THESIS

OPTIMIZATION OF OVERHEAD ENCLOSURE MONITORING SOFTWARE IN A RODENT MODEL OF OSTEOARTHRITIS

Submitted by

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ABSTRACT

OPTIMIZATION OF OVERHEAD ENCLOSURE MONITORING SOFTWARE

IN A RODENT MODEL OF OSTEOARTHRITIS

Osteoarthritis (OA) is a degenerative joint disease characterized by pain, inflammation, and decreased range of motion, leading to impaired activities of daily living and reduced quality of life.[21] OA affects between 250 and 500 million people worldwide, contributing to a substantial and sustained economic burden.[22,23,39] Given the global pervasiveness of this poorly understood disease process, *in vivo* OA research relies on both naturally occurring and induced animal models for its study.[35] The Dunkin Hartley guinea pig spontaneously develops degenerative joint disease as early as 3 months of age and represents a well-characterized animal model of primary OA with pathological progression similar to humans.[6,37,52] In contrast, secondary OA is caused by non-idiopathic factors, including trauma, and animal models of secondary OA rely on chemical, surgical and non-surgical induction of instability.[27]

Open-field testing (OFT) is a behavioral tool which provides objective measurements of mobility outcomes for animals enrolled in musculoskeletal studies and can be paired with overhead monitoring software to non-invasively track voluntary animal movement through the designated arena. However, established protocols for OFT have not been published in the guinea pig. The overarching goal of this project was to optimize OFT in the guinea pig to reduce environmental variability in behavioral testing conditions. The results of this project

provided a framework to ensure accurate and reproducible data collection in subsequent studies involving therapeutic interventions to both spontaneous OA and traumatic OA.

A hallmark symptom of OA is pain and, as such, the second portion of this work was dedicated to researching cannabidiol (CBD) as an alternative interventional therapeutic to analgesia. Specifically, mobility outcomes assessments were performed during a pharmacokinetic safety study as well as a chronic oral CBD dosing study. Significant differences were analyzed both on baseline (pre-treatment) and on treatment intervention in each phase of this two-part study pertaining to OFT. The results of these studies identified time-of-day effects exist when testing guinea pigs in the open-field and provided preliminary evidence that no adverse short-term behavioral effects exist after oral administration of CBD.

The final goal of this project was to design of bioreactor to establish a non-surgical animal model of post-traumatic osteoarthritis (PTOA) in the guinea pig through precision rupture of the anterior cruciate ligament (ACL) by tibial compression and displacement. While this model has been characterized in other rodents, it has not been described in guinea pigs. Work from this portion of the project helped produce a functional bioreactor which will be used initially on cadavers and will ultimately promote *in vivo* research of interventional treatments for PTOA by establishing reproducible ligament lesions with subsequent degenerative joint pathology.

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CHAPTER I.

TIME-OF-DAY EFFECTS AND TESTING DURATION OPTIMIZATION FOR OVERHEAD ENCLOSURE MONITORING IN THE DUNKIN HARTLEY GUINEA PIG[1]

1. Introduction

Open-field testing (OFT) is used in research to provide objective behavioral and mobility outcome measurements and can be paired with enclosure monitoring software to non-invasively track animals throughout a designated arena.[36] While initially developed for use in rats, OFT has since been used across numerous rodent species to assess anxiety, cognition, exploratory behavior, and locomotion.[15] In guinea pigs, OFT has been used extensively to assess behavioral and mobility outcomes in a variety of research fields, including cognitive development,[47] neurotoxicity,[33] nutrition,[44] osteoarthritis,[46] and pharmacology,[40] among others.

It is optimal to control for both environmental and biological variables when designing behavioral studies that will use OFT. Common environmental variables considered include housing, lighting, and novelty of the open-field; typical biological variables controlled for include age, sex, strain, and body mass.[15] Given its influence on physiologic parameters, time-of-day is also an important biological variable to consider. However, it can often be overlooked

[1] A version of this manuscript has been submitted to the Journal of the American Association for Laboratory Animal Science: **Helbling JE, Spittler AP, Sadar MJ, Santangelo KS.** 2022. Time-Of-Day Effects and Testing Duration Optimization for Overhead Enclosure Monitoring in the Dunkin Hartley Guinea Pig. JAALAS.

during study design and analyses, potentially affecting both consistency of results and reproducibility.[38]

Time-of-day can affect processes such as learning and memory, sensation and perception, along with numerous behaviors including mating, aggression, and drug-seeking.[38] For nocturnal animals such as mice and rats, it is generally more suitable to conduct behavioral testing during the dark phase when they will be most active.[13] However, some studies have shown that mice appear to be unaffected by circadian cycles during OFT.[5,49] Methods of testing these species during their active cycle include placing animals on a reverse light-dark cycle and testing during the dark phase of the daily illumination cycle.[38] Lastly, the use of continuous home cage monitoring to minimize confounding variables has also been shown to be a reliable method for conducting behavioral analyses and is gaining in popularity.[4,51]

Given the above, one objective of this study was to determine whether a relationship between time-of-day and open-field mobility outcomes exist in the Dunkin Hartley guinea pig. Given the above, one objective of this study was to determine whether a relationship between time-of-day and open-field mobility outcomes exist in the Dunkin Hartley guinea pig. It was hypothesized guinea pigs would display periods of heightened activity during the earliest testing period, given their crepuscular nature (i.e., being most active at dawn and dusk). However, a study in laboratory guinea pigs found they traveled over twice as much distance in the dark phase but did not demonstrate evidence of nocturnal rhythms. Further, peak locomotor activity occurred around 1900 with secondary peaks occurring every 6-8 hours.[30] This suggests that laboratory guinea pigs may not display natural or expected behaviors when exposed to controlled lighting conditions.

Prior to the development of automated video-tracking software, testing duration for OFT in rodents ranged from 2 to 10 minutes, primarily due to the time-intensiveness of manual data acquisition.[15] Recently, durations for OFT in mice and rats have ranged from 5 to 30 minutes.[1,3,19,43,48] In guinea pigs, published durations for OFT vary greatly, ranging from as short as 5 to 10 minutes in novel enclosures to as long as 22 hours within home cages.[9,12,30,40]

Therefore, inefficiencies may exist for guinea pigs undergoing prolonged open-field enclosure monitoring. Thus, the second objective of this study was to optimize and refine the open-field enclosure monitoring procedure for the guinea pig. We hypothesized that a testing duration of 10 minutes would suffice for capturing representative movement samples.

2. Materials and Methods

2.1 Animals

Ten 5-month-old intact male Dunkin Hartley guinea pigs sourced from Charles River Laboratories (Wilmington, MA) participated in this study. Guinea pigs were singly housed in #6 Thoren (30.80 cm x 59.37 cm x 22.86 cm) conventional static isolator cages (Maxi-Miser Interchangeable IVC Caging, Thoren, Hazelton, PA) with 0.125-in. corncob bedding and red hut shelters (Bio-Serv, French Town, NJ). Hay cubes (PMI Nutrition International, Brentwood, MO) were provided daily as enrichment. Caging was changed 2 times weekly. Animal rooms were maintained on a 12:12-h light:dark cycle (lights on from 0600:1800) between 20-26°C with 30-70% room humidity. Teklad Global Guinea Pig Diet 2040 (Envigo, Madison, WI) and filter-sterilized water were provided without restriction. Guinea pigs were free of Sendai virus, lymphocytic choriomeningitis virus, pneumonia virus of mice, guinea pig adenovirus, guinea pig reovirus, *Helicobacter spp*., *Mycoplasma pulmonis*, and ectoparasites. All procedures were

approved by the University IACUC and conducted in accordance with the Animal Welfare Act[2] and the *Guide for the Care and Use of Laboratory Animals*[24].

2.2 Open-field Enclosure Monitoring

Data for time-of-day effects and testing duration optimization were collected concurrently as animals were permitted voluntary movement in the enclosure for 14 minutes at four different times of day. Guinea pigs were transported in their home cages to the testing room 15 minutes prior to testing. The room was consistently dimly lit at 15 lux (Light Meter LM-3000, Lightray Innovation GmbH) to provide contrast and to minimize video tracking error. The open-field enclosure was a circular blue plastic bin measuring 114 cm in diameter and 15 cm in height, with a red hut shelter placed in the center. The enclosure was surrounded by a wire pen to prevent animals from escaping. A standard high definition 720p webcam (Logitech, Newark, CA) was placed above the enclosure to non-invasively record video. Animals were acclimated to the enclosure for 15 minutes for two days prior to data collection. Following acclimation, guinea pigs were randomly selected, placed in the center of the apparatus, and allowed to move freely for 14 minutes for data collection. Observers were positioned adjacent to the enclosure to ensure animal safety and remained still and silent for the duration of each test. No background noise was provided. The enclosure was cleaned with dilute soapy water between tests to ensure removal of olfactory stimuli from previous subjects.

2.3 Behavioral Tracking

Mobility outcomes were assessed using ANY-maze behavioral tracking software (Stoelting Co., Wood Dale, IL). ANY-maze software was programmed to divide the total 14-minute testing duration into 2-minute bins without interruption of behavior. Recordings were

performed between four time periods (0530-0700, 0930-1100, 1130-1300, and 1530-1700), which were dictated by the requirements for the primary study in which the animals were involved. Additional justifications for these time periods included assessments that would occur during a typical working day and minimization of overall animal testing burden. Mobility measures tracked included total distance traveled, average speed while mobile, total time mobile, and total time in the red hut shelter. Additionally, rearing and chewing on enclosure wire bars were observed behaviors that were video coded and analyzed. Data from each animal was collected once in each of the above periods sequentially on the same day. Animals were returned to their home cage between testing events. No treatments were performed. All recordings were performed by the same handlers (APS and JEH).

2.4 Statistical Analysis

Data were analyzed using Prism version 9.0.0 for Windows (GraphPad Software, San Diego, CA). Group size was determined using G*Power (version 3.1) from pilot work whereby the primary outcome was total distance traveled during a 10-minute collection period. Using an a priori f test for repeated measures (RM) ANOVA with 4 measurements and an effect size of 0.5, power associated with an alpha level of 0.05 was 0.95 with a sample size of 10 animals per group (PMID: 2106931).[34] Normality was confirmed using the D'Agostino-Pearson normality test, $\alpha = 0.05$. Statistical analysis for time-of-day effects was performed using repeated-measures RM one-way ANOVA. Differences in testing periods and bins were analyzed using RM two-way ANOVA with Geisser-Greenhouse correction. Post-hoc testing was performed for both analyses using Tukey's multiple comparisons test. The data were primarily right skewed and, as

such, mobility outcomes were reported as median and interquartile range (25^{th} and 75^{th} percentiles) unless otherwise noted.

3. Results

3.1 Time-of-Day Analysis

Animals were placed in an open-field test using ANY-maze enclosure monitoring software to quantify time mobile, distance traveled, average speed while mobile, and time in red hut shelter. Rearing and chewing on bars were also analyzed.

3.1.1 Total time mobile

Significant differences existed among periods for time mobile (P = 0.0013, Table 1.1), with animals spending the greatest time mobile during the 0530-0700 testing period. Significant differences existed between the 0530-0700 timepoint and all others (Figures 1.1). Median time mobile during the 14-minute testing interval for the 0530-0700 timepoint was 17.84% (IQR: 12.05% to 25.67%) compared to 1.88% (IQR: 0.01% to 9.61%, P = 0.0228) in the 0930-1100 timepoint, 1.90% (IQR: 0.74% to 7.32%, P = 0.0173) in the 1130-1300 timepoint, and 0.90% (IQR: 0.20% to 7.50%, P = 0.0306) in the 1530-1700 timepoint. No significant differences were present among the later time periods.

