624-8_10

Hartsel, J.A., Eades, J., Hickory, B., Makriyannis, A. 2016. Cannabis sativa and Hemp. In R.C. Gupta (Ed.), *Nutraceuticals Efficacy, Safety and Toxicity* 735-751. DOI: 10.1016/B978-0-12-802147-7.00053-X.

Hazekamp, A. 2018. The Trouble with CBD Oil. *Medical cannabis and cannabinoids* 1:65-72. DOI: 10.1159/000489287

Hegde, V.L., Nagarkatti, P.S., Nagarkatti, M. 2011. Role of Myeloid-Derived Suppressor Cells in Amelioration of Experimental Autoimmune Hepatitis Following Activation of TRPV1 Receptors by Cannabidiol. *PLoS ONE* 6(4):e18281. DOI: 10.1371/journal.pone.0018281

Herkenham, M., Lynn, A.B., Johnson, M.R., Melvin, L.S., de Costa, B.R., Rice, K.C. 1991. Characterization and Localization of Cannabinoid Receptors in Raat Brain: A Quantitative *in vitro* Autoradiographic Study. *Journ. Of Neuroscience* 11(2): 563-583. DOI: 10.1523/JNEUROSCI

Hessle, A., Eriksson M., Nadeau, E., Turner, T., Johansson, B. 2008. Cold-pressed hempseed cake as a protein feed for growing cattle. *Acta agriculturae Scand* 58(a) 136-145

Hill, A. J., Williams, C.M., Whalley, B.J., Stephens, G.J. 2012. Phytocannabinoids as novel therapeutic agents in CNS disorders. *Pharmacol Therap* 133(1): 79-97. Retrieved from: http://centaur.reading.ac.uk/24227/1/PT_review_final_revised.pdf

Holland, J.L., Kronfeld, D.S., Meacham, T.N. 1996. Behavior of Horses Is Affected by Soy Lecithin and Corn Oil in the Diet. *J Anim Sci* 74:1252-1255. DOI: 10.2527/1996.7461252x

Hosten, A.O. 1990. BUN and Creatinine. In: Walker, H.K., Hall, W.D., Hurst, J.W., editors. Clinical Methods: The History, Physical, and Laboratory Examinations. 3rd Edition. Boston: Butterworths. Chapter 193. Retrieved from: https://www.ncbi.nlm.nigh.gov/books/NBK305.

Houpt, K.A. 1981. Equine Behavior Problems in Relation to Humane Management. *Int J Study Anim Prob* 2(6): 329-337. Retrieved July 6, 2020 from: https://animalstudiesrepository.org/cgi/viewcontent.cgi?article=1004&context=acwp_ewp

Houpt, K., S.M. McDonnell. 1993. Equine Stereotypies. *Comp Cont Educ Pract Vet* 15(9): 1265-1271. Retrieved from: https://www.paardenwelzijnscheck.nl/app/webroot/files/ckeditor_files/files/Voeding%20en%20Water/Houpt%20%26%20McDonnell%20(1993)%20Equine%20stereotypies.pdf

Howlett, A.C., Barth, F., Bonner, T.I., Cabral, G., Casellas, P., Devane, W.A., et al. 2002. International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev* 54:161-202. Retrieved from: https://www.researchgate.net/profile/Francis_Barth/publication/11335792_International Union_of_Pharmacology_XXVII_Classification_of_Cannabinoid_Receptors/links/09e415012443d588ec000000.pdf

Ivanov I., Borchert, P., Hinz, B. 2015. A simple method for simultaneous determination of *N*-palmitoylethanolamine and 2-arachidonoylglycerol in human cells. *Anal Bioanal Chem* 407:1781-1787. DOI 10.1007/s00216-014-8384-5

Jamshidi, N., Taylor, D.A. 2009. Anandamide administration into the ventromedial hypothalamus stimulates appetite in rats. *Br J Pharmacol* 134(6): 1151-1154. DOI: 10.1038/sj.bjp.0704379