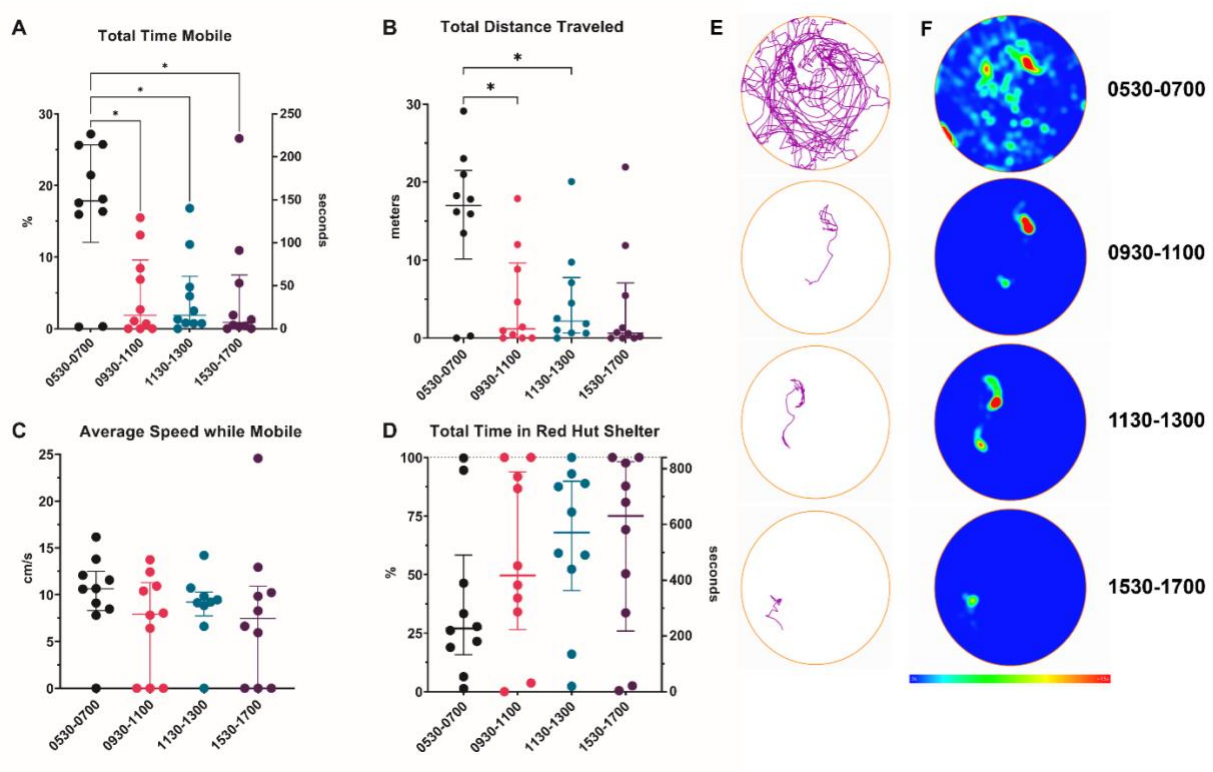

Figure 1.1. Time-of-day analysis in open-field enclosure monitoring in untreated animals.

Guinea pig activity is highest in the early morning, as evidenced by increased time mobile and distance traveled, with shorter durations spent in the red hut shelter. (A) Total time mobile as a percentage of total testing duration (left Y axis) and seconds (right Y axis) in 10 5-mo old guinea pigs. Significant differences were found between times of day (P = 0.0013), with animals demonstrating the greatest mobility during the 0530-0700 testing period. (B) Total distance traveled in meters also differed significantly (P = 0.0047) among time-of-day, with animals traveling further in the 0530-0700 period. (C) Average speed while mobile in cm/s did not vary significantly between time-of-day. (D) Total time in red hut approached significance (P = 0.0769) with animals spending less time in the shelter during the morning periods; dashed line represents maximum testing duration (840 seconds). Representative guinea pig shows increased mobility during the 0530-0700 time-period by track plot of distance traveled (E) and heat map spectrum (F) of the animal's head position for the test duration, bar scale 0 seconds (blue) to 15 seconds (red). RM one-way ANOVA with Tukey's multiple comparisons post-hoc analysis. All data presented as median (IQR) *, P ≤ 0.05.

3.1.2 Total distance traveled

Significant differences were also identified among timepoints for distance traveled (*P* = 0.0047, Table 1.1). Significant differences existed between the 0530-0700 timepoint and the

two subsequent timepoints with median distance traveled in the 0530-0700 timepoint of 17.00 m (IQR: 10.15 m to 21.50 m) compared to 1.91 m (IQR: 0.00 m to 9.62 m, *P* = 0.0459) in the 0930-1100 timepoint and 2.19 m (IQR: 0.65 m to 7.68 m, *P* = 0.0397) in the 1130-1300 timepoint (Figure 1.1). An association between the 0530-0700 timepoint and the 1530-1700 was observed with animals traveling 0.64 m (IQR: 0.00 m to 7.06 m, *P* = 0.0508).

3.1.3 Average speed while mobile

Animals maintained consistent average speeds during all timepoints, with no significant differences observed (Figure 1.1).

3.1.4 Total time in red hut shelter

Time spent in red hut shelter approached significance (*P* = 0.0769), with animals spending greater time in the shelter during the later testing periods (Figure 1.1). Median percentage of testing bout spent in the red hut shelter during the 0530-0700 timepoint was 26.98% (IQR: 15.79% to 58.37%) compared to 49.66% (IQR: 26.46% to 93.87%) at the 0930-1100 timepoint, 67.92% (IQR: 43.21% to 89.91%) at the 1130-1300 timepoint, and 75.03% (IQR: 25.86% to 98.24%) at the 1530-1700 timepoint.

3.1.5 Rearing

Time spent rearing approached significance (*P* = 0.0561), with animals spending the most time rearing in the 0530-0700 timepoint (Table 1.1).

3.1.6 Chewing on enclosure wire bars

Time spent chewing on enclosure wire bars approached significance (*P* = 0.0633), with animals spending the most time chewing on bars in the 0530-0700 timepoint (Table 1.1).

Table 1.1. Descriptive statistics for mobility and behavioral outcome parameters in untreated animals.

	0530-0700	0930-1100	1130-1300	1530-1700	P Value
Total Time Mobile (sec)	149.9 (114.5)	15.8 (80.7)	16.0 (55.2)	7.6 (61.3)	0.0013
Time Mobile (%)	17.8 (13.6)	1.9 (9.6)	1.9 (6.6)	0.9 (7.3)	
Total Distance Traveled (m)	17.0 (11.4)	1.9 (9.6)	2.2 (7.1)	0.6 (7.1)	0.0047
Avg Speed while Mobile (cm/s)	10.6 (4.2)	7.9 (11.3)	9.2 (2.5)	7.5 (10.9)	0.5662
Time in Red Hut Shelter (sec)	226.6 (357.7)	417.1 (566.3)	570.6 (392.3)	630.3 (608.0)	0.0769
Time in Red Hut Shelter (%)	27.0 (42.6)	49.7 (67.4)	67.9 (46.7)	75.0 (72.4)	
Time Rearing (sec)	122.8 (191.7)	0.0 (63.0)	4.7 (63.6)	0.0 (61.1)	0.0561
Time Rearing (%)	14.6 (22.8)	0.0 (7.6)	0.6 (7.6)	0.0 (7.3)	
Time Chewing on Bars (sec)	32.0 (223.3)	0.0 (10.1)	0.0 (5.4)	0.0 (4.5)	0.0633
Time Chewing on Bars (%)	3.8 (26.6)	0.0 (1.2)	0.0 (0.6)	0.0 (0.5)	

Data are shown as median (IQR).

3.2 Testing Optimization Analysis

The 14-minute testing duration was divided into 2-minute bins to identify patterns in activity. While median values across 2-minute bins did not demonstrate significant differences for any measured mobility outcomes (Figure 1.2), an association (P = 0.0506) existed between trial and time-of-day for time spent in the red hut shelter. Additionally, median cumulative total distance traveled within the first 10 minutes of the 14-minute testing period was 88.75% for the

0530-0700 timepoint and 100.00% at subsequent timepoints, suggesting diminishing returns of data collection beyond 10 minutes of total testing duration (Table 1.2).

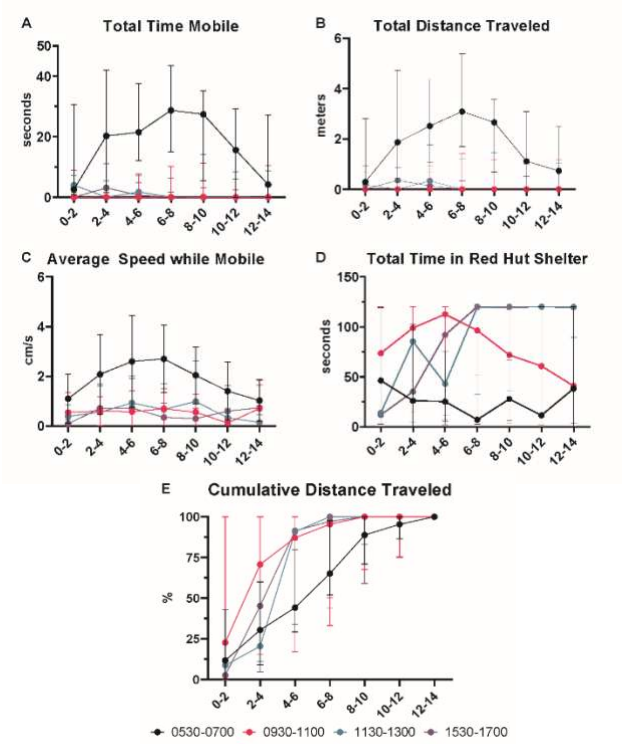

Figure 1.2. Mobility outcome parameters divided into distinct 2-minute bins within 14-minute testing interval.

Open-field enclosure monitoring software, programmed to create distinct 2-minute bins (x-axis) within 14-minute testing period. (A) Total time mobile in seconds (median with IQR), in 10 5-mo-old guinea pigs. Mean values across 2-minute intervals were not statistically significant for (A) Time mobile, (B) Total distance traveled in meters, or (C) Average speed while mobile in cm/s (median with IQR). As per data provided in Figure 1, 0530-0700 demonstrated increased activity compared to other time of day comparison (P = 0.0013). (D) Total time in red hut in seconds (median with IQR) showed no significant differences overall; however, during both the 1130-1300 and 1530-1700 timepoints, animals spent extensive time in the red hut. (E) Median cumulative percent distance traveled approached 90% in all timepoints within the first 10-minutes of the 14-minute testing interval. RM two-way ANOVA with Geisser-Greenhouse correction, Tukey's multiple comparisons post-hoc analysis.

Table 1.2. Cumulative percent distance traveled by 2-minute bin.