Jeong, M., Cho, J., Shin, J., Jeon, Y.J., Kim, J.H., Lee, S.J., Kim, E.S., Lee, K. 2014. Hempseed oil induces reactive oxygen species-and C/EBP homologous protein-mediated apoptosis in MH7A human rheumatoid arthritis fibroblast-like synovial cells. *J Ethnopharmacol* 154: 745-752. DOI: 10.1016/j.jep.2014.04.052

Kaiser L., Heleski, C., Siegford, J., Smith, K. 2006. Stress-related behaviors among horses used in a therapeutic riding program. *JAVMA* 228(1): 39-45. DOI: 10.2460/javma.228.1.39

Kandel, E.R., Schwartz, J.H., Jessell, T.M., Siegelbaum, S.A., Hudspeth, A.J. 2013. Principles of Neural Science (5th ed. pp. 210-235). The McGraw-Hill Companies, Inc.

Karimafer, M., Salesi, M., Farajzadegan, Z. 2012. The association of anti-CCP1 antibodies with disease activity score 28 (DAS-28) in rheumatoid arthritis. *Adv Biomed Res* 1:30. DOI: 10.4103/2277-9175.98156

Kawcak, C.E. 2001. Current and Future Diagnostic Means to Better Characterize Osteoarthritis in the Horse-Imaging. *AAEP Proceedings* 47: 164-170. Retrieved

from:http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.464.3474&rep=rep1&type=pdf

Keegan, K.G. 2012. Objective measures of lameness evaluation. *American College of Veterinary Surgeons*. Retrieved June 17, 2020, from https://pdfs.semanticscholar.org/b924/95e9c8701b7a423c571d4066d6d96e3c04d4.pdf

KER. 2016. Measuring Arthritic Inflammation in Equine Joints. Retrieved June 17, 2020, from https://ker.com/equinews/measuring-arthritic-inflammation-equine-joints/

King, C., Mansmann, R. 1997. Lameness: recognizing and treating the horse's most common ailment. *Equine Research* 29-31,162-169

Koenig, T.J., Dart, A.J., Mcllwraith, C.W., Horadagoda, N. 2014. Treatment of Experimentally Induced Osteoarthritis in Horses Using an Intravenous Combination of Sodium Pentosan Polysulfate, N-Acetyl Glucosamine, and Sodium Hyaluronan. *Vet Surg* 43(5). DOI: 10.1111/j.1532-950X.2014.12203.x

Kogan, L. R., Hellver, P.W., Robinson, G. 2016 Consumers' perceptions of hemp products for animals. *Journal of American Holistic Veterinary Association* 42: 40-50. Retrieved from: http://www.le-comptoir-malin.com/medias/files/cbd-animaux-ahvma-2016-v42-hemp-article.pdf

Kriese, U., Schumann, E., Weber, W.E., Beyer, M., Brühl, L., Matthäus, B. 2004. Oil content, tocopherol composition and fatty acid patterns of the seeds of 51 *Cannabis sativa* L. genotypes. *Euphytica* 137: 339-351

La Porta, C. 2014. Involvement of the endocannabinoid system in osteoarthritis pain. 2015. Doctoral Thesis. Universitat Pompeu Fabra Barcelona

Landa, L., Sulcova, A. and Gbelec, P. 2016. The use of cannabinoids in animals and therapeutic implications for veterinary medicine: a review. *Vet Med* 61:111-122. DOI: 10.17221/8762-VETMED

Latif, S., Anwar, F. 2009. Physicochemical studies of hemp (*Cannabis sativa)* seed oil using enzyme assisted cold-pressing. *Eur J Lipid Sci Technol* 111:1042-1048. DOI: 10.1002/ejlt.200900008

Leedy, L.D., J. E. Ormrod. 2013. Practical Research Planning and Design. 10th ed. Pearson, p. 220-221

Leiner, L., Fendt, M. 2011. Behavioral fear and heart rate responses of horses after exposure to novel objects: Effects of habituation. *Appl Anim Behav Sci* 131 (3-4): 104-109. DOI: 10.1016/j.applanim.2011.02.004

Leizer, C., D. Ribnicky, A. Poulev, S. Dushenkob, I. Raskin. 2000. The Composition of Hemp Seed Oil and Its Potential as an Important Source of Nutrition. *Journal of Nutraceuticals, Functional & Medicinal Foods* 2:4, 35-53. DOI:10.1300/J133v02n04_04

Lindholst, C. 2010. Long term stability of cannabis resin and cannabis extracts. *Aust J Forensic Sci* 42(3): 181-190. DOI: 10.1080/00450610903258144

Lutge, E.E., Gray, A., Siegfried, N. 2013. The medicinal use of cannabis for reduced morbidity and mortality in patients with HIV/AIDS. *Cochrane Database Syst Rev* 4. DOI: 10.1002/14651858.CD005175.pub3/pdf/standard

Licka, T., M. Kapuan, C. Peham. 2004. Influence of rider on lameness in trotting horses. *Equine Vet J* 36(8): 734-736. DOI: 10.2746/0425164044848028

Liu, W.M., Duan, E.K., Cao, Y.J. 2002. Anandamide on embryo implantation in the mouse. *Life Sci* 71:1623-1632. DOI: 10.1016/S0024-3205(02)01928-8

Lodzki, M., Godin, B., Rakou, L., Mechoulam, R., Gallily, R., Touitou, E. 2003. Cannabidiol- transdermal delivery and anti-inflammatory effect in a murine model. *J Controlled Release 93*: 377-387. DOI: 10.1016/j.jconrel.2003.09.001

Luk, T., Wenzhen, J., Zvonok, A., Lu, D., Lin, X.Z., Chavkin, C., Makriyannis, A., Mackie, K. 2009. Identification of a potent and highly efficacious, yet slowly desensitizing CB1 cannabinoid receptor agonist. *Br J Pharmacol* 142(3): 495-500. DOI:10.1038/sj.bjp.0705792

Mackie, K. 2006. Part 1: The Endocannabinoid System, Mechanisms of Action and Functions, Mechanisms of CB1 receptor signaling: endocannabinoid modulation of synaptic strength. *IJO* 30: S19-S23. DOI:10.1038/sj.ijo.0803273

Mackie, K. 2008. Cannabinoid receptors: where they are and what they do. *J Neuroendocrinol* 20 Suppl 1:10-14. DOI: 10.1111/j.1365-2826.2008.01671.x

Malfait, A.M., Gallily, R., Sumariwalla, P.F., Malik, A.S., Andreakos, E., Mechoulam, R., Feldmann, M. 2000. The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritic therapeutic in murine collagen-induced arthritis. *Proceedings of the National Academy of Sciences* 97 (17) 9561-9566. DOI: 10.1073/pnas.160105897

Mayo, L.M., Asratian, A., Lindé, J., Holm, L., Nätt, D., Augier, G., Stensson, N., Vecchiarelli, H.A., Balsevich, G., Aukema, R.J., Ghafouri, B., Spagnolo, P.A., Lee, F.S., Hill, M.N., Heilig, M. 2020. Protective effects of elevated anandamide on stress and fear-related behaviors: translational evidence from humans and mice. *Mol Psychiatry* 25: 993-1005. DOI: 10.1038/s41380-018-0215-1

McPartland, J.M., Matia, I., Di Marzo, V., Glass, M. 2005. Evolutionary origins of the endocannabinoid system. *Gene* 370:64-74. DOI: 10.1016/j.gene.2005.11.004

Mechoulam, J.M., Agraval, J., Glesson, D., Heasman, K., Glass, M. 2006. Cannabinoid receptors in invertebrates. *J Evol Biol* 19: 366-373. DOI: 10.1111/j.1420-9101.2005.01028.x

Mechoulam, R., Peters, M., Murillo-Rodriguez, E., Hanuš L.O. 2007. Cannabidiol-Recent Advances. *Chem Biodivers* 4: 1678-1692. DOI: 10.1002/cbdv.200790147

Morgane, P.J., Galler, J.R., Mokler, D.J. 2005. A Review of Systems and Networks of the Limbic forebrain/limbic Midbrain. *Prog Neurobiol* 75(2): 143-160. DOI: 10.1016/j.pneurobio.2005.01.001