Bin (min)	0530-0700	0930-1100	1130-1300	1530-1700
0-2	11.73 [0.40 to 42.87]	22.65 [0.00 to 100.00]	8.73 [0.00 to 100.00]	2.55 [0.00 to 100.00]
2-4	30.39 [9.00 to 59.96]	70.70 [15.56 to 100.00]	20.46 [11.17 to 100.00]	45.30 [4.65 to 100.00
4-6	44.16 [29.25 to 79.74]	87.07 [17.00 to 100.00]	90.84 [17.21 to 100.00]	91.35 [33.92 to 100.00]
6-8	65.10 [51.88 to 97.65]	95.44 [33.22 to 100.00]	100.00 [43.86 to 100.00]	97.20 [50.29 to 100.00]
8-10	88.75 [71.03 to 100.00]	100.00 [67.62 to 100.00]	100.00 [83.00 to 100.00]	100.00 [59.04 to 100.00]
10-12	95.37 [86.44 to 100.00]	100.00 [75.18 to 100.00]	100.00 [99.81 to 100.00]	100.00 [75.33 to 100.00]
12-14	100.00	100.00	100.00	100.00

Data are shown as median [range].

4. Discussion

The purpose of this study was to identify whether Dunkin Hartley guinea pigs displayed time-of-day variation in activity patterns and to investigate potential refinements in overhead enclosure monitoring duration. Animals exhibited significantly higher mobility outcomes, including time mobile and distance traveled during the 0530-0700 testing period, but did not display heightened activity in the subsequent testing periods. These findings supported the hypothesis that animals would be more active in the early morning period. However, it should be noted that due to the requirements for the animals' primary study, the present study did not include a timepoint beyond 1700. Therefore, incorporation of a later timepoint should be considered for future studies.

Lack of heightened activity in the late afternoon may have been due to facility constraints, with lighting abruptly producing complete light and darkness at 0600 and 1800, respectively, rather than transitory changes in light intensity as guinea pigs would experience in nature. A previous study using continuous home cage monitoring identified juvenile pair

housed Hartley guinea pigs to be most active around 1900, the time which the dark cycle began in that facility.[30] As such, common lighting conditions in vivaria may impact the natural behavior of animals. This is further supported by an early study in laboratory guinea pigs which found they remained active throughout the day and night unlike wild guinea pigs which demonstrate crepuscular behavior.[54] Research has suggested that introducing timers with gradual transitions to simulate dawn and dusk may be beneficial for studying behavioral and mobility outcomes in multiple species.[53]

An important behavioral consideration for the present study is presence of observers in the room during testing. Observers were seated directly adjacent to the enclosure to ensure animal safety, which may have negatively influenced natural exploratory behaviors.[15,47] Guinea pigs prefer ambulating along the cage perimeter, which was directly in the observer's field of view.[7,13] Animals experiencing stress to novel environments may become immobile, demonstrate darting behavior, or seek shelter.[13] As such, home cage testing in behavioral and mobility research is becoming more commonplace, especially in smaller rodents, to eliminate the variable of observer effects.[16]

An advantage of testing in the home cage environment is the novelty of the open field is drastically decreased, which is important when using OFT for mobility and behavioral outcomes. To reduce the likelihood of decreased mobility outcomes due to novelty of a new enclosure, animals were acclimated to the enclosure for 15 minutes per day in the two days preceding testing. An overhead enclosure monitoring study of guinea pigs in their home cage found they spent 65.6 ± 1.5% (mean ± SEM) of their time in the shelter.[30] We found animals

spent similar time in the red hut during the later testing periods, however animals spent only 37.6 ± 10.7% (mean ± SEM) in the red hut during the 0530-0700 timepoint.

The present findings may indicate animals had an increased exploratory drive in the early morning, and that the effects of novel enclosures may be negated with acclimation prior to testing. It has previously been shown that bedding the open-field with similar substrate to the home cage can improve the spontaneous exploratory behavior of guinea pigs.[33] The enclosure in this study was not bedded with any material to ensure sanitation and reduce presence of olfactory cues from previous subjects, which may have contributed to decreased activity in some animals.

Novel exploration also appears to decrease as the duration of the test increases, suggesting data may be collected more effectively. A recent study in rats that received 30 minutes of acclimation 24 hours prior to OFT progressively decreased ambulatory activity from minutes 1 to 10, and showed no significant changes from that point until the test concluded at 20 minutes.[43] Mice have shown gradual progression of decreased ambulatory activity over time, reaching steady state in 30-60 minutes.[15] Analysis agreed with our hypothesis that a 10-minute testing period would adequately capture representative data, as animals completed nearly 90% of their total distance traveled within the first 10-minutes of the 14-minute trial (Table 1.2).

In conclusion, the results of this study found that time-of-day effects significantly influenced mobility outcomes in Dunkin Hartley guinea pigs. Therefore, it is recommended to standardize the testing period to limit the impact of variability on locomotion parameters. Additionally, this study identified a potential refinement to the overhead enclosure monitoring

procedure that may result in decreased testing burden for both animals and researchers. It is likely that a testing duration of 10 minutes will suffice when collecting OFT data in the Dunkin Hartley guinea pig. Continuous home cage monitoring may be considered in future studies to identify periods of heightened activity over a 24-hour period while reducing potential variables such as noise, observer influence, and enclosure novelty. Additional studies are needed to analyze the time-of-day effects and duration of OFT in different strains, ages, and sexes of guinea pigs.

CHAPTER II.

MOBILITY OUTCOMES ASSESSMENTS IN CONTROL AND CBD TREATED DUNKIN HARTLEY GUINEA PIGS[2]

This two-phase prospective study allowed the opportunity to incorporate insights from the above protocol. Phase I of the study was a short-term pharmacokinetic cohort study which consisted of two groups of guinea pigs receiving a single dose of either 25 mg/kg or 50 mg/kg of cannabidiol (CBD) orally. Phase II of the study was a 3-month randomized controlled trial investigating the effects of twice daily oral dosing of two CBD doses (50 mg/kg and 100 mg/kg), along with an almond oil vehicle control and an untreated control, on mobility outcomes.

PHASE I: BEHAVIORAL EFFECTS OF SINGLE ORAL CBD ADMINISTRATION IN GUINEA PIGS

1. Phase I Introduction

Cannabidiol (CBD), the non-psychotropic component of the cannabis plant, has gained widespread popularity over the past several years as a treatment for osteoarthritis (OA) in both human and veterinary medicine.[29] Evidence suggests that CBD exerts anti-inflammatory and pain-modulating effects by acting on the endocannabinoid system, a biochemical signaling network that is thought to play a role in OA pathogenesis and pain control. Experimentally, CBD

[2] Data from this research were published in the Journal of Veterinary Pharmacology and Therapeutics: **Spittler AP, Helbling JE, McGrath S, Gustafson DL, Santangelo KS, Sadar MJ.** 2021. Plasma and joint tissue pharmacokinetics of two doses of oral cannabidiol oil in guinea pigs (Cavia porcellus). J Vet Pharmacol Ther.

has been shown to have analgesic and anti-inflammatory effects in laboratory mouse and rat models of rheumatoid arthritis,[32] inflammation, [10,11] and joint degeneration,[20,42] but efficacy in guinea pigs with naturally-occurring OA has yet to be determined.

The objective of this study was to assess behavioral effects of CBD following one-time oral administration in Dunkin Hartley guinea pigs. This guinea pig strain spontaneously develops OA at 3 months of age, making it an attractive model to study primary OA. We hypothesized that there would be no short-term adverse behavioral effects associated with oral administration of a single dose of CBD.

2. Phase I Materials and Methods

2.1 Animals

Ten, 5-month-old intact male Dunkin Hartley guinea pigs participated in this study; animals were sourced from Charles River Laboratories (Wilmington, MA) with a 2F catheter surgically implanted in the right jugular vein. The mean ± SD body weight was 918.97 ± 79.12 g. All guinea pigs were deemed healthy based on the results of physical exam and complete blood count (CBC) and serum biochemistry profile performed prior to the start of the experiment. Guinea pigs were singly housed in #6 Thoren (30.80 cm x 59.37 cm x 22.86 cm) conventional static isolator cages (Maxi-Miser Interchangeable IVC Caging, Thoren, Hazelton, PA) with 0.125-in. corncob bedding and red hut shelters (Bio-Serv, French Town, NJ). Hay cubes (PMI Nutrition International, Brentwood, MO) were provided daily as enrichment. Caging was changed 2 times weekly. Animal rooms were maintained on a 12:12-h light:dark cycle (lights on from 0600:1800) between 20-26°C with 30-70% room humidity. Teklad Global Guinea Pig Diet 2040 (Envigo, Madison, WI) and filter-sterilized water were provided without restriction. Guinea pigs were

free of Sendai virus, lymphocytic choriomeningitis virus, pneumonia virus of mice, guinea pig adenovirus, guinea pig reovirus, *Helicobacter spp*., *Mycoplasma pulmonis*, and ectoparasites. All procedures were approved by the University IACUC and conducted in accordance with the Animal Welfare Act[2] and the *Guide for the Care and Use of Laboratory Animals*[24].

2.2 CBD Administration

A 100 mg/mL CBD suspension in almond oil was formulated and provided by Canopy Animal Health (Toronto, Ontario). Guinea pigs were randomly assigned (by drawing numbers out of a container) into one of two CBD dosing groups (25 mg/kg or 50 mg/kg). Animals were unfasted prior to CBD dosing.

2.3 Open-field Enclosure Monitoring

Data for time-of-day effects and testing duration optimization were collected concurrently as animals were permitted voluntary movement in the enclosure for 14 minutes at four different times of day. Guinea pigs were transported in their home cages to the testing room 15 minutes prior to testing. The room was consistently dimly lit at 15 lux (Light Meter LM-3000, Lightray Innovation GmbH) to provide contrast and to minimize video tracking error. The open-field enclosure was a circular blue plastic bin measuring 114 cm in diameter and 15 cm in height, with a red hut shelter placed in the center. The enclosure was surrounded by a wire pen to prevent animals from escaping. A standard high definition 720p webcam (Logitech, Newark, CA) was placed above the enclosure to non-invasively record video. Animals were acclimated to the enclosure for 15 minutes for two days prior to data collection. All recordings were performed by the same handlers (APS, JEH). As time of day may affect guinea pig behavior and mobility, baseline recordings were performed the day prior to CBD administration from 0530-

0700, 0900-1130, 1130-1300, and 1530-1700. Subsequent recordings were performed the following day after CBD administration at corresponding time intervals between blood collections. The 0530-0700 recording post-CBD was performed prior to the 24-hour blood collection.

2.5 Statistical analysis

Data were analyzed using Prism version 9.0.0 for Windows (GraphPad Software, San Diego, CA). Group size was determined using G*Power (version 3.1) from pilot work whereby the primary outcome was total distance traveled during a 10-minute collection period. Using an a priori f test for repeated measures (RM) ANOVA with 4 measurements and an effect size of 0.5, power associated with an alpha level of 0.05 was 0.95 with a sample size of 10 animals per group (PMID: 2106931).[34] Normality was confirmed using the D'Agostino-Pearson normality test, $\alpha = 0.05$. To account for time-of-day effects on mobility and behavior, enclosure monitoring parameters within each timepoint (i.e., 0530 pre-CBD vs. 0530 post-CBD) were analyzed by paired t-tests or Wilcoxon matched pairs signed-rank tests as dictated by normality. Correlation was determined by Pearson coefficient. Significance was set at a value of $p < 0.05$.

3. Phase I Results

3.1 Animals

All guinea pigs remained healthy with no clinically apparent adverse effects from drug administration during the study period. Detailed methods used for pharmacokinetic analyses and quantification of plasma and tissue levels of CBD in animals can be found in a previously published manuscript (PMID: 34658021).[46]

3.2 Open-field enclosure monitoring

To assess potential side effects on activity levels from CBD administration, mobility parameters of guinea pigs were monitored in an open-field apparatus before and after treatment.