Mustafa, A.F., McKinnon, J.J., Christensen, D.A.1999. The nutritive value of hemp meal for ruminants. *Can J Anim Sci* 79(1): 91-95. DOI: 10.4141/A98-031

Nasri, H., Baradaran, A., Shirzad, H., Rafieian-Kopaei, M. 2014. New Concepts in Nutraceuticals as Alternative for Pharmaceuticals. *Int J Perv Med* 5(12): 1487-1499. Retrieved July 8, 2020 from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4336979/

National Research Council. 2007. *Nutrient Requirements of Horses: Sixth Revised Edition.* Washington, DC: The National Academics Press. DOI: 10.17226.11653

Navarro, M., Hernández, E., Muñoz, R.M., del Arco, I., Villanúa, M.A., Carrera, M.R., Rodríguez de Fonseca, F. 1997. Acute administration of the CB_1 cannabinoid receptor antagonist SR 141716A induces anxiety-like responses in the rat. *Neuropharmacology and neurotoxicology* 8(2): 491-496. Retrieved from: https://journals.lww.com/neuroreport/Abstract/1997/01200/Acute_administration_of_theCB1_cannabinoid.23.aspx

Noble, G.K., Brockwell, Y.M., Munn, K.J., Harris, P.A., Davidson, H.P.B., Li, X., Zhang, D. 2008. Effects of a commercial dose of L-tryptophan on plasma tryptophan concentrations and behavior in horses. *Equine Vet J* 40(1): 51-56. DOI: 10.2746/042516407X238503

Pagotto, U., Marscicano, G., Cota, D., Lutz, B., Pasquali, R. 2006. The Emerging Role of the Endocannabinoid System in Endocrine Regulation and Energy Balance. *Endocrine Reviews*. 27(1): 73-100. DOI: 10.1210/er.2005-0009

Palazzoli, F., Citti, C., Licata, M., Vilella, A., Manca, L., Zoli, M., Vandelli, M. A., Forni, F., Cannazza, G. 2018. Development of a simple and sensitive liquid chromatography triple quadrupole mass spectrometry (LC-MS/MS) method for the determination of cannabidiol (CBD), delta-9 tetrahydrocannibinol (THC), and

its metabolites in rat whole blood after oral administration of a single high dose of CBD. *J Pharmaceut Biomed* 150: 25-32. DOI:10.1016/j.jpba.2017.11.054

Pearson, W., MacNicol, J. 2017. Acute Effects of a Single-Dose Nutrition Product on Stress Response and Task Completion in Horses. *J Equine Vet Sci* 51: 86-91. DOI: 10.1016/j.jevs.2017.01.001

Pertwee, R.G. 2000. Cannabinoid receptor ligands: clinical and neuropharmacological considerations, relevant to future drug discovery and development. *Exp Opin Invest Drugs* 9:1553-1571. Abstract. DOI:10.1517/13543784.9.7.1553

Pertwee, R.G. 2004. Novel Pharmacological Targets for Cannabinoids. *Neuropharmacology* 2(1): 9-29. DOI: 10.2174/1570159043476927

Pertwee, R.G. 2006. The pharmacology of cannabinoid receptors and their ligands: an overview. *Int J Obes* 30:S13-S18. DOI: 10.1038/sj.ijo.0803272

Pertwee, R.G. 2008. Ligands that target cannabinoid receptors in the brain: from THC to anandamide and beyond. *Addict Biol* 13:147-159. DOI: 10.1111/j.1369-1600.2008.00108.x

Philpott, H.T., O'Brien, M., McDougall, J.J. 2017. Attenuation of early phase inflammation by cannabidiol prevents pain and nerve damage in rat osteoarthritis. *Pain* 158: 2224-2451. DOI: 10.1097/j.pain.0000000000001052

Procuik, M.A., Edel, A.L., Richard, M.N., Gavel, N.T., Ander, B.P., Dupasquier, C.M.C., Pierce, G.N. 2008. Cholesterol-induced stimulation of platelet aggregation is prevented by a hempseed-enriched diet. *Can J Physiol. Pharmacology* 86: 153-159. DOI: 10.1139/Y08-011

Resstel LBM, Tavares RF, Lisboa SFS, Joca SRL, Corrêa MA, Cuimarães FS. 2009. 5-HT_{1A} receptors are involved in the cannabidiol induced attenuation of behavioural and cardiovascular responses to acute restraint stress in rats." *Br J Pharmacol* 156: 181-8. DOI: 10.1111/j.1476-5381.2008.00046.x

Romano, B., Borrelli, F., Fasolino, I., Capasso, R., Piscitelli, F., Cascio, M.G., Pertwee, R.G., Coppola, D., Vassallo, L., Orlando, P., Di Marzo, V., Izzo, A.A. 2013. The cannabinoid TRPA1 agonist cannabichromene inhibits nitric oxide production in macrophages and ameliorates murine colitis. *Br J Pharmacol* 169: 213-229. DOI:10.1111/bph.12120

Romano, L.L., Hazekamp, A. 2013. Cannabis Oil: chemical evaluation of an upcoming cannabis-based medicine. *Cannabinoids*. 7(1): 1-11. Retrieved August 8, 2020 from: https://bedrocan.com/wp-content/uploads/2013-cannabis-oil-chemical-evaluation-of-an-upcoming-cannabis-based-medicine_romano.pdf

Ross, D.J., Roberts, J.L. 2018. Equine Calming Products: A Short Survey Into Their Use, Effect, and Knowledge Using a Small Sample of Horse Owners in the North of Scotland, UK. *J Equine Vet Sci* 68: 63-67. DOI: 10.1016/j.jevs.2018.05.208

Rossdale, P., Hopes, R., Digby, N. 1985. Epidemiology study of wastage among racehorses 1982 and 1983. *Vet Rec* 116:66-69. DOI: 10.1136/vr.116.3.66

Russo, E.B. 2011. Review. Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. *Brit J Pharmacol* 163:1344-1364. DOI: 10.1111/j.1476-5381.2011.01238.x

Russo, R. 2007. History of Cannabis and Its Preperations in Saga, Science, and Sobriquet. *Chem Biodivers* 4: 1614-1648. DOI: 10.1002/cbdv.200790144

Rosenbaum D.M., Rasmussen, S.G.F., Kobilka, B.K. 2009. The structure and function of G-protein-coupled receptors. *Nature* 459(7245): 356-363. DOI: 10.1038/nature08144

Ryberg, E., Larsson, N., Sjögren, S., Hjorth, S., Hermanssonm N.O., Leonova J., Elebring, T., Nilsson, K., Drmota, T., Greasley, P.J. 2007. The orphan receptor GPR55 is a novel cannabinoid receptor. *Br J Pharmacol* 152(7):1092-1101. DOI:10.1038/sj.bjp.0707460

Sastre-Garriga, J., Vila, C., Clissold, S., Montalban, X. 2011. THC and CBD oromucosal spray (Sativex ®) in the management of spasticity associated with multiple sclerosis. *Expert Rev Neurother* 11(5): 627-637. DOI: 10.1586/ern.11.47

Sallaberry, C.A., Astern, L. 2018. The Endocannabinoid System, Our Universal Regulator. *JYI* 34(6): 48-55. DOI: 10.22186/jyi.34.5.48-55

Samara, E., Bialer, M., Mecholulam, R. 1988. Pharmacokinetics of cannabidiol in dogs. *Drug Metab Dispos* 16(3):469-472. Retrieved August 8, 2020 from: http://dmd.aspetjournals.org/content/16/3/469.short

Samara, E., Bialer, M., Harvey, D.J. 1990. Pharmacokinetics of urinary metabolites of cannabidiol in the dog. *Biopharm Drug Dispos* 11(9): 785-795. DOI: 10.1002/bdd.2510110906

Sarrafchi, A., Blokhuis, H.J. 2013. Equine stereotypic behaviors: Causation, occurrence, and prevention. *J Vet Behav* 8: 386-394. DOI: 10.1016/j.jveb.2013.04.068

Schlueter, A.E., Orth, M.W. 2004. Equine osteoarthritis: a brief review of the disease and its causes. *Equine and Comparative Exercise Physiology* 1(4); 221-231