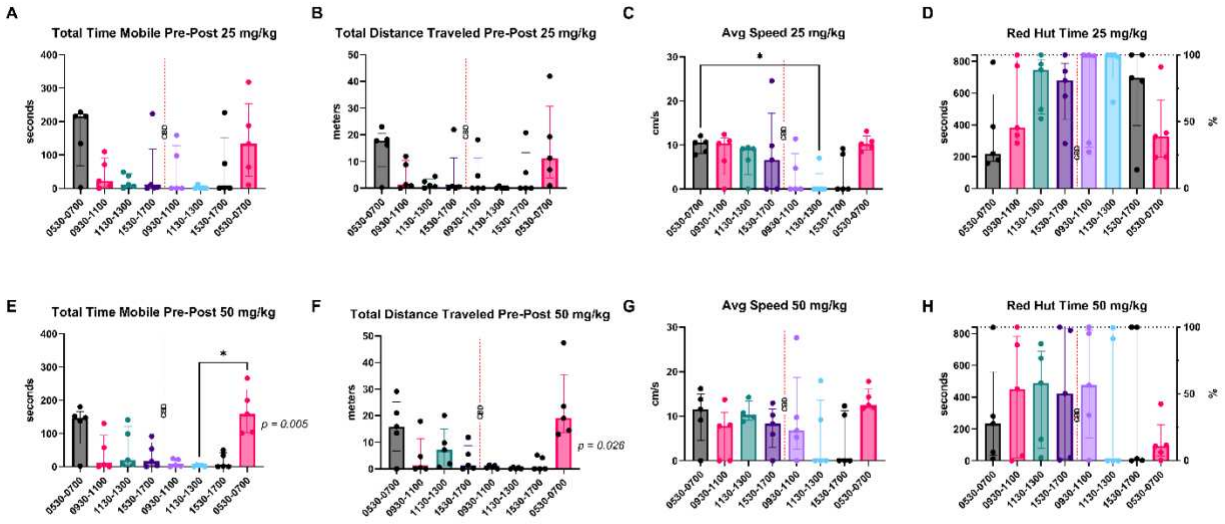

Figure 2.1. Mobility outcome parameters by time-of-day, pre- and post-dosing of CBD.

Mobility outcomes differed among times of day for certain parameters, including (C) average speed while mobile 25 mg/kg, (E) total time mobile 50 mg/kg, and (F) total distance traveled 50 mg/kg. As such, analyses of dosing effects were time matched according to the respective baseline and assessed for significance using paired t-tests or Wilcoxon matched pairs signed-rank test based on normality (Figure 2.2).

No significant differences were present in total distance traveled, average speed, or time in red hut between baseline and post-CBD timepoints (Figure 2.2). Strong positive correlations ($r > 0.7$) were observed in final distance traveled and final concentrations of CBD in the articular cartilage and infrapatellar fat pad (IFP) in the 25 mg/kg cohort (Table 2.1). Strong negative correlations ($r < -0.7$) were observed in final red hut time and final concentrations of

CBD in the articular cartilage and IFP in the same cohort. Therefore, an association between single administration of CBD at the 25 mg/kg dose and mobility outcomes did exist.

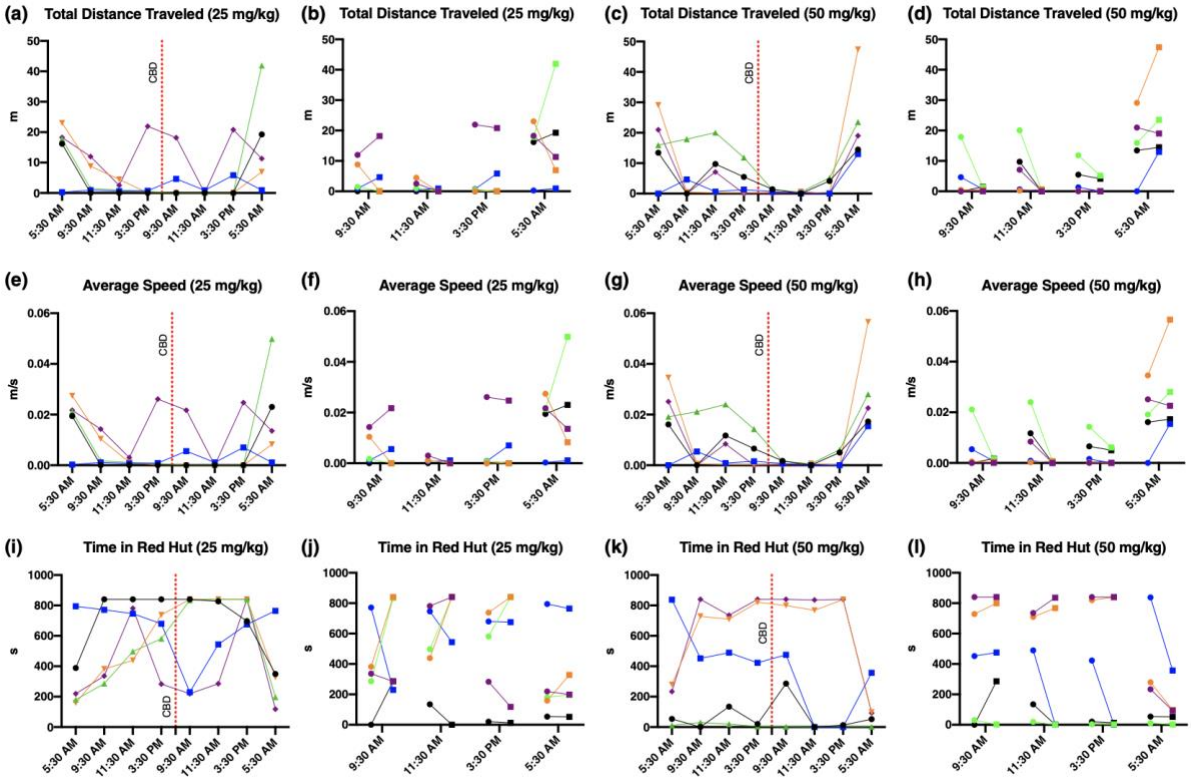

Figure 2.2. Longitudinal and before-after graphs of open-field enclosure monitoring parameters for guinea pigs that received a single oral dose of 25 or 50 mg/kg CBD.

Longitudinal graphs show total distance traveled (a, c), average speed (e, g), and time in red hut (i, k) values from baseline to post-CBD timepoints. Before-after graphs show total distance traveled (b, d), average speed (f, h), and time in red hut (j, l) between pre- and post-CBD timepoints. Circles represent pre-CBD values, and squares represent post-CBD values.

Table 2.1. Pearson correlation between final distance traveled, final red hut time, and CBD concentrations.

	Animal	Final Distance (m)	Final Red Hut Time (s)	Plasma Concentration (ng/mL)	Articular Cartilage Concentration (ng/g)	IFP Concentration (ng/g)
25 mg/kg	2002	19.256	349.9	1.2	14	141
	2003	0.957	764.9	7.15	7.06	54.7
	2175	41.917	196.9	4.14	146	151
	2176	6.929	328.2	1.83	75.7	92.9
	2178	11.327	198.7	0.169	72.5	151
r Plasma		-0.08	0.77			
r Articular		0.75	-0.72			
r IFP		0.72	-0.90[a]			
50 mg/kg	2001	13.441	52.2	3.89	33.8	225
	2004	0	357.4	13.4	55.1	311
	2005	15.922	2.4	15.8	53.6	189
	2174	29.115	98.3	5.2	75.9	136
	2177	20.987	92.6	10.5	185	433
r Plasma		-0.45	0.22			
r Articular		0.38	-0.07			
r IFP		-0.30	0.32			

[a] $p = 0.04$

4. Phase I Discussion

Administration of CBD oil as a single 25 or 50 mg/kg dose produced no adverse effects in guinea pigs. Behavior and activity parameters did not significantly differ from baseline timepoints after CBD administration. However, positive correlations between final distance traveled and articular and IFP concentrations were observed, along with negative correlations for final time in red hut in the 25 mg/kg CBD treatment group. As such, those guinea pigs in the 25 mg/kg cohort experiencing higher plasma and IFP concentrations of CBD may have demonstrated a dose-dependent reduction in anxiety, leading to less time spent in red hut shelter and increased willingness to explore the arena. A recent study in a neuropathic pain

model in rats found that animals treated with CBD (3 mg/kg, IP) showed decreased anxiety related behaviors when placed in an open field test.[45] Similarly, a bell-shaped dose-dependent relationship in anxiolytic effects of CBD has been shown in rats using an elevated plus maze, with animals receiving 5 mg/kg CBD IP showing the highest entry ratio of all doses (2.5, 5, 10, and 20 mg/kg).[17]

The results of this study provide preliminary data for the use of CBD for OA in guinea pigs. Further studies are still needed to determine long-term safety, therapeutic doses, and the efficacy of CBD for the treatment of OA in both male and female guinea pigs.

PHASE 2: INVESTIGATING EFFECTS OF CHRONIC CBD ADMINISTRATION IN GUINEA PIGS

1. Phase II Introduction

The objective of Phase 2 of the study was to assess behavioral effects of chronic oral CBD dosing in the Dunkin Hartley guinea pig. Given the above findings, we hypothesized that time-of-day effects would be present in pre-treatment baseline data collections and that there would be no short-term adverse behavioral effects associated with oral administration of CBD.

2. Phase II Materials and Methods

2.1 Animals

Thirty-two, 2-month-old intact male Dunkin Hartley guinea pigs participated in this study, sourced from Charles River Laboratories (Wilmington, MA). Guinea pigs were singly housed in #6 Thoren (30.80 cm x 59.37 cm x 22.86 cm) conventional static isolator cages (Maxi-Miser Interchangeable IVC Caging, Thoren, Hazelton, PA) with 0.125-in. corncob bedding and red hut shelters (Bio-Serv, French Town, NJ). Hay cubes (PMI Nutrition International,

Brentwood, MO) were provided daily as enrichment. Caging was changed 2 times weekly. Animal rooms were maintained on a 12:12-h light:dark cycle (lights on from 0600:1800) between 20-26°C with 30-70% room humidity. Teklad Global Guinea Pig Diet 2040 (Envigo, Madison, WI) and filter-sterilized water were provided without restriction. Guinea pigs were free of Sendai virus, lymphocytic choriomeningitis virus, pneumonia virus of mice, guinea pig adenovirus, guinea pig reovirus, *Helicobacter spp*., *Mycoplasma pulmonis*, and ectoparasites. All procedures were approved by the University IACUC and conducted in accordance with the Animal Welfare Act[2] and the *Guide for the Care and Use of Laboratory Animals*[24].

2.2 CBD Administration

A 100 mg/mL CBD suspension in almond oil was formulated and provided by Canopy Animal Health (Toronto, Ontario). Animals were randomly assigned to receive 50 mg/kg CBD (n=8), 100 mg/kg (n=8), vehicle control oil (n=8), or were untreated (n=8).

2.3 Open-field Enclosure Monitoring

Data for time-of-day effects and testing duration optimization were collected concurrently as animals were permitted voluntary movement in the enclosure for 14 minutes at four different times of day. Guinea pigs were transported in their home cages to the testing room 15 minutes prior to testing. The room was consistently dimly lit at 15 lux (Light Meter LM-3000, Lightray Innovation GmbH) to provide contrast and to minimize video tracking error. The open-field enclosure was a circular blue plastic bin measuring 114 cm in diameter and 15 cm in height, with a red hut shelter placed in the center. The enclosure was surrounded by a wire pen to prevent animals from escaping. A standard high definition 720p webcam (Logitech, Newark, CA) was placed above the enclosure to non-invasively record video. Animals were acclimated to

the enclosure for 15 minutes for four days prior to data collection. All recordings were performed by the same handlers (APS, JEH). As time of day may affect guinea pig behavior and mobility, baseline recordings were performed prior to administration of treatment.