Schmidt, A., Möstel, E., Wehnert, C., Aurich, J., Müller, J., Aurich, C. 2010. Cortisol release and heart rate variability in horses during road transport. *Horm Behav* 57: 209-215. DOI: 10.1016.j.yhbeh.2009.11.003

Scuderi, C., Filippis, D., Juvone, T., Blasio, A., Steardo, A., Esposito, G. 2009. Cannabidiol in Medicine: A Review of its Therapeutic Potential in CNS Disorders. *Phytother Res* 23: 597-602. DOI: 10.1002/ptr.2625

Seidman, A.J., Limaiem, F. 2019. Synovial Fluid Analysis. In: StatPearls. Treasure Island, FL: StatPearls Publishing. Retrieved from: https://ncbi.nlm.nih.gov/books/NBK537114/

Silversides, F.G., Lefrançois, M.R. 2005. The effect of feeding hempseed meal to laying hens. *Br Poult Sci* 46(2): 231-235. DOI: 10.1080/00716605000066183

Simopoulos, A.P. 2002. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed Pharmacother* 56: 365-379. Retrieved from: https://www.essentialnutrition.com.br/media/artigos/gamalift/21.pdf

Slatkin, N.E. 2007. Cannabinoids in the treatment of chemotherapy-induced nausea and vomiting: beyond prevention of acute emesis. *J Support Oncol* 5(3):1-9. Retrieved August 8, 2020 from: https://europepmc.org/article/med/17566383

Small, E., Jui, P.Y., Lefkovitch. 1976. A Numerical Taxonomic Analysis of Cannabis with Special Reference to Species Delimitation. *Systemic Botany* 1:67-84. DOI: 63.150.211.93

Snider, M.A. 2020. Incorporation of Hempseed in the Broiler Chicken Diet. *Murray State University Theses and Dissertations* 166. Retrieved July 7, 2020 from: https://digitalcommons.murraystate.edu/etd/162

Staton, P.C., Hatcher, J.P., Walker, D.J., Morrison, A.D., Shapland, E.M., Hughes, J.P., Chong, E., Mander, P.K., Green, P.J., Billinton, A., Fulleylove, M., Lancaster, H.C., Smith, J.C., Bailey, L.T., Wise, A., Brown, A.J., Richardson, J.C., Chessel, I.P. 2008. The putative cannabinoid receptor GPR55 plays a role in mechanical hyperalgesia associated with inflammatory and neuropathic pain. *Pain* 139(1): 225-236. DOI:10.1016/j.pain.2008.04.006

Swirsley, N., Spooner, H., Hoffman, R. 2017. Supplement use and perceptions: a study of US horse owners. *J Equine Vet Sci.* 59: 34-39. DOI: 10.1016/j.jevs.2017.08.021

Thomas, A., Baillie, G.L., Phillips, R.K., Razdan, R.K., Ross, R.A., Pertwee, R.G. 2007. Cannabidiol displays unexpectedly high potency as an anatagonist of CB1 and CB2 receptor agonist *in vitro*. *Br J Pharmacol* 150(5): 613-623. DOI:10.1038/sj.bjp.0707133

Tulamo, R., Bramlage, L.R., Gabel, A.A. 1989. Sequential clinical and synovial fluid changes associated with acute infectious arthritis in the horse. *Equine Vet J* 21(5): 317-387. DOI: 10.1111/j.2042-3306.1989.tb02681.x

Van der Stelt, M., Trevisani, M., Vellani, V., De Petrocellis, L., Moriello, A.S., Campi, B., McNaughton, P. Geppetti, P., Di Marzo, V. 2005. Anandamide acts as an intracellular messenger amplifying Ca^{2+} influx via TRPV1 channels. *The EMBO J* 25: 3026-3037. DOI: 10.1038/sj.emboj.7600784

Wade, D.T., Makela, P., Robson, P., House, H., Bateman, C. 2004. Do cannabis-based medicinal extracts have general or specific effects on symptoms in multiple sclerosis? A double-blind, randomized, placebo-controlled study on 160 patients. *Multiple Sclerosis* 10:434-441. DOI: 10.1191/1352458504ms1082oa