2.5 Statistical analysis

Data were analyzed using Prism version 9.0.0 for Windows (GraphPad Software, San Diego, CA). Group size and power were determined using the statistical software at http://www.stat.uiowa.edu/~rlenth/Power, as histologic assessment of OA was the primary outcome of interest in the larger body of work. Power was calculated at 0.9 with a sample size of 8 per experimental group. Normality was confirmed using the D'Agostino-Pearson normality test, $\alpha = 0.05$. Statistical analysis for time-of-day effects was performed using repeated-measures RM one-way ANOVA. Differences in testing periods and bins were analyzed using RM two-way ANOVA with Geisser-Greenhouse correction. Post-hoc testing was performed for both analyses using Tukey's multiple comparisons test. Significance was set at a value of $p < 0.05$.

3a. Phase II Baseline Results

3a.1 Time-of-Day Analysis

To repeat and confirm findings presented in Chapter 1, baseline data was collected prior to administering CBD and vehicle treatments. Animals were placed in an open-field test using ANY-maze enclosure monitoring software to quantify time mobile, distance traveled, average speed while mobile, and time in red hut shelter.

3a.1.1 Total time mobile

Significant differences existed among periods for time mobile ($P = 0.0489$, Figure 2.3A), with animals spending the greatest time mobile during the 0530-0700 testing period. Median

time mobile during the 14-minute testing interval for the 0530-0700 timepoint was 22.46% (IQR: 13.46% to 29.56%) compared to 10.64% (IQR: 2.08% to 19.94%) in the 0930-1100 timepoint, 14.15% (IQR: 4.15% to 21.89%) in the 1130-1300 timepoint, and 18.67% (IQR: 3.73% to 50.74%) in the 1530-1700 timepoint (Table 2.2).

3a.1.2 Total distance traveled

Mean differences among timepoints for total distance traveled approached significance (P = 0.0553). A significant difference existed between the 0530-0700 timepoint and the 1530-1700 timepoint (P = 0.0344, Figure 2.3B) with median distance traveled in the 0530-0700 timepoint of 15.66 m (IQR: 7.84 m to 24.30 m) compared to 7.60 m (IQR: 3.07 m to 16.60 m) in the 1530-1700 timepoint (Table 2.2).

3a.1.3 Average speed while mobile

Animals maintained consistent average speeds during all timepoints, with no significant differences observed (P = 0.2573, Figure 2.3C).

3a.1.4 Total time in red hut shelter

Animals spent greater time in the shelter during the later testing periods (Figure 2.3D). Median percentage of testing bout spent in the red hut shelter during the 0530-0700 timepoint was 58.48% (IQR: 46.15% to 67.08%) compared to 69.40% (IQR: 34.99% to 91.36%) at the 0930-1100 timepoint, 66.87% (IQR: 51.00% to 93.96%) at the 1130-1300 timepoint, and 71.46% (IQR: 44.42% to 93.23%) at the 1530-1700 timepoint (Table 2.2).

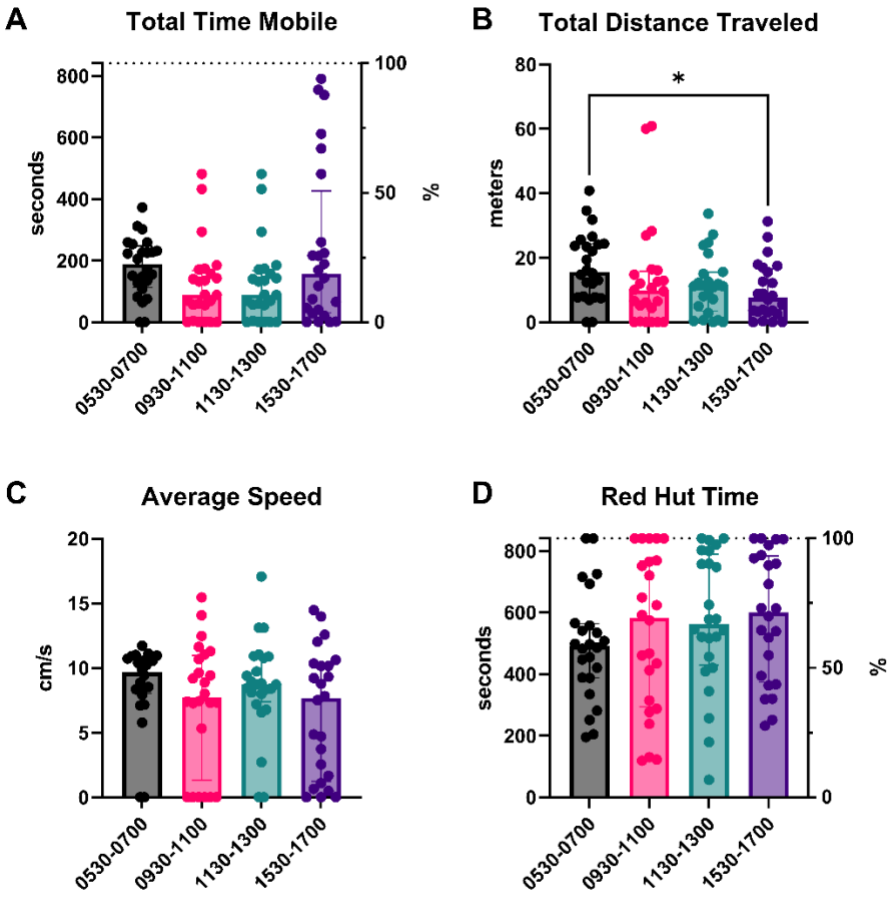

Figure 2.3. Phase II baseline mobility outcome parameters.

Data revealed activity is highest in the early morning, as evidenced by increased time mobile and distance traveled, with shorter duration spent in the red hut shelter. (A) Total time mobile in seconds (left Y axis) and as a percentage of total testing duration (right Y axis) in twenty-four 2-mo old guinea pigs. Total time mobile differed significantly between times of day ($P = 0.0489$), with animals demonstrating the greatest mobility during the 0530-0700 testing period. (B) Mean differences in total distance traveled approached significance ($P = 0.0553$) with animals traveling further in the 0530-0700 period. (C) Average speed while mobile in cm/s and (D) Total time in red hut did not vary significantly between time-of-day. However, animals did spend the least time in red hut during the 0530-0700 timepoint; dashed line represents maximum testing duration (840 seconds). RM one-way ANOVA with Tukey's multiple comparisons post-hoc analysis. All data presented as median (IQR) *, $P \leq 0.05$.

Table 2.2. Descriptive statistics for Phase II baseline mobility outcome parameters.

	0530-0700	0930-1100	1130-1300	1530-1700	P Value
Total Time Mobile (sec)	188.7 (135.2)	89.4 (150.1)	118.9 (149.1)	156.9 (394.9)	0.0489
Time Mobile (%)	22.5 (16.1)	10.6 (17.9)	14.2 (17.7)	18.7 (47.0)	
Total Distance Traveled (m)	15.7 (16.5)	9.8 (14.5)	11.2 (12.1)	7.6 (13.5)	0.0553
Avg Speed while Mobile (cm/s)	9.7 (2.6)	7.7 (9.6)	8.7 (3.5)	7.7 (9.1)	0.2573
Time in Red Hut Shelter (sec)	491.2 (175.8)	583.0 (473.5)	561.7 (360.9)	600.3 (410.0)	0.2430
Time in Red Hut Shelter (%)	58.5 (20.9)	69.4 (56.4)	66.9 (43.0)	71.5 (48.8)	

Data are shown as median (IQR).

3a.2 Testing Optimization Analysis

Similar to the methods utilized in Chapter 1, the 14-minute testing duration was divided into 2-minute bins to identify patterns in activity. Significant differences ($P < 0.0001$) were observed for all measured mobility outcomes between 2-minute bins (Figure 2.3). Significant interactions between trial and time-of-day were also observed for total time mobile ($P = 0.0086$), total distance traveled ($P = 0.0381$), average speed while mobile ($P = 0.0212$), and total time in red hut shelter ($P = 0.0417$). Median cumulative total distance traveled within the first 10 minutes of the 14-minute testing period was 86.99% for the 1130-1300 timepoint and approached 100.00% at other timepoints (Figure 2.4), suggesting diminishing returns of data collection beyond 10 minutes of total testing duration (Table 2.3).

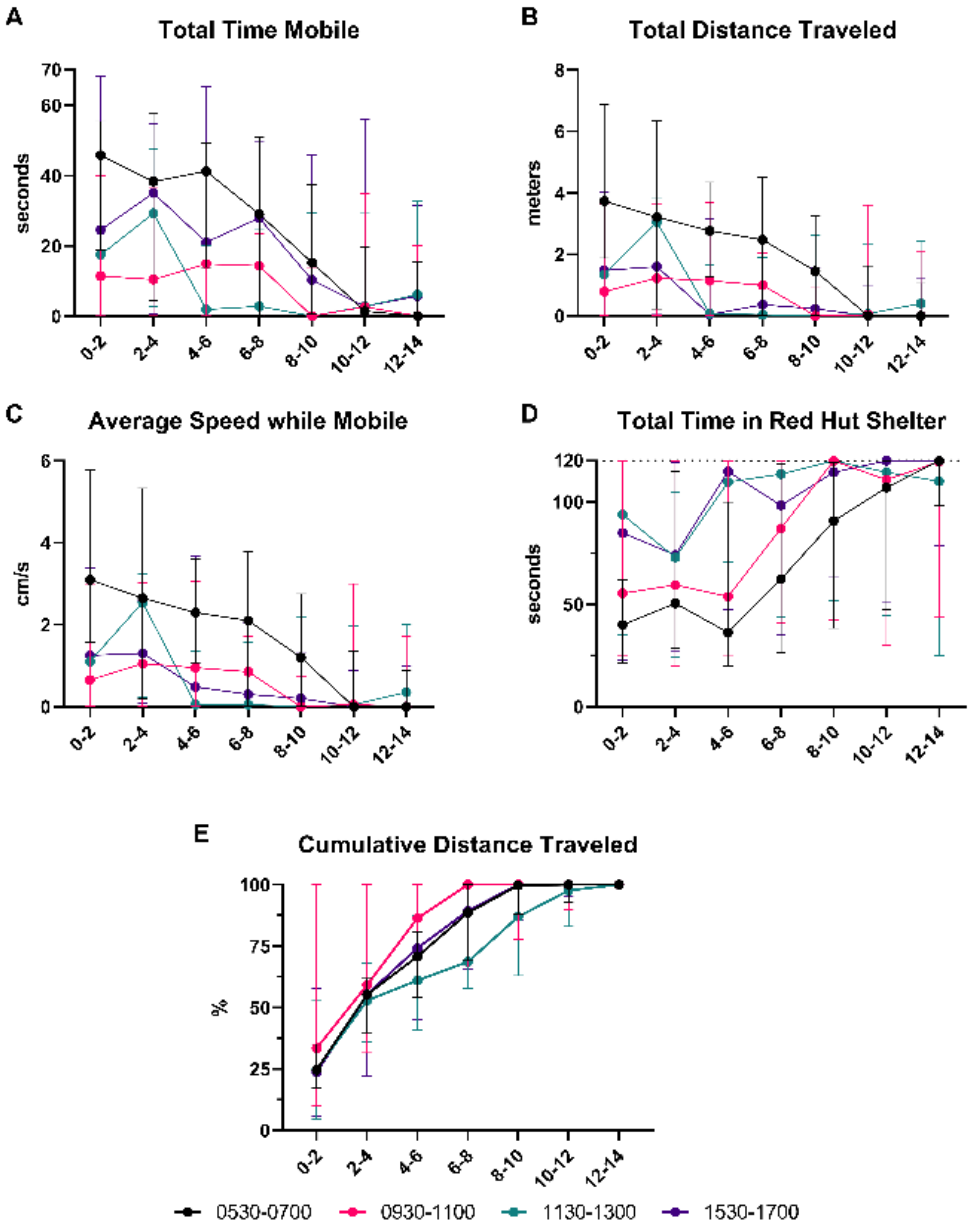

Figure 2.4. Mobility outcomes divided into distinct 2-minute bins within 14-minute testing period.

(A) Total time mobile in seconds (median with IQR), in 24 2-mo-old guinea pigs. Mean values across 2-minute intervals were statistically significant ($P < 0.0001$) for (A) Time mobile, (B) Total distance traveled in meters, (C) Average speed while mobile in cm/s and (D) Total time in red hut in seconds. (E) Median cumulative percent distance traveled approached 90% in all timepoints within the first 10-minutes of the 14-minute testing interval. RM two-way ANOVA with Geisser-Greenhouse correction, Tukey's multiple comparisons post-hoc analysis. All data presented as median and IQR.

Table 2.3. Cumulative percent distance traveled by 2-minute bin.

Bin (min)	0530-0700	0930-1100	1130-1300	1530-1700
0-2	24.60 [0.68 to 100.00]	33.42 [0.00 to 100.00]	24.76 [0.00 to 100.00]	23.60 [0.00 to 100.00]
2-4	55.33 [0.68 to 100.00]	59.19 [0.00 to 100.00]	52.91 [0.00 to 100.00]	55.41 [0.00 to 100.00]
4-6	70.76 [20.25 to 100.00]	86.34 [0.00 to 100.00]	61.08 [0.00 to 100.00]	74.18 [0.00 to 100.00]
6-8	88.53 [35.60 to 100.00]	100.00 [34.39 to 100.00]	68.67 [0.00 to 100.00]	89.41 [0.00 to 100.00]
8-10	99.74 [51.96 to 100.00]	100.00 [34.39 to 100.00]	86.99 [0.00 to 100.00]	100.00 [0.00 to 100.00]
10-12	100.00 [82.63 to 100.00]	100.00 [69.43 to 100.00]	97.66 [0.00 to 100.00]	100.00 [47.96 to 100.00]
12-14	100.00	100.00	100.00	100.00

Data are shown as median [range].

3b. Phase II Treatment Results

To assess treatment effects, data were analyzed using a mixed-effects analysis, Tukey's multiple comparisons post-hoc analysis. No significant differences were observed in any mobility outcomes for indicated Any-Maze parameters when normalized to baseline (Figure 2.7).

3b.1.1 Total time mobile

Significant differences in total time mobile were observed longitudinally within the untreated control group between 14 and 16 weeks of age (P = 0.0382) and again between age 16 and 18 weeks (P = 0.0468). A significant interaction was also observed between age and treatment (P = 0.0295). Individual longitudinal data for total time mobile for each treatment group have been provided below (Figure 2.5A-D). Significant differences were observed at 16 weeks of age between the untreated control and both the vehicle control (P = 0.0092) and the 50 mg/kg CBD (P = 0.0166) groups (Figure 2.6A).

3b.1.2 Total distance traveled

Significant differences were not observed longitudinally within treatment groups for total distance traveled (*P* = 0.2225). Additionally, no fixed effects were observed based on age (*P* = 0.3121). Individual longitudinal data for total distance traveled for each treatment group have been provided below (Figure 2.5E-H). Significant differences were observed at 16 weeks of age between the untreated control and both the vehicle control (*P* = 0.089) and the 50 mg/kg CBD (*P* = 0.0134) groups (Figure 2.6B).

3b.1.3 Average speed while mobile

A significant difference in average speed while mobile was observed longitudinally between 16 and 21 weeks old in the untreated control group (*P* = 0.0019). Individual longitudinal data for average speed while mobile for each treatment group have been provided below (Figure 2.5I-L). A significant difference (*P* = 0.0074) was also observed between the untreated and vehicle control groups at 21 weeks of age (Figure 2.6C).

3b.1.4 Total time in red hut shelter

A significant difference (*P* = 0.0122) was observed longitudinally between 14 and 16 weeks old for the untreated control group. Individual longitudinal data for average speed while mobile for each treatment group have been provided below (Figure 2.5M-P). Significant interactions were observed for age (*P* = 0.0016), treatment (*P* = 0.0424), and age x treatment (*P* = 0.0196). Additionally, significant differences in total time spent in the red hut shelter existed between the untreated control group and the 50 mg/kg CBD group at both 16 and 21 weeks of age (*P* = 0.0027 and 0.0480, respectively) and the 100 mg/kg CBD group at 16 weeks (*P* = 0.0143, Figure 2.6C).

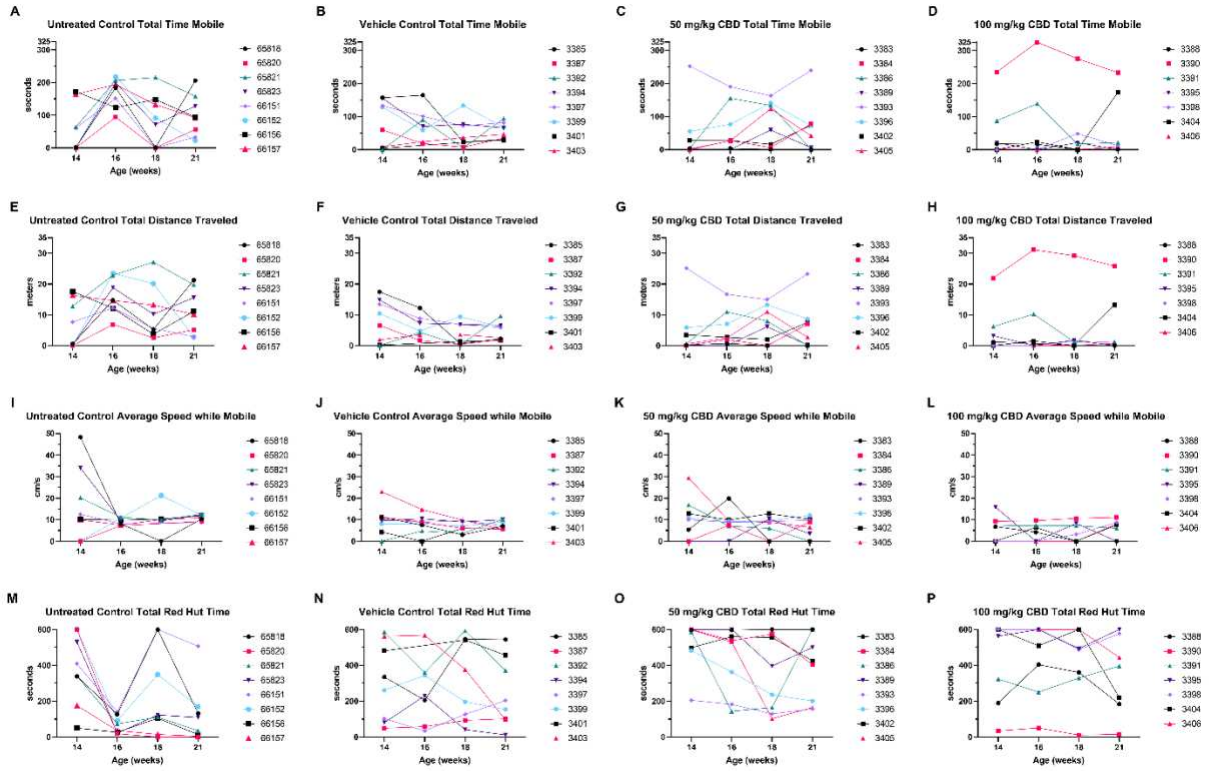

Figure 2.5. Longitudinal graphs representing open-field enclosure monitoring parameters by treatment.

Longitudinal graphs show total time mobile (A, B, C, D), total distance traveled (E, F, G, H), average speed while mobile (I, J, K, L), and total red hut time (M, N, O, P) for individual animals in each treatment and control group.

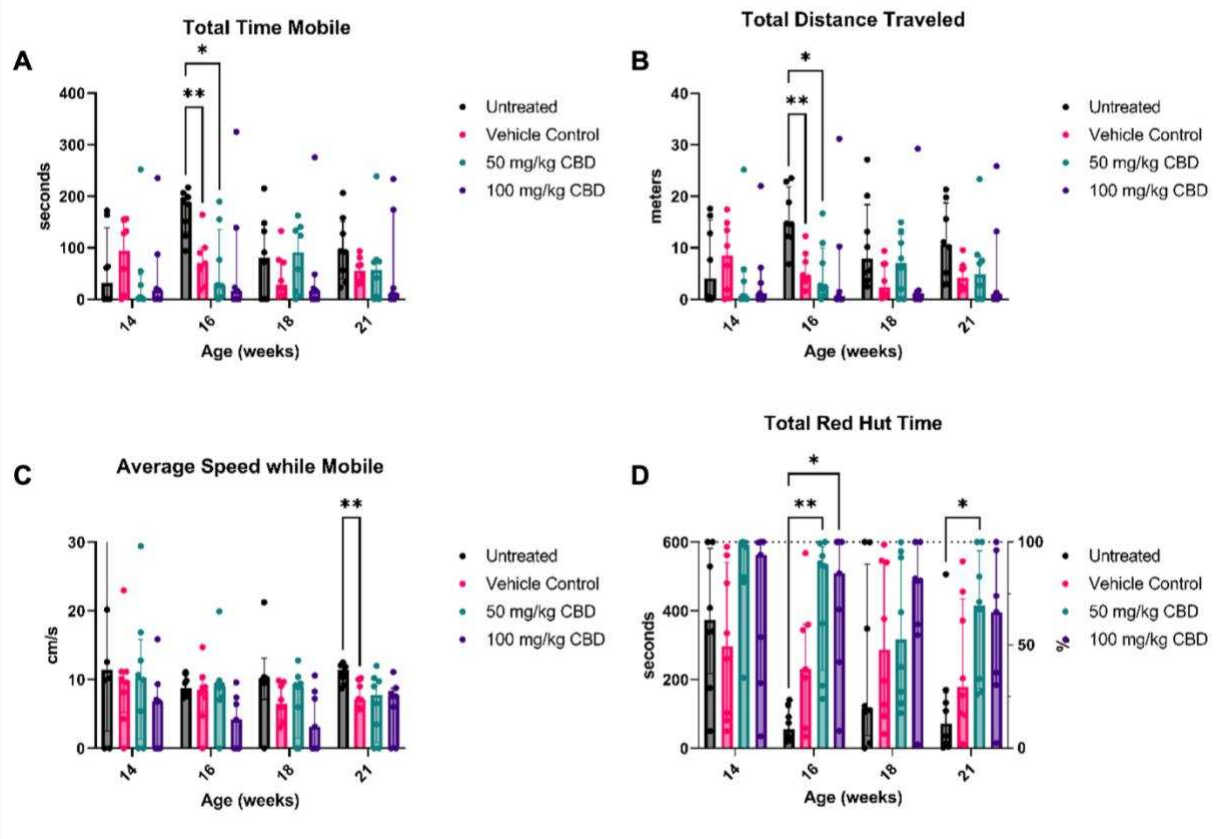

Figure 2.6. Longitudinal graphs demonstrating differences between treatment and control groups for mobility outcomes.