Wiley, J.L., Burston, J.J., Leggett, D.C., Alekseeva, O.O., Razdan, R.K., Mahadevan, A., Martin, B.R. 2009. CB_1 cannabinoid receptor-mediated modulation of food intake in mice. *Br J Pharmacol* 145(3): 293-300. DOI: 10.1038/sj.bjp.0706157

Wirth, P.W., Watson, E.S., ElSohly, M., Turner, C.E., Murphy, J.C. 1980. Anti-inflammatory properties of cannabichromene. *Life Sci* 26(23): 1991-1995. DOI:10.1016/0024-3205(80)90631-1

Zuardi, A.W. 2006. History of *cannabis* as a medicine: a review. *Braz J Psychiat* 28(2): 152-157. DOI:10.1590/S1516-44462006000200015

Appendix A

IACUC Approval

MURRAY STATE UNIVERSITY

Institutional Animal Care and Use Committee

328 Wells Hall
Murray, KY 42071-2393
270-809-3534 • 270-809-3535 fax

January 6, 2020

Dr. C.A. Shea Porr
Animal/Equine Science
Murray State University
Murray, KY 42071

Dear Dr. Porr:

The Murray State University Institutional Animal Care and Use Committee (IACUC) has approved your research protocol with revisions for the project titled, "Safety and Efficacy Evaluation of Extended Oral CBD Treatment in Horses."

The protocol timeline is approved through May 15, 2020. Please use the Animal Use Report (attached) to keep up-to-date information about the animals. At the termination of the protocol, you will need to complete the Conclusion Report (attached) and list final information concerning the animals.

If you have any questions, please contact me at 270-809-3534

Sincerely,

Kristi Stockdale
IACUC Coordinator

cc:
IACUC File

Appendix B

Instructor Surveys

Evaluator:
Horse:
Date:
Please rate **behavior** according to the behavior scale provided here:

Behavior Scale:
1: Positive, quiet (ears forward, relaxed, pleasant; absence of negative behaviors)
2: Somewhat positive (1-2 instances of negative behavior, mostly pleasant)
3: Moderate (2-3 displays of negative behavior; may only be one form)
4: Somewhat negative (3-4 instances of negative behavior; mostly negative)
5: Negative, poor (pinning ears, biting, kicking, pulling back; 5+ instances displayed of negative behavior, multiple forms of display)

Behavior: **Please rate the following scenarios as fitting to this horse:**					
1. Horse behavior during tack-up	1	2	3	4	5
2. Handling on the ground	1	2	3	4	5
3. Behavior when tied	1	2	3	4	5
4. Work ethic in classes	1	2	3	4	5
5. Work ethic in practices	1	2	3	4	5
6. Attitude towards other horses in the class	1	2	3	4	5
7. Provide and rate any unlisted behavior:	1	2	3	4	5

Please rate variable influencers and **movement** according to the scale provided here:

Variable Influencers and Movement Scale	
1:	Very high
2:	High
3:	Moderate
4:	Low
5:	Very low

Variable Influencers:					
1. Reactivity to environment	1	2	3	4	5
2. Ability to adapt to the rider	1	2	3	4	5
Movement:					
Please answer the following relative to performance:					
1. Suppleness on a circle left	1	2	3	4	5
2. Suppleness on a circle right	1	2	3	4	5
3. Ability to track up	1	2	3	4	5
4. Willingness to take the right lead	1	2	3	4	5
5. Willingness to take the left lead	1	2	3	4	5
6. Ability to track a straight line off the rail	1	2	3	4	5
Does the horse appear sound during sessions? (Circle one)		Yes		No	
If no, please explain below:					

Are there other aspects of behavior or movement that you feel have not been addressed here? If so, please explain:

Appendix C

Definition of Terms

Cannabis Sativa- Hemp

CBC- Complete blood count

CBD- Cannabidiol

Flexion Test- A standard test used by veterinarians for soundness evaluation. After the joint is flexed for 30 to 60 seconds, the horse is asked to trot off immediately. Typically, a joint that is at risk will result in the horse favoring that limb in the flexion test. These are intended as an attempt to localize the pain within a certain area (King and Mansmann, 1997). Often the flexion test will be used in conjunction with the equine lameness scale to place a numerical value to the degree of lameness.