(A) Significant differences were observed at 16 weeks of age in total time mobile between the untreated control and both the vehicle control (*P* = 0.0092) and the 50 mg/kg CBD (*P* = 0.0166) groups. (B) Significant differences were observed in total distance traveled at 16 weeks of age between the untreated control and both the vehicle control (*P* = 0.089) and the 50 mg/kg CBD (*P* = 0.0134) groups. (C) A significant difference (*P* = 0.0074) was also observed in average speed while mobile between the untreated and vehicle control groups at 21 weeks of age. (D) Significant differences in total time spent in the red hut shelter existed between the untreated control group and the 50 mg/kg CBD group at both 16 and 21 weeks of age (*P* = 0.0027 and 0.0480, respectively) and the 100 mg/kg CBD group at 16 weeks (*P* = 0.0143). Mixed-effects analysis, Tukey's multiple comparisons post-hoc analysis, data presented as median and interquartile range.

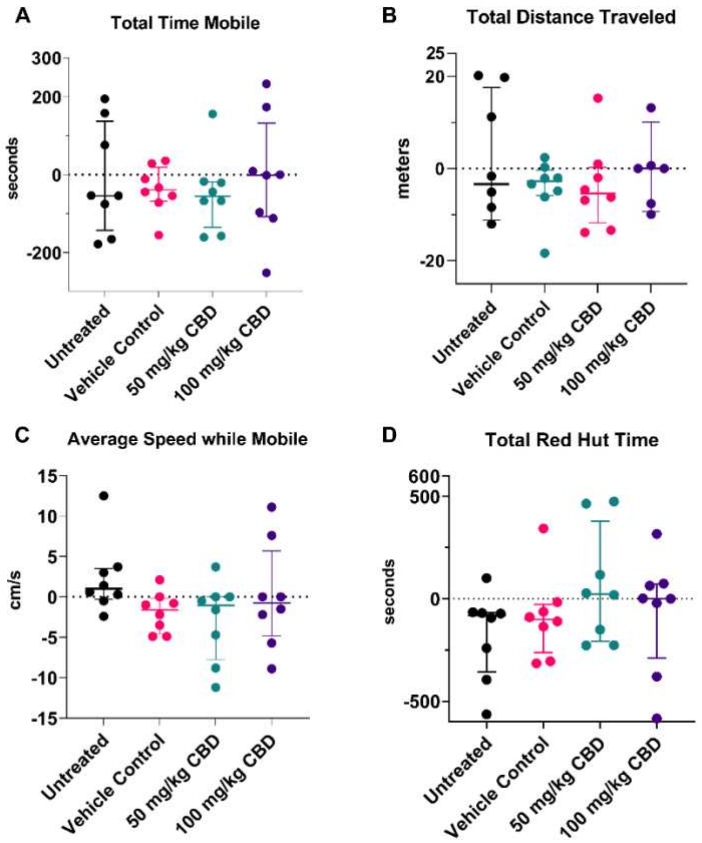

Figure 2.7. Final minus baseline for mobility outcomes.

No significant differences were seen within treatment groups for (A) total time mobile, (B) total distance traveled, (C) average speed while mobile, or (D) time in red hut shelter.

4. Phase II Discussion

As for findings presented in Chapter 1, pre-treatment baseline data collected revealed animals demonstrate increased activity patterns in the early morning and demonstrated diminishing returns of data collection beyond a 10-minute testing interval in the OFT. As such, when designing studies involving OFT in guinea pigs, time-of-day should be accounted for and controlled for when possible. Significant interactions ($P < 0.0001$) were observed between 2-minute bins and all mobility outcome parameters, and a testing duration of greater than 10

minutes is likely unnecessary as animals completed at least 86% of total distance traveled within the first 10 minutes of the 14-minute testing duration.

Within group differences were seen in the untreated control group longitudinally for total time mobile, with peak activity occurring at 16 weeks. Following 16 weeks of age, total time mobile decreased and demonstrated less variability. As such, age may be an important consideration when designing studies involving OFT in the Dunkin Hartley guinea pig, which spontaneously develops OA near 3 months of age. No treatment effects were observed within groups when assessing final minus baseline data for any mobility outcomes (Figure 2.7).

Importantly, no significant differences were detected between the almond oil vehicle control and the CBD treatment group. However, significant between group differences were observed in the vehicle control and 50 mg/kg CBD groups when compared to the untreated control group for total time mobile and total distance traveled at 16 weeks of age, with animals in the untreated control group spending more time mobile and traveling a greater distance. Animals in the 100 mg/kg CBD group demonstrated a similar mean difference in distance traveled as the 50 mg/kg CBD group when compared to the untreated control at 16 weeks of age but had increased variability among animals. Animals in the untreated control group also spent significantly less time in the red hut shelter at both 16 weeks and 21 weeks of age compared to the CBD treatment groups. Given no differences were observed between the almond oil control and the CBD treatments, but that these groups did differ significantly from the untreated control, the almond oil vehicle may have contributed to decreased time mobile and distance traveled. Additionally, CBD treatments may have experienced decreased

exploratory interest as they spent increased time in the red hut shelter compared to the untreated control.

A recent study in mice found that CBD dosed at 5 mg/kg intraperitoneally significantly increased the time spent in the center of the open field, indicating they may experience reduced anxiety and/or somnolence.[26] In another study, rats receiving an oral dose of 10-30 mg/kg CBD once demonstrated hypolocomotion at 30 minutes, and a sex-dependent relationship was observed at 270 minutes post-dosing, with females having a lower level of activity. Lastly, a recent placebo-controlled study in humans found lethargy as the most common reported side effect after receiving CBD.[14]

As such, animals receiving CBD may be less inclined to ambulate and more likely to rest in the red hut shelter during OFT. Given these findings, the current work should be broadened to include animals of varying sexes while controlling for age and time of day. Additional considerations regarding the vehicle and fasting status should be considered when designing future studies for chronic oral dosing of CBD.

CHAPTER III.

DESIGN OF A MECHANICAL BIOREACTOR FOR VALIDATION OF NON-SURGICAL GUINEA PIG MODEL OF POST-TRAUMATIC OSTEOARTHRITIS

While spontaneous animal models have clear advantages when researching primary osteoarthritis (OA), they do not closely reflect the pathology experienced in secondary OA. In both humans and animals, post-traumatic osteoarthritis (PTOA) is one of the most common forms of secondary OA. As such, animal models for post-traumatic osteoarthritis (PTOA) have been well-characterized and have been detailed below. However, while guinea pigs continue to be a commonly used species in OA research, a guinea pig model of externally induced PTOA has not been described.

3.1 Background

3.1.1 Classification of PTOA Models

Surgical – Various methods of surgical induction of OA exist, each resulting in rapid progression, making the technique ideal for short-term studies. While long-term economic costs can be avoided, such rapid progression can present challenges to studying early phases of disease.

Anterior cruciate ligament transection (ACLT) – The ACLT model is the most common surgical model of PTOA and results in joint destabilization and secondary mechanical trauma to chondrocytes, leading to degradation of articular cartilage.[28]

Meniscectomy – Similar to ACLT, meniscectomies lead to rapid onset of OA through joint instability and mechanical trauma but cause more severe degenerative changes.[25] Guinea pigs have increased load bearing on the medial meniscus like humans, making them a favorable model of PTOA through medial meniscectomy.[27]

Medial meniscal tear – Guinea pigs and rats are well-described models of PTOA by medial meniscal tear, which is achieved through medial collateral ligament transection.[35] An advantage of the guinea pig model is the predilection of natural development of primary OA in the contralateral limb.[6]

Ovariectomy – Given that post-menopausal women experience osteoporosis, and OA as a sequela, it is theorized that estrogen serves a protective function against the development of OA. While guinea pigs have been used to study this disease progression[55], the procedure is typically used from a proof-of-principle perspective to further the understanding of the unknown pathophysiology of OA.

Chemical – Chemically induced models of PTOA involve the direct injection of toxic or inflammatory compounds into the stifle. Described chemicals include sodium monoiodoacetate (MIA)[18,50], papain[8], quinolone[6], and collagenase[31]. While less invasive than surgery, chemical induction does not reflect the natural pathophysiology that occurs in traumatic OA. As such, chemical induction of PTOA has commonly been used to study the underlying pain mechanisms and identify potential therapeutic and analgesic candidates rather than histopathological changes.

Non-surgical – Surgical induction of OA can have complications such as lack of reproducibility between, and within, surgeons and may alter the inflammatory pathway through the surgery

itself and infection. To avoid these confounding variables, non-surgical models which produce an external insult to the joint have been developed. As the injuries rely on mechanical induction, the reproducibility and precision can be much higher than invasive surgical models. Another advantage is that human injury commonly occurs after external joint trauma, and non-surgical animal models of PTOA closely replicate the natural disease conditions and pathophysiology. Several non-surgical PTOA animal models exist, including intra-articular tibial plateau fracture (IATPF), cyclic articular cartilage tibial compression (CACTC), tibial compression overload, and transarticular impact. In the present study, the tibial compression model was chosen, as it closely replicates trauma caused in athletic injuries.

3.2 Current Bioreactor Design and Progress

The goal of this project was to scale an existing bioreactor for use in a mouse model of non-surgical PTOA to be compatible with both rats and guinea pigs. The present bioreactor can be programmed to produce chronic overuse changes by performing cyclic compressions over a period or as a single compressive overload to produce ACL rupture secondary to anterior subluxation of the tibia relative to the femur through tibial compression. Aside from accounting for morphologic variability between species, special consideration was given to the degree of force required to consistently produce ACL ruptures.

3.2.1 Morphometric measurements

Design of the bioreactor components began by collecting data on the external anatomical features of both guinea pigs and rats. While the non-surgical rat model of PTOA has been described, the current project included development of components to ensure compatibility of the bioreactor between studies using both species. Morphometric data (Table

3.1) were collected from three 5 ½ month old male guinea pigs and four mixed-sex rats with an average age of 3 ½ months using a precision mechanical caliper.

Table 3.1. Morphometric measurements of representative guinea pigs and rats.

	Sex	Age (mo)	Weight (g)	Hip width (mm)	Nose-rump length (mm)	Stifle diameter (mm)	Femur length (mm)	Foot length (mm)	Foot width (mm)	Foot thickness (mm)
GUINEA PIG	M	5.5	1186	127	305	21	61	51	12	13
	M	5.5	850	108	305	18	65	43	11	12
	M	5.5	1106	102	298	16	65	53	13	11
	AVG	5.5	1047	112	303	18	64	49	12	12
RAT	F	4	258	64	178	13	30	40	12	7
	F	4	350	57	184	14	43	41	11	7
	M	3	415	76	178	14	47	47	9	7
	M	3	420	89	197	12	50	44	9	7
	AVG	3.5	361	71	184	13	42	43	10	7

3.2.2 Computer-Aided Design (CAD)

Following morphometric data collection, Autodesk Fusion 360 (San Rafael, CA) CAD software was used to generate dimensional sketches of bioreactor components (Figure 3.1) and ultimately scaled orthogonal views of the completed assembly (Figure 3.2). Components were designed to be modular, such that parts could be quickly interchanged based on the selected limb and species. The functional components rest on a frame of machined aluminum and include i) an electric hydraulic digital servo-actuator ii) a load cell iii) stifle rest and iv) foot cup. Additionally, an actuator carriage with a hand brake was incorporated to control the stifle angle between animals of varying sizes.

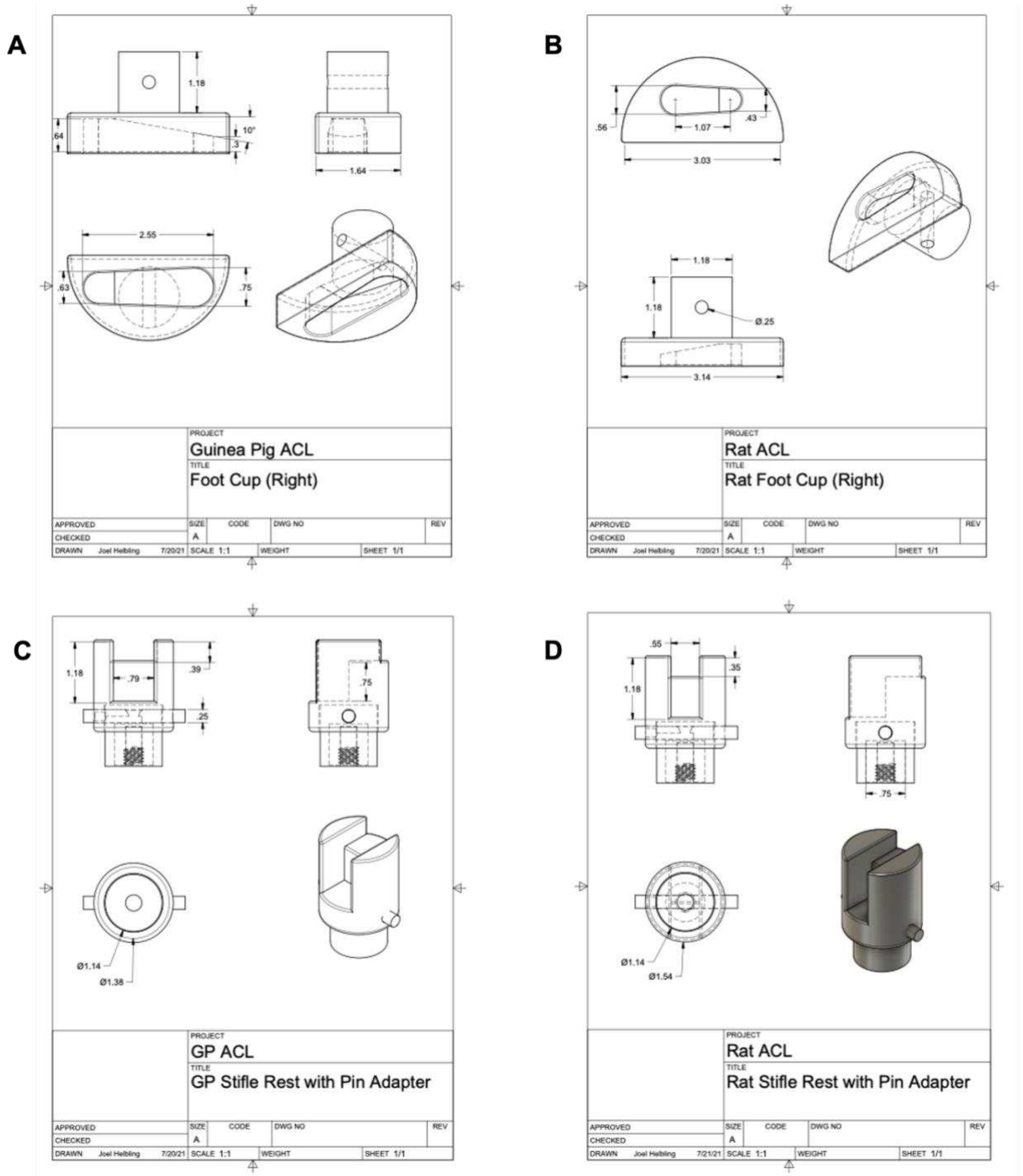

Figure 3.1. Dimensional drawings of guinea pig and rat foot cup and stifle rest.

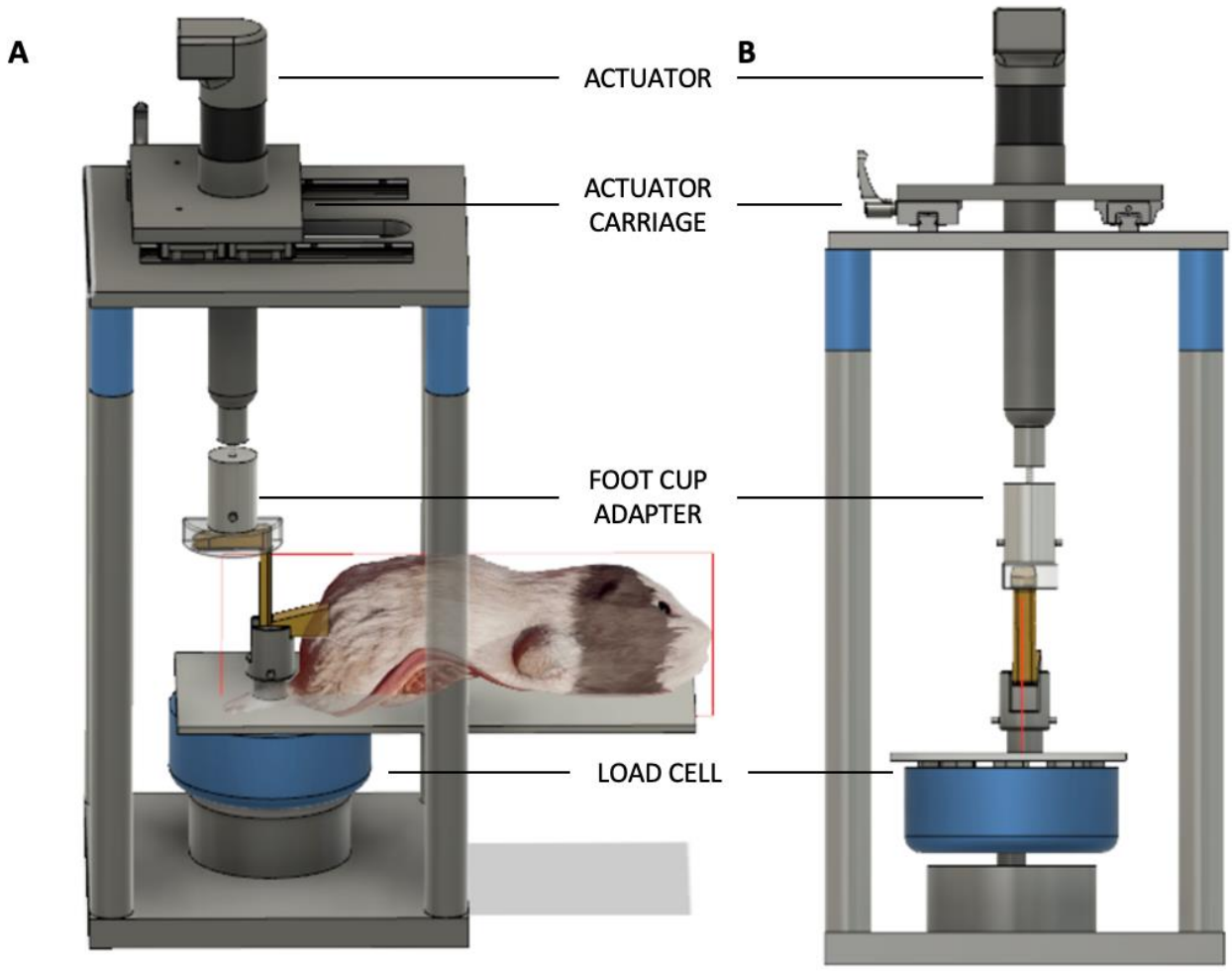

Figure 3.2. Orthogonal views of the completed bioreactor assembly.

3.2.3 Rapid prototyping and manufacturing

Both the foot cup and stifle rest were 3D-printed using a fused deposition modeling (FDM) printer (Lulzbot Mini 2 Desktop, Fargo Additive Manufacturing Equipment, Fargo, ND) with polylactic acid (PLA), a thermoplastic monomer. The foot cup adapter and the platform on which anesthetized animals rest in sternal recumbency were milled using machined aluminum.

3.3 Future Work

Immediate work is being conducted on both rat and guinea pig cadavers to validate the bioreactor and animal model prior to beginning *in vivo* research. Specifically, optimization of the force and displacement distance required, along with the duration and frequency of cyclic compression is needed. After assessing external mechanical rupture efficacy in each species, and characterizing features of the ACL tear, both grossly and histologically, future directions include comparing existing surgical ACL transection models to the present non-surgical model to assess clinical progression of PTOA, as well as researching therapeutic interventions.

CHAPTER IV.

CONCLUSION

The overarching goal of this project was to optimize open-field testing (OFT) in the guinea pig to ensure accurate and reproducible data collection in subsequent studies involving therapeutic interventions to both spontaneous and traumatic osteoarthritis (OA). First, a study was completed analyzing the effects of time-of-day and testing duration on mobility outcomes. While the Dunkin Hartley guinea pig is commonly used in OA research, published testing intervals for OFT vary greatly, which may result in unnecessary testing burden for both animals and personnel. Animals demonstrated increased mobility outcomes in the earliest testing period (0530-0700) and 10-minutes was found to be sufficient for a total testing period. These factors should be considered when conducting future behavioral or mobility research using OFT in the Dunkin Hartley guinea pig.

Using this information as a reference, a two-phase prospective study was performed to investigate the effects of oral cannabidiol (CBD) on mobility outcomes. During the Phase I pharmacokinetic portion of the study, mobility data was collected at four times-of-day (0530-0700, 0900-1100, 1130-1300, and 1530-1700) the day prior to dosing, and repeated the following day at the same timepoints. Additionally, data at 0530-0700 was collected at 24-hours post-dosing prior to euthanasia. The preliminary pharmacokinetic study found no significant behavioral effects following oral CBD administration at either 25 mg/kg or 50 mg/kg. This

suggests no immediate short-term effects occur following oral CBD administration at these doses.

Parenteral dosing of guinea pigs with medications can be challenging; therefore, the second phase of this study was conducted to assess oral dosing of CBD for practical and clinical considerations. In this study, 32 two-month-old male guinea pigs were divided into four groups of 8. Animals were placed into the untreated control group or randomly selected to receive one of three interventions, almond oil vehicle, 50 mg/kg CBD in vehicle, and 100 mg/kg CBD in vehicle. Each treatment was dosed orally twice daily for three months. OFT was performed once weekly until the study endpoint. Pre-treatment baseline data collected from these animals demonstrated periods of heightened activity in the earliest testing period (0530-0700) and found decreased activity after 10-minutes in the OFT. These findings agreed closely with data collected in Chapter 1. Assessment of the longitudinal effects of CBD treatments found no significant changes in mobility outcomes when analyzing final minus baseline data from any group. Age related changes in outcomes were observed within the untreated control group and between the untreated control group and CBD treated groups during certain periods.

Lastly, a mechanical bioreactor was designed to produce non-surgical precision rupture of the anterior cruciate ligament (ACL) in anesthetized guinea pigs. Cadavers have been collected for validation of mechanical bioreactor and future directions include comparing the pathology and progression of OA in guinea pigs receiving external ACL rupture compared to surgical transection of the ligament. Identifying the model that most closely compares to human OA progression will allow interventional and analgesic therapies to have the greatest chance at helping people suffering from this degenerative disease.

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