G-Gelding; a male horse, at least 2 years old and unable to reproduce

g- Gram

kg- Kilograms

Lameness Grading System- A 1 through 5 numerical scale used to create a standard of comparison on degrees of lameness. Scoring a 1 would coincide with minimal lameness, inconsistently seen. The degree of lameness increases to 5, quantifying a severely lame horse where lameness is observed standing at rest (King and Mansmann, 1997).

lbs- Pounds

M-mare; a female horse at least 2 years old

mo- Month

Nutraceutical- Defined as an oral substance that is recognized for improving the overall health of consumers. DeFelice coined the term in 1989 by joining nutrient and pharmaceutical Hartsel et al., 2019).

yr- Year(s)

Δ^9-THC- Delta 9 tetrahydrocannabinol

Appendix D

Limitations and Assumptions

The following limitations create restraints on the research:

1) The sample size of horses is limited to the population provided at Murray State University. This included only 2 horses in the pilot, 18 in Project 2 and 24 in Project 3. Project 3 required the population to be further reduced statistical analysis, including only 17 horses for the majority of tests and 15 for the survey responses.
2) Radiographs are not included within the methods of Project 3, thus proof of improvement relies upon video observation and survey responses.
3) Although the AAEP recommended flexion test is accepted as a standard for equine lameness evaluation, it can be subject to human error. Completion of the test by a licensed veterinarian serves as a means of reducing this subjectivity.
4) While novel object tests associated with surveys attempt to provide a form of standard in behavior evaluation, this can also be subject to human error due to its reliance upon honest and accurate observation of the individual involved.
5) All phases were conducted under applied research settings, lacking the controlled environment of a laboratory setting.

The research was conducted under the following assumptions:

1) All subjective evaluations such as lameness and reactivity assessments were completed as honest evaluations following the rubric of the designed scales. A licensed veterinarian completed lameness evaluations to minimize variability.
2) Current technologies within CBD analytical labs are providing accurate and consistent cannabinoid readings.
3) CBD products used within the study are being administered correctly, ensuring the horse fully consumed the product.
4) Blood samples were being properly stored and shipped.
5) All management changes or additional products administered to study horses were documented throughout the study.

Appendix E

Budget

The total budget, excluding the value of donated items was $32,982.00. A cost breakdown for each project is provided below. Supplies calculations include materials for blood sampling, and organization. Estimated costs of donated materials were included and marked with an asterisk. The centrifuge and freezer for blood storage were available prior to the start of the projects.

Project 1		Project 2		Project 3	
Description	Cost	Description	Cost	Description	Cost
Initial blood analysis	$367	Blood analysis-CBD	$8,640	Graduate Assistant	$7,050.00
Second blood analysis	$540	Sample shipping	$160	Dartfish program	$240.00
Shipping fees	$100	Blood analysis-SC	$823	Feed analysis	$220
Supplies	$10	Blood analysis-CBC	$378	Shipping	$580
Internal feed analysis	$1750	Supplies	$250	Blood analysis-CBD	$5,760
External feed analysis	$110	EVS Pharm CBD pellets*	$300	Blood analysis-SC	$756
EVS Pharm CBD pellets*	$75	Catheter insertion-Dr. Tony Hicks*	$132	Blood analysis-CBC	$1098
EVS Pharm CBD oil*	$150			Lameness evaluations	$3,600
				Supplies	$250
				EVS Pharm CBD pellets*	$4500
				Folium Biosciences CBD*	$1100

Note. Asterisk (*) represents donated materials.

Appendix F

Time Schedule

The comprehensive time scale expanded over a 12 mo period. Project 1 was completed in 4 hr. This included time to place horses in stalls, administer treatments, collect and evaluate blood, and return the subjects to their normal locations. Project 2 was organized to occur over 12 hr. Project 3 was the most time intensive of the three studies, occurring over an 8 wk period and serving as the primary focus. Following Project 3 conclusion, 3 mo were taken for data analysis. The thesis defense was completed in the fall of 2020